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Pozycja troficzna wazonkowca białego (*Enchytraeus albidus*) w kontekście badań molekularnych

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Katowice 2024

A wizard is never late, Frodo Baggins. Nor is he early. He arrives precisely when he means to.

- The Lord of the Rings: The Fellowship of the Ring (2001)

Podziękowania

Chciałbym serdecznie podziękować Panu Profesorowi dr. hab. Piotrowi Świątkowi za opiekę w trakcie moich studiów doktoranckich. Dziękuję za zaufanie, jakim darzył Pan moją osobę, oraz wiarę, jaką pokładał Pan w moje zdolności. Jestem wdzięczny za niezwykle dużą swobodę naukową, mobilizację, kiedy było to konieczne oraz za pokłady cierpliwości. Dziękuję za możliwość szczerej rozmowy i okazane wsparcie.

Chciałbym serdecznie podziękować Pani Profesor dr hab. Agacie Daszkowskiej-Golec za opiekę merytoryczną, słowa wsparcia i inspirację oraz konstruktywną krytykę, jakże przydatną każdemu naukowcowi, aby ten osiągał wyższe standardy naukowe i był precyzyjniejszy w formułowaniu myśli.

Podziękowania składam również dr hab. Annie Urbisz za okazane zrozumienie, empatię i wsparcie.

Kierownikowi studiów doktoranckich, Pani Profesor dr hab. Katarzynie Hupert-Kocurek, oraz specjaliście Pani Zofii Kuglin dziękuję za zrozumienie, cierpliwość i udzieloną pomoc w trudnych chwilach podczas studiów doktoranckich.

Mojej koleżance mgr Dominice Raś i mgr. Miłoszowi Morawskiemu dziękuję za wiarę we mnie, pomoc i wspólną pracę w laboratorium wydziałowym do późnych godzin nocnych.

Szczególnie chciałbym podziękować również moim przyjaciołom: dr. Szymonowi Gorgoniowi, dr. n. med. Damianowi Gojowemu, dr n. med. Sylwii Dudzicz-Gojowy, lic. Karolowi Trontowi oraz mgr. Bartkowi Zajuszowi. Dziękuję za przyjaźń, okazane wsparcie, wiarę we mnie i udostępnienie swoich platform sprzętowych w czasie pandemii COVID-19, abym mógł szybciej składać te wszystkie interesujące transkryptomy.

Wreszcie chciałbym podziękować mojej Żonie, Iwonie Śliż, za to, że przejęła na swoje barki część moich obowiązków domowych, abym mógł realizować swoją pasję, którą jest nauka. Dziękuję Ci również za to, że każdego ranka czekała na mnie na biurku świeżo przygotowana kawa.

Pracę dedykuję moim córkom, Róży i Lenie, aby wiedziały, że są tak samo mocno uparte jak ja, a wszystko jest tylko kwestią czasu. Wszystko, co dobre, wymaga czasu.

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[2] Gajda, Ł.; Daszkowska-Golec, A.; Świątek, P. 2024a. Discovery and characterization of the α-amylases cDNAs from *Enchytraeus albidus* shed light on the Evolution of "*Enchytraeus-Eisenia* type" Amy homologs in Annelida. Biochimie, 221, 38–59. https://doi.org/10.1016/j.biochi.2024.01.008 63

[3] Gajda, Ł.; Daszkowska-Golec, A.; Świątek, P. 2024b. Trophic position of the white worm (*Enchytraeus albidus*) in the context of digestive enzyme genes revealed by transcriptomics Analysis. Int. J. Mol. Sci., 25, 4685. https://doi.org/10.3390/ijms25094685 85

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I. AUTOREFERAT ROZPRAWY

1. Wprowadzenie

1.1. Charakterystyka rodziny Enchytraeidae oraz jej rola w przyrodzie

Wazonkowce (Enchytraeidae), zwane również doniczkowcami, są szeroko rozpowszechnioną rodziną małych, dżdżownicokształtnych pierścienic (Annelida) zaliczanych do siodełkowców (Clitellata). Znanych jest ponad 700 gatunków, z czego większość to gatunki lądowe (Schmelz i in., 2013; Schmelz i Collado, 2015). Niektórzy przedstawiciele Enchytraeidae zasiedlają skrajne środowiska, począwszy od osadów dennych mórz, a kończąc na śniegu i lodowcach (Shain i in., 2001; Torii, 2015; Prantoni i in., 2017; Lee i in., 2019). Generalnie jednak wazonkowce preferują siedliska bogate w materię organiczną, gdzie mogą występować masowo, na przykład w pokładach obumierających makroglonów i roślin w strefie przybrzeżnej mórz (czyli w tzw. kidzinie), w niektórych glebach towarzyszących specyficznym formacjom roślinnym, czy w kompoście (Boros, 2010; Dózsa-Farkas, 1978; Giere, 1975; Springett, 1967). Ich zagęszczenie może sięgać ponad 300 000 osobników/m^2 (Bardgett i Van Der Putten, 2014). Wazonkowce są zaangażowane w wiele ważnych procesów glebowych, między innymi w dekompozycję martwej materii organicznej i powstawanie próchnicy, tworzenie struktury gleby oraz regulację aktywności i dyspersję mikroorganizmów (Didden, 1990; Wolters, 1988). Mogą wykazywać większy wpływ na strukturę gleby niż dżdżownice, np. w ekosystemach gleb rolnych (Topoliantz, 2000), a nawet prawie całkowicie przejąć ekofunkcjonalną rolę tego ostatniego taksonu w kontekście formowania struktury gleby w suchych, kwaśnych lasach sosnowych (Räty i Huhta, 2003). Pomimo odgrywania znaczącej roli w wielu ekosystemach glebowych i faktu, że wazonkowce są znane nauce od ponad 150 lat (Udekem, 1855), pozostają one nadal słabo poznaną grupą zwierząt pod względem szczegółowej biologii i ekologii troficznej.

1.2. Krótka charakterystyka budowy układu pokarmowego wazonkowców

Budowa układu pokarmowego wazonkowców jest zgodna z ogólnym planem budowy układu pokarmowego skąposzczetów, czyli z tzw. „modelem rury w rurze" (Schmelz i Collado, 2013). Układ pokarmowy przebiega prosto lub nieco kręto przez całą długość ciała zwierzęcia, od otworu gębowego do odbytu, i jest zawieszony na przegrodach międzysegmentalnych oraz utrzymywany dodatkowo poprzez wewnątrzsegmentalne więzadła brzuszne lub grzbietowe (Kasprzak, 1986). Funkcjonalnie można podzielić go na jamę gębową, gardziel, przełyk, jelito

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i odbyt (Gelder, 1984; Kasprzak, 1986; Mothes-Wagner i in., 1996; Schmelz i Collado, 2013). Rozwojowo w układzie pokarmowym można wyróżnić jelito przednie (obejmujące jamę gębową i gardziel), jelito środkowe (obejmujące przełyk i jelito) oraz jelito tylne (z odbytem) (Schmelz i Collado, 2013). W gardzieli (segment III) po stronie grzbietowej położony jest charakterystyczny wysoki nabłonek palisadowy współtworzący tzw. organ gardzielowy (ang. pharyngeal pad). Organ ten poprzez zwarty i skomplikowany system mięśni gardzieli może być wysuwany i wciągany podczas pobierania pokarmu (Kasprzak, 1986; Purschke, 2003; Schmelz i Collado, 2010, 2013). W segmentach przednich ciała wazonkowców występuje od dwóch do kilku par (u *Enchytraeus* trzy pary) gruczołów septalnych (gardzielowych). Przypisuje się im funkcję produkcji mucyny oraz enzymów trawiennych (Schmelz i Collado, 2010). Są one rozwinięte na przegrodach międzysegmentalnych IV/V, V/VI oraz VI/VII, rzadziej dalszych. Przednie pary gruczołów septalnych mają postać owalnych, płatowatych i zbitych struktur bez światła. Tylna para jest często wydłużona. Poszczególne pary mogą być w różnym stopniu połączone ze sobą (bądź nie) na stronie grzbietowej (Kasprzak, 1986). Z kolei płaty różnych gruczołów po każdej stronie są na ogół połączone podłużnie poprzez wentro-lateralne pasmo komórek. W segmentach III i IV pasma te mogą być dobrze widoczne i wznoszą się w kierunku grzbietowym, kontaktując się z dorsalną częścią organu gardzielowego. Poza podstawowymi (pierwszorzędowymi) gruczołami septalnymi mogą również występować wyraźnie mniejsze, dodatkowe (drugorzędowe) gruczoły septalne (Kasprzak, 1986; Schmelz i Collado, 2010). W miejscu przejścia gardzieli w przełyk u części gatunków wazonkowców, w tym u przedstawicieli rodzaju *Enchytraeus* otwierają się ujścia peptonefrydiów – słabo poznanego narządu. Właściwy organ znajduje się z reguły pomiędzy segmentami III a VII. Peptonefrydia wykazują zróżnicowaną morfologię u różnych gatunków, mogą być parzystym lub nieparzystym organem, rozgałęzionym bądź nierozgałęzionym. Ich światło ma zwykle ciągłość ze światłem przełyku (Schmelz i Westheide, 2000). U *Enchytraeus* peptonefrydia przyjmują postać parzystych, nierozgałęzionych i ślepo zakończonych rurek. W literaturze peptonefrydia nazywane są również gruczołami ślinowymi lub przydatkami przełyku. Ta pierwsza, starsza nazwa jest niedokładna i myląca, gdyż funkcją peptonefrydiów nie jest wspomaganie trawienia poprzez produkcję enzymów, lecz najprawdopodobniej udział w utrzymaniu homeostazy wodnej i jonowej organizmu oraz ewentualne nawilżanie przesuwającej się dalej treści pokarmowej (Kasprzak, 1986; Schmelz i Westheide, 2000). Przełyk u Enchytraeidae stopniowo lub gwałtownie przechodzi w jelito, a przejściu temu może towarzyszyć występowanie uchyłków (kieszeni) jelitowych (Schmelz i Collado, 2013). Jelito otoczone jest wieńcem tkanki chloragogenowej z mięśniówką, które tworzą na znacznej długości zwartą strukturę dookoła

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narządu. Tkanka chloragogenowa pełni funkcje podobne do wątroby kręgowców (Varute i More, 1973; Cornelius, 1985). Pomiędzy nabłonkiem jelita a warstwą komórek chloragogenowych z mięśniówką (ta ostatnia skierowana do wewnątrz), usytuowana jest okołojelitowa zatoka krwionośna (Schmelz i Collado, 2010, 2013). W obrębie tylnego odcinka jelita środkowego u wazonkowców opisano tylko kilka modyfikacji jego budowy. Można tutaj wspomnieć o *pars tumida* (odcinek jelita z powiększonym i pęcherzykowatym nabłonkiem, zwykle ograniczonym do części wentralnej) oraz o rurkach Čejki (ang. Čejkaian tubules). Te ostatnie są cienkimi, wydłużonymi strukturami zbudowanymi z nabłonka, które biegną w obrębie nabłonka jelita środkowego, równolegle do długiej osi narządu, na długości kilku segmentów. Rurki Čejki są ślepo zakończone a pory prowadzące do nich znajdują się na granicy jelita środkowego i tylnego. Pierwotnie sugerowano ich rolę w wydzielaniu enzymów i trawieniu, jednak najprawdopodobniej uczestniczą jedynie w resorpcji wody (Schmelz i Collado, 2013). Poza powyższymi modyfikacjami opisano również, u pojedynczych gatunków (*Lumbricillus lineatus* i *Enchytraeus coronatus*), histologiczną dywersyfikację jelita środkowego na regiony o zróżnicowanej aktywności enzymatycznej (Gelder, 1984; Mothes-Wagner i in., 1996). Warto tutaj zaznaczyć, że jak dotąd detekcja enzymów w tkankach układu pokarmowego Enchytraeidae była prowadzona w ograniczonym zakresie (Ude, 1975; Gelder, 1984; Mothes-Wagner i in., 1996) i pomimo nazywania ich często na wyrost w wielu pracach „enzymami trawiennymi", dotyczyła tak naprawdę tylko aktywności ogólnych markerów histologicznych, takich jak fosfataza zasadowa, fosfataza kwaśna, esterazy A i C, czy β-N-acetyloglukozaminidaza (Cima, 2017). Typowe enzymy trawienne w ujęciu fizjologicznym, takie jak np. amylaza czy celulaza, nie były przedmiotem histolokalizacji (wyjątkiem jest detekcja aktywności β-galaktozydazy u *L. lineatus*; Gelder, 1984).

1.3. Znaczenie taksonomiczne, naukowe i ekonomiczne wazonkowca białego

Wazonkowiec biały (*Enchytraeus albidus* Henle, 1837) jest naukowo i ekonomicznie istotnym gatunkiem. Był jednym z pierwszych opisanych przedstawicieli rodziny Enchytraeidae i został wyznaczony za gatunek typowym dla rodzaju *Enchytraeus* (Erséus i in., 2019). Występuje powszechnie w różnych częściach Europy oraz Ameryce Północnej, w tym na Grenlandii (Dai i in., 2021). Uważany jest za gatunek oportunistyczny, zasiedlający zarówno habitaty ściśle lądowe (gleby rolne, kompost) jak i w wodno-lądowe (w strefie przybrzeżnej mórz). W Polsce znany głównie z terenów nizinnych, spotykany rzadko w naturalnych stanowiskach, jednakże związany z żyznymi, bogatymi w szczątki organiczne glebami ogrodowymi (Kasprzak, 1986).

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W warunkach domowych bywa hodowany przez zapalonych akwarystów jako atrakcyjna forma żywego pokarmu dla ryb ozdobnych.

Niedawne badania taksonomiczne potwierdziły, że *Enchytraeus albidus sensu lato* tworzył kompleks gatunków kryptycznych, co sugerowano już wcześniej (Erséus i Gustafsson, 2009; Schmelz i Collado, 2010). Obecnie z taksonu wyróżniono 9 odrębnych gatunków, które wykazują jedynie subtelne różnice morfologiczne, lecz wyraźnie różnią się od siebie pod względem genetycznym (Erséus i in., 2019; Nagy i in., 2023). W związku z tym, efektywną i precyzyjną metodą identyfikacji gatunku dla niespecjalistów z zakresu morfologii pozostaje barkoding DNA.

Enchytraeus albidus jest wykorzystywany jako organizm modelowy w badaniach fizjologicznych (Dai i in., 2021; de Boer i in., 2018), ekotoksykologicznych (w tym w teście OECD nr 220) (Kovačević i in., 2022) oraz w biologii rozwoju (Urbisz i in., 2017, 2022). Ponadto, wykorzystuje się go do produkcji żywego, wysokobiałkowego pokarmu w makroskali na potrzeby akwakultury, szczególne w hodowli ryb jesiotrowatych (Chebanov i Galich, 2013). Obecnie prowadzi się już zaawansowane prace wdrożeniowe w wykorzystaniu *E. albidus* w akwakulturze ryb ściśle morskich, głównie fląder, w tym także w biotechnologicznie nowoczesnych systemach recyrkulacyjnych (Walsh i in., 2015; Fairchild i in., 2017; Holmstrup i in., 2022).

1.4. Tajemnica bioróżnorodności w glebowych sieciach troficznych

Nadal aktualnym problemem badawczym poruszonym już w 1975 roku przez J. M. Andersona w artykule „The Enigma of Soil Animal Species Diversity" pozostaje pytanie: co powoduje, że pomimo wysokiej bioróżnorodność organizmów glebowych ich specjalizacja pokarmowa jest relatywnie niewielka? Fakt ten niejako stoi w sprzeczności z ekologiczną teorią konkurencji międzygatunkowej zakładającą konkurencyjne wypieranie gatunków, których nisze ekologiczne się pokrywają (Łuczak, 1956). Anderson (1975) w swoim artykule zaproponował hipotezy mogące tłumaczyć, przynajmniej częściowo, obserwowany fenomen. Jedna z nich postulowała, że występują nieodkryte różnice w wykorzystaniu zasobów pokarmowych między gatunkami (tj. nieujawnione, konkretne preferencje pokarmowe). Druga z kolei wskazywała na to, że istnieją nieodkryte różnice w zamieszkiwanych mikrośrodowiskach pomiędzy gatunkami (Anderson, 1975). W późniejszym czasie w odniesieniu do problemu bioróżnorodności organizmów glebowych zaproponowano również różne hipotezy dotyczące mechanizmu zależności między charakterystyką ekosystemu a liczbą poziomów troficznych w glebowej sieci troficznej, a które stały się przedmiotem sporu naukowców. Zaproponowano bowiem, że

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liczba poziomów troficznych rośnie wraz z produktywnością i dostępnością zasobów (Persson i in., 1992), lub wręcz odwrotnie, że to w ubogich w składniki odżywcze ekosystemach duża liczba interakcji między gatunkami skutkuje większą liczbą poziomów troficznych (Vander Zanden i in., 1999). Późniejsze badania nawiązujące do hipotezy mikrośrodowiskowej Andersona dostarczyły dowodów, że wysoka bioróżnorodność organizmów glebowych może być determinowana poprzez mikrostrukturalną heterogeniczność gleby, jednakże efekt ten zależny jest od skali, a także od wielkości organizmów (Nielsen i in., 2010). W związku z tym sposób, w jaki duża liczba gatunków zwierząt glebowych zajmujących ten sam poziom troficzny, jak na przykład saprofagi, może koegzystować w jednej sieci pokarmowej pozostaje dalej nierozwiązanym problem badawczym. Nawiązując do hipotezy nieujawnionych preferencji pokarmowych Andersona (1975), w zrozumieniu tajemnicy bioróżnorodności zwierząt glebowych przeszkodę stanowią między innymi ograniczenia tradycyjnie stosowanych metod badawczych. Sam proces odżywiania ma złożony mechanizm i obejmuje szereg procesów składowych, w tym pobór pokarmu, trawienie, asymilację składników odżywczych oraz ich retencję (Potapov i in., 2021, 2022). Dlatego też, status troficzny wielu grup bezkręgowców glebowych pozostaje niepewny lub konceptualny, ponieważ klasyczne metody badawcze, takie jak bezpośrednia obserwacja behawioru odżywiania, analiza mikroskopowa zawartości przewodu pokarmowego, analiza aktywności enzymów w homogenatach tkankowych, hodowle na różnych źródłach pożywienia lub testy wyboru źródła pokarmu zwykle dostarczają jedynie ograniczonej informacji o jednym bądź kilku procesach składowych procesu odżywiania (Maraun i in., 2023). W przypadku saprofagów takich jak na przykład dżdżownice, których przewód pokarmowy wypełnia mieszanina najróżniejszych materiałów i organizmów, praktycznie niemożliwe jest określenie, które ze składowych stanowią podstawę diety tej grupy zwierząt (Scheu, 2002). Ważnym jest, aby odróżnić pobierany pokarm od tego, co jest faktycznie asymilowane (Anderson, 1975). Ponadto, w przypadku wielu bezkręgowców mezofauny, w tym np. wazonkowców, trzeba rozważyć również potencjalny wkład aparatu enzymatycznego mikrobioty w procesy trawienne gospodarza (Krištůfek i in., 1999; Herrera i in., 2017). Przeszkodę w badaniu relacji troficznych może stanowić również elastyczność pokarmowa wykazywana przez niektóre zwierzęta, tj. zdolność do operowania na więcej niż jednym poziomie troficznym w zależności od środowiska i dostępności pokarmu (Scheu, 2002). Sprawę komplikuje również fakt istnienia kryptycznej różnorodności w wielu grupach bezkręgowców (Martinsson i Erséus, 2014). Oczekuje się, że gatunki podobne morfologicznie będą podobne ekologicznie, ale podobieństwo morfologiczne niekoniecznie ilustruje ekologiczną równoważność. Gatunki kryptyczne bowiem mogą różnić

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się pomiędzy sobą w niektórych właściwościach ekologicznych i fizjologicznych (Martinsson i Erséus, 2014; Cabrol i in., 2015).

W sieciach pokarmowych podstawowymi jednostkami nie są zazwyczaj pojedyncze gatunki, lecz całe grupy troficzne (gildie troficzne, trofogatunki) składające się z gatunków, które uznaje się za funkcjonalnie równoważne. W konceptualnym modelu glebowej sieci troficznej (zob. Figura 1) można wyróżnić saprofagi pierwszorzędowe (ang. primary decomposers), saprofagi drugorzędowe (ang. secondary decomposers) oraz drapieżniki. Ponadto dla dokładności opisu modelu, należy wyróżnić i uwzględnić szczątki roślinne, detrytus (częściowo rozłożona martwa materia organiczna) oraz mikroorganizmy (Scheu, 2002). Zgodnie z definicją saprofagi pierwszorzędowe do odżywiania wykorzystują materiał roślinny słabo skolonizowany przez mikroorganizmy, podczas gdy saprofagi drugorzędowe polegają na resztkach roślinnych wstępnie zdegradowanych (≈ detrytus) przez mikroflorę i/lub na mikroorganizmach jako źródłach pożywienia (Illig i in., 2005; Puppe i in., 2012). W wysoce bioróżnorodnych społecznościach organizmów, takich jak te występujące w glebie, agregowanie gatunków do grup troficznych jest nieuniknione. Niemniej jednak stwierdzono, że właściwości sieci pokarmowych mogą w dużym stopniu zależeć od rozdzielczości sieci, tj. od stopnia agregacji gatunków w jej obrębie (Scheu, 2002). W związku z tym poszukuje się nowych metod do badania zależności pokarmowych w sieciach troficznych, które zapewniają większą dokładność i lepszą rozdzielczość analizowanych sieci pokarmowych. Pierwotnie, wśród takich metod rozważano analizę stabilnych izotopów węgla (^{13}C) i azotu (^{15}N) oraz metody molekularne (np. fluorescencyjną hybrydyzację *in situ*) (Scheu, 2002). Obecnie analiza stabilnych izotopów prawie całkowicie zdominowała badania dotyczące ekologii troficznej, jednakże metoda ta nie jest pozbawiona pewnych ograniczeń. Interpretacja uzyskanych wyników bywa trudna, gdyż szereg czynników ontogenetycznych, fizjologicznych i biochemicznych może wpływać na izotopowy skład tkanek zwierzęcych (Briones, 2014). Ponadto, rozróżnienie między odżywianiem się różnymi taksonami mikroorganizmów, jest trudne i często niemożliwe przy użyciu samej tylko analizy stabilnych izotopów. Niezbędna zatem wydaje się być wielowymiarowa analiza niszy pokarmowej z wykorzystaniem wielu różnych metod (Potapov i in., 2021, 2022).

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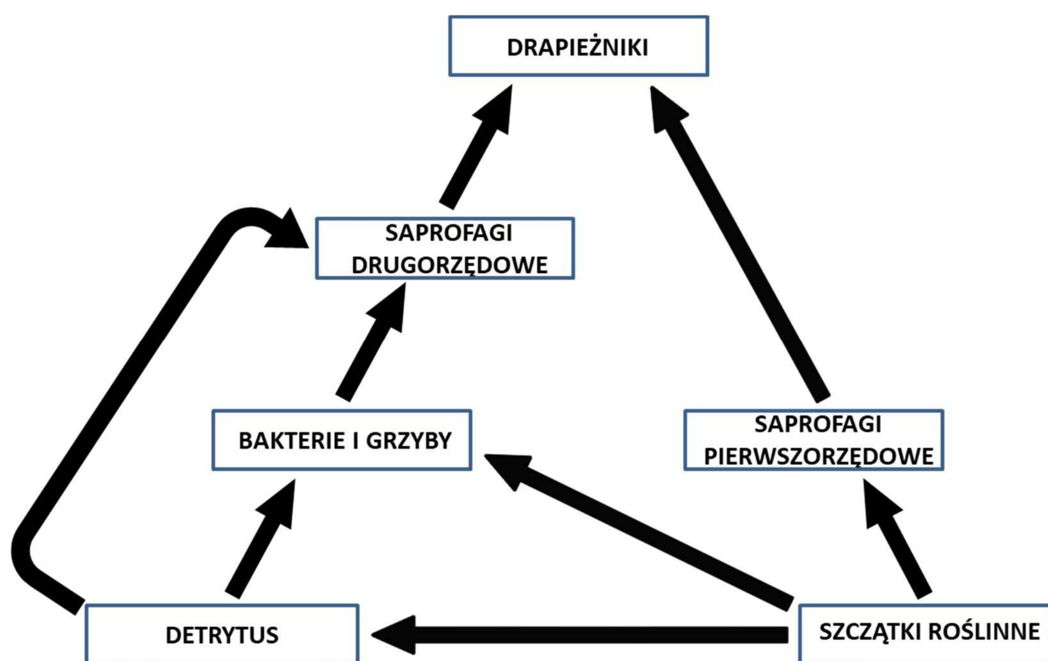

Figura 1. Konceptualny model glebowej sieci troficznej z rozróżnieniem saprofagów pierwszo- i drugorzędowych (na podstawie Scheu, 2002).

2. Cel rozprawy doktorskiej, hipotezy badawcze i etapy prac badawczych

Celem rozprawy doktorskiej było ustalenie pozycji troficznej wazonkowca białego (*Enchytraeus albidus*) na podstawie informacji dotyczących jego zdolności trawiennych, uzyskanych dzięki wykorzystaniu klasycznych, jak i nowoczesnych technik biologii molekularnej, w tym dzięki profilowaniu transkryptomu.

W pierwszym etapie prac badawczych nad rozprawą doktorską przeprowadzono krytyczną analizę obecnego na ten czas stanu wiedzy na temat preferencji pokarmowych rodziny Enchytraeidae i pozycji troficznej jej przedstawicieli. W przygotowanym i opublikowanym artykule przeglądowym (Gajda i in., 2017) dokonano ekstensywnej syntezy dostępnej wiedzy, w tym opisu, oceny i klasyfikacji potencjalnych źródeł pokarmu dla Enchytraeidae. Rozważano między innymi zagadnienia takie jak: selektywność w poborze pokarmu, proces trawienia oraz czynniki wpływające na wybór pokarmu. Przybliżono stan wiedzy na temat aktywności enzymatycznej wazonkowców, ze szczególnym uwzględnieniem zdolności celulolitycznych. Zidentyfikowano luki w istniejącej wiedzy oraz sprzeczności pomiędzy wynikami niektórych badań. W ramach pracy przeglądowej powtórzono również jeden ze starszych eksperymentów (Reichert i in., 1996) i wykazano niejednoznaczność pierwotnego rezultatu oraz nieadekwatność jego oryginalnej interpretacji. W wyniku przeprowadzonej analizy i przeglądu literatury zaproponowano wstępny podział przedstawicieli Enchytraeidae na dwie grupy troficzne: (1) saprofagi pierwszorzędowe oraz (2)

10:1146465866

saprofagi drugorzędowe. Jednocześnie zwrócono uwagę na konieczność prowadzenia dalszych, szczegółowych badań na pojedynczych gatunkach, co było sugerowane już wcześniej przez szereg innych autorów.

Przeprowadzenie pierwszego etapu prac badawczych umożliwiło postawienie hipotez badawczych, a następnie ich weryfikację w dalszych etapach prac. Postawione hipotezy badawcze brzmiały następująco:

H1: *Enchytraeus albidus* nie wykazuje endogennej ekspresji genów, które kodują celulazy, dlatego nie należy do grupy saprofagów pierwszorzędowych.

H2: *Enchytraeus albidus* wykazuje ekspresję genów, które kodują enzymy trawienne zaangażowane w troficzną lizę bakterii lub grzybów, takie jak np. hydrolazy mureinowe lub chitynazy, i dlatego należy do grupy saprofagów drugorzędowych.

W drugim etapie prac badawczych podjęto próbę amplifikacji transkryptów wybranych genów enzymów trawiennych u *E. albidus* za pomocą metody PCR z odwrotną transkrypcją (reverse transcription-PCR), z zastosowaniem podejścia „gene fishing", tj. przy użyciu wysoce zdegenerowanych starterów zaprojektowanych na podstawie sekwencji podobnych, dostępnych w bazach danych dla innych organizmów. Początkowo uzyskana częściowa sekwencja kodująca dla α-amylazy I (tj. *Amy I*) została w pełni scharakteryzowana dzięki metodom umożliwiającym poznanie oraz amplifikację końców 5' i 3' cDNA (5' cRACE i one-sided PCR). Przeprowadzono również klonowanie uzyskanej kompletnej sekwencji kodującej *Amy I* w celu separacji poszczególnych alleli i w celu scharakteryzowania polimorfizmu genu.

W trzecim etapie prac badawczych wyprowadzono hodowlę *E. albidus* z pojedynczego kokonu – monohaplotypową linię laboratoryjną PL-A – jednorodną pod względem mitochondrialnego genu podjednostki I oksydazy cytochromu c (COI). Uzyskano również transkryptom z osobników pochodzący z tej hodowli, a także przeprowadzono jego szczegółową analizę bioinformatyczną. Dzięki otrzymanym danym transkryptomicznym ujawniono ekspresję drugiego genu α-amylazy (tj. *Amy II*), a następnie również cały profil enzymatyczny badanego gatunku. Amplifikacja sekwencji kodujących *Amy I* i *Amy II* za pomocą metody PCR posłużyła do walidacji złożonego transkryptomu. Wyniki badań podjętych w etapach II i III opublikowano w pracach oryginalnych Gajda i inni (2024a, b).

Wszystkie powyższe etapy przyczyniły się do realizacji założonego celu badawczego. Ponadto, wiedza i umiejętności nabyte w trakcie jego realizacji umożliwiły prezentację

11:2715852989

uzyskanych wyników w szerszym kontekście, tj. w kontekście filogenetycznym i ewolucyjnym, dotyczącym wybranych genów kodujących enzymy trawienne w taksonach, takich jak Annelida i Clitellata. Pomimo faktu, że analiza filogenetyczna nie była bezpośrednim celem rozprawy doktorskiej rozumianym *per se*, to należy uznać ją za integralną część tej pracy, gdyż przyczyniła się do sformułowania bezpośrednio głębszych konkluzji, dotyczących zidentyfikowanych enzymów trawiennych, w tym także w odniesieniu do hipotezy nieujawnionych preferencji pokarmowych Andersona (1975). Rozszerzenie analiz korzystnie wpłynęło również na poszerzone możliwości publikacyjne uzyskanych wyników i wytyczyło potencjalne ścieżki pod nowe projekty badawcze.

3. Materialy i metody

3.1. Materiał zwierzęcy i warunki hodowli

Materiałem wykorzystanym w niniejszych badaniach były osobniki *Enchytraeus albidus*. Wszystkie początkowe hodowle, w tym również ta, z której pochodziły osobniki wykorzystane w pierwszym etapie prac badawczych, zostały założone z „porcji zarodowych" (tj. niewielka liczba osobników wraz z podłożem) zakupionych na platformie zakupowej Allegro, od prywatnego sprzedawcy z dwudziestoletnim doświadczeniem w hodowli wazonkowców. W drugim etapie prac badawczych ustanowiono tzw. „hodowlę mieszaną" (zawierającą osobniki o różnych haplotypach COI) z pojedynczej porcji zarodowej. Zwierzęta były przechowywane w temperaturze pokojowej w plastikowym pojemniku z defaunizowaną glebą ogrodową i karmione głównie płatkami dla ryb (Tropical) dwa razy w tygodniu. Losowe okazy z tej hodowli zostały poddane barkodingowi DNA oraz zostały przeanalizowane za pomocą analizy polimorfizmu konformacji pojedynczych nici fragmentów restrykcyjnych DNA (PCR-RF-SSCP, ang. polymerase chain reaction-restriction fragments-single strand conformation polymorphism analysis). W trzecim etapie prac badawczych wyprowadzono hodowlę (linię) monohaplotypową, jednorodną pod względem COI (szczep PL-A; numer dostępowy GenBank MK044803). Hodowla monohaplotypowa została ustanowiona z pojedynczego kokonu pochodzącego z hodowli mieszanej, przeniesionego na szalkę Petriego zawierająca 0,8%-1% agarozę (o czystości odpowiedniej dla biologii molekularnej) w wodzie kranowej (podłoże pozbawione składników odżywczych). Po wykluciu i wzroście osobniki *E. albidus* zostały przeniesione do pojemnika z defaunizowaną ziemią ogrodową i utrzymywane jak opisano wcześniej. Czystość genetyczną ustanowionej hodowli

12:7739439356

monohaplotypowej potwierdzono poprzez amplifikację i sekwencjonowanie fragmentu genu COI z losowych osobników.

3.2. Izolacja DNA oraz barkoding osobników

Mając na uwadze starsze doniesienia (Erséus i Gustafsson, 2009; Schmelz i Collado, 2010) sugerujące potencjalną kryptyczną różnorodność *Enchytraeus albidus*, losowe osobniki pochodzące z hodowli poddano barkodowaniu DNA w oparciu o amplifikację fragmentu genu COI (Hebert i in., 2003) (tzw. fragment Folmera; u pierścienic o długości 658 pz, nie wliczając starterów) w celu potwierdzenia przynależności taksonomicznej i na wypadek ewentualnej rewizji gatunku. Izolację DNA z osobników przeprowadzono metodą kolumienkową z trawieniem proteinazą K za pomocą zestawu GeneMATRIX Tissue DNA Purification Kit (EURx) według instrukcji producenta. Reakcje PCR przeprowadzono przy użyciu EURx Color OptiTaq PCR Master Mix (2×), starterów LCO1490 (0,4 µM w objętości końcowej) i HCO2198 (0,4 µM w objętości końcowej) oraz z 2 µl izolatu DNA jako matrycy, uzupełniając wodą wolną od nukleaz do finalnej objętości 50 µl. Reakcje przeprowadzano w termocyklerze Biometra TProfessional Basic Gradient, stosując warunki podane w Tabeli 1, zaadaptowane z pracy Martinsson i Erséus (2014). Rozdział elektroforetyczny produktów mieszaniny poreakcyjnej przeprowadzono w żelu agarozowym (1,2%) w buforze TBE z dodatkiem SimplySafe (EURx) i analizowano przy użyciu transiluminatora ETX (Vilber Lourmat). Uzyskane produkty PCR zostały przesłane do Genomed (Warszawa) i zsekwencjonowane w obu kierunkach. Nowo uzyskane sekwencje COI (haplotypy PL-A, PL-B i PL-C) zostały zdeponowane w bazie danych GenBank pod numerami dostępowymi: MK044803–MK044805.

Tabela 1. **„**Uniwersalne" warunki termalne PCR stosowane w barkodingu DNA oraz do amplifikacji większości innych wybranych sekwencji z tej pracy. Warunki termalne, które oryginalnie zaadaptowano z pracy Martinsson i Erséus (2014) zostały zmodyfikowane na podstawie doświadczeń własnych autora rozprawy z metodą PCR.

<table>
<tr><th></th><th>Temperatura [°C]</th><th>Czas [s]</th><th>Liczba cykli</th></tr>
<tr><td>Denaturacja wstępna</td><td>95</td><td>260</td><td>1 (pierwszy)</td></tr>
<tr><td>Denaturacja</td><td>95</td><td>40</td><td rowspan="3">35</td></tr>
<tr><td>Annealing</td><td>45</td><td>45</td></tr>
<tr><td>Elongacja</td><td>72</td><td>60</td></tr>
<tr><td>Elongacja końcowa</td><td>72</td><td>120</td><td>1 (ostatni)</td></tr>
</table>

3.3. Analiza PCR-RF-SSCP

Analiza PCR-RF-SSCP została przeprowadzona na podstawie protokołu zaadaptowanego z pracy Rakus i inni (2008). Aby uzyskać fragmenty DNA o odpowiedniej długości do analizy (< 300 pz), uzyskane amplikony COI zostały pocięte za pomocą enzymu restrykcyjnego HpyCH4V, który rozpoznaje miejsce restrykcyjne (5'…TG^CA…3'). Teoretyczny wzór cięcia wybranych amplikonów COI przedstawia Figura 2. Wyboru enzymu restrykcyjnego dokonano przy pomocy programu Gene Runner 6.5.52 (http://www.generunner.net). Analizę PCR-RF-SSCP przeprowadzono następująco: 10 µl danego produktu PCR poddano trawieniu za pomocą 1U HpyCH4V (New England Biolabs) w obecności 1,5 µl buforu reakcyjnego CutSmart NEBuffer (10×) oraz z dodatkiem wody wolnej od nukleaz w łącznej objętości 15 µl; całość inkubowano w temperaturze 37 °C przez 24 godziny w termocyklerze. Reakcję zatrzymano w temperaturze 65°C przez 20 minut. Po trawieniu, 7,5 µl mieszaniny poreakcyjnej zmieszano z 14 µl buforu obciążającego do SSCP (95% formamid, 0,1% błękit bromofenolowy, 0,1% ksylenocyjanol FF, 1 mM EDTA, 10 mM NaOH). Próbki denaturowano w temperaturze 95 °C przez 10 minut, przenoszono natychmiast na lód, a następnie ładowano na żel poliakrylamidowy (grubość 0,7 mm) o stężeniu 9% z dodatkiem 5% glicerolu. Rozdział elektroforetyczny w aparacie DNA Pointer (Kucharczyk) z chłodzoną cyrkulacyjną łaźnią wodną Hoefer RCB 300 przeprowadzono w 1-krotnym buforze TBE po uprzedniej pre-elektroforezie (100V, 15 min, bez próbek). Właściwa elektroforeza miała miejsce w następujących warunkach: 100V przez 15 minut w 5 °C, a następnie 600 V przez 135 minut w tej samej temperaturze. Po zakończonym rozdziale elektroforetycznym żel wysrebrzano według następującej procedury: żel traktowano 10-procentowym roztworem etanolu przez 15 minut, następnie 1-procentowym kwasem azotowym (V) przez 10 minut, azotanem (V) srebra (0,01 M) z dodaną na świeżo formaliną (1000 µl na litr $AgNO_3$) przez 30 minut, przepłukując krótko wodą destylowaną po każdym z etapów. Następnie, żel był traktowany niewielkimi porcjami świeżo przygotowanego roztworu 3-procentowego węglanu sodu z dodatkiem formaliny (500 µl na litr Na_2CO_3), aż do rozwinięcia widocznych prążków. W ostatnim kroku, żel został utrwalony za pomocą 10-procentowego kwasu octowego przez 15 minut i sfotografowany.

14:2984549576

Figura 2. Przewidywane miejsca cięcia sekwencji COI z ujawnionych holotypów (PL-A, PL-B oraz PL-C) z hodowli mieszanej przez enzym restrykcyjny HpyCH4V. Sposób cięcia sekwencji COI do fragmentów o tępych końcach został zaznaczony pionową czarną linią w obrębie niebieskich pudełek (sekwencja rozpoznawana przez restryktazę) na obu niciach sekwencji konsensusowej (sensownej i antysensownej). Polimorfizmy pojedynczych nukleotydów (SNPs) zostały zaznaczone w zielonych pudełkach w obrębie sekwencji haplotypów COI z przypisanymi im numerami dostępowymi. W analizie uwzględniono sekwencje starterów.

3.4. Izolacja RNA oraz dwuetapowy RT-PCR

Do izolacji całkowitego RNA wykorzystano odpowiednio od jednego do pięciu osobników *E. albidus* na próbkę (5 próbek oznaczonych E1-E5, 15 osobników łącznie). RNA izolowano przy użyciu metody kolumienkowej za pomocą zestawu GeneMATRIX Universal RNA Purification Kit (EURx, Gdańsk, Polska), zgodnie z instrukcją producenta, bez dodatkowego trawienia DNazą. Stężenie i jakość wyizolowanego RNA zostały ocenione przy użyciu mikrospektrofotometru NanoDrop 2000 (NanoDrop Technologies). Deklarację producenta zestawu o braku konieczności traktowania DNazą oraz poprawność wykonanej izolacji pod kątem kontaminacji DNA zwalidowano za pomocą kontrolnych reakcji PCR, które targetowały gen COI, używając 1 µl izolatu z danej próbki jako matrycy (w reakcjach tych nie uzyskano produktów amplifikacji). Wyizolowany całkowity RNA został przepisany na cDNA przy użyciu zestawu do odwrotnej transkrypcji NG dART RT kit (EURx) oraz startera oligo(dT)$_{20}$ targetującego transkrypty poliadenylowane. Procedurę przeprowadzono zgodnie z instrukcją producenta. Reakcję odwrotnej transkrypcji zatrzymano inkubując próbki w 85 °C przez 5 minut.

15:9400011955

Podjęto próbę amplifikacji transkryptów wybranych genów enzymów trawiennych (amylaza, chitynaza, celulaza, trehalaza i β-galaktozydaza) z wykorzystaniem zaprojektowanych par starterów o wysokim stopniu zdegenerowania. Sukcesem zakończyła się jedynie reakcja PCR targetująca α-amylazę I (*Amy I*). Amplifikacja rejonu rdzennego (tj. wewnętrznego) sekwencji kodującej (CDS) *Amy I* przeprowadzono przy użyciu EURx Color OptiTaq PCR Master Mix (2×), odpowiednich par starterów (0,6 µM w objętości końcowej każdy) oraz z zastosowaniem 1 µl cDNA dla danej próbki jako matrycy, uzupełniając wodą wolną od nukleaz do finalnej objętości 50 µl. Początkowo amplifikacja rejonu rdzennego CDS *Amy I* została przeprowadzona przy użyciu pary zdegenerowanych starterów: AmyF i AmyR, a następnie, przy użyciu pary starterów o niższym stopniu zdegenerowania: tgAmyF i agAmyR. Startery z obu par okazały się częściowo niedopasowane do matrycy, jednakże amplifikacja CDS była możliwa przy zadanej temperaturze annealingu (warunki reakcji takie jak podano w Tabeli 1), lecz nie w podwyższonej do 50 °C. Produkty PCR rozdzielono elektroforetycznie w 1,2% żelu agarozowym TBE z dodatkiem SimplySafe (EURx) i analizowano za pomocą transiluminatora UV ETX. Amplikony uzyskane w reakcji PCR z wykorzystaniem pary starterów tgAmyF i agAmyR zostały zsekwencjonowany (Genomed, Warszawa). Na podstawie uzyskanych odczytów niskiej jakości zaprojektowano serię nowych starterów wewnętrznych, które umożliwiły dokładne poznanie rejonu rdzennego, a także dalszą charakterystykę kompletnej sekwencji α-amylazy I przy wykorzystaniu 5’ cRACE oraz one-sided PCR (zob. Figura 3 oraz Tabela 2).

16:1056238072

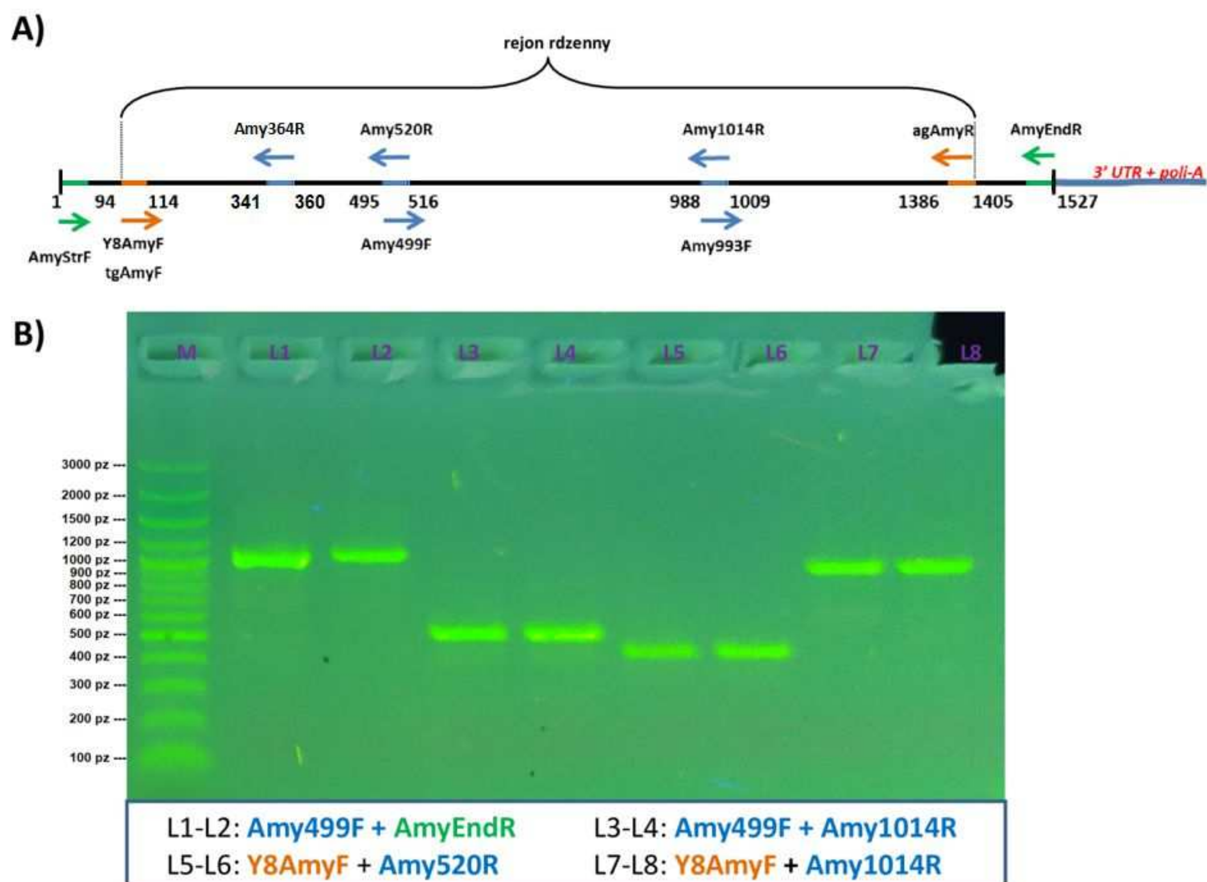

Figura 3. Amplifikacja sekwencji kodującej α-amylazy I. A) Schematyczna mapa z pozycjami przyłączania starterów w obrębie sekwencji CDS *Amy I*. B) Test wybranych starterów umożliwiających amplifikację różnych fragmentów sekwencji kodującej *Amy I.* Przedstawiono wynik rozdziału elektroforetycznego produktów PCR. Startery oznaczone na pomarańczowo były częściowo niedopasowane (pomimo zdegenerowania), lecz umożliwiały amplifikację przy obniżonej temperaturze annealingu. Startery oznaczone na niebiesko to niezdegenerowane startery doskonale wiążące się z matrycą, które zostały opracowane na podstawie odczytów z pary tgAmy + agAmyR. Startery oznaczone na zielono to startery, których zaprojektowanie było możliwe dzięki określeniu sekwencji końców 5' lub 3' za pomocą metod opisanych poniżej (zob. rozdział 3.5). Studzienka M zawierała marker wielkości DNA GPB3000bp (GenoPlast). Jako matrycę w powyższych testowych reakcjach PCR wykorzystano cDNA odpowiadający próbkom: E2 oraz E3.

Tabela 2. Startery wykorzystane w identyfikacji sekwencji kodującej α-amylazy I.

Nazwa startera	Orientacja startera	długość (nt)	sekwencja 5'->3'	Tm [°C]
AmyF	sensowna	21	ATsGTsCAyyTsTTyGArTGG	48,5 - 56,3
AmyR	antysensowna	20	CmvGArATvACrTCrCArTA	41,5 - 55,9
tgAmyF	sensowna	21	ATsGTsCAyyTsTTTGAGTGG	50,5 - 54,4
agAmyR	antysensowna	20	CmvGArATvACrTCACAGTA	43,6 - 53,8
Y8AmyF	sensowna	21	ATCGTCCAyTTGTTTGAGTGG	50,5 - 52,4
Amy499F	sensowna	22	TTACAACGATGCAAACCAAGTC	51,1
Amy520R	antysensowna	22	GACTTGGTTTGCATCGTTGTAA	51,1
Amy993F	sensowna	22	ACCTTCTTTGAGGCAAACATGT	51,1
Amy1014R	antysensowna	22	ACATGTTTGCCTCAAAGAAGGT	51,1
AmyStrF	sensowna	25	ATGCTGTCACTGATTGTGTTTTGTC	54,4
AmyEndR	antysensowna	23	TCAGACATGTAGAGCAATCATGG	50,5
Amy364R	antysensowna	20	AGTCATGTGGTTGAATACCC	49,7

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3.5. Charakterystyka nieznanych końców cDNA

W celu określenia pełnej sekwencji kodującej α-amylazy I zastosowano metody umożliwiające amplifikację nieznanych końców 5' i 3' wybranego cDNA.

3.5.1. Identyfikacja końca 3' cDNA za pomocą one-sided PCR

Koniec 3' sekwencji kodującej *Amy I* został powielony za pomocą techniki nazywanej jednostronną reakcją PCR (ang. one-sided PCR). Amplifikacja ta została oparta na uproszczonym przez autora rozprawy protokole dla one-sided PCR. Protokół dla tej techniki pierwotnie zostały opracowany przez Oharę i in. (1989), a później zaktualizowany przez Dorita i Oharę (1992). Aby przeprowadzić amplifikację PCR końca 3' cDNA *Amy I* zastosowano starter Amy993F (wykorzystany uprzednio w charakterystyce rejonu rdzennego) specyficzny dla genu *Amy I* oraz niespecyficzny starter oligo$(dT)_{30}$ komplementarny do sekwencji ogona poli-A obecnego w transkrypcie (i przepisanego wraz z pozostałą częścią sekwencji na cDNA). Modyfikacja protokołu dokonana przez autora rozprawy polegała na podniesieniu temperatury annealingu z zalecanej $\leq$ 42 °C (Dorit i Ohara, 1992) do 45 °C i użyciu jako startera antysensownego oligo$(dT)_{30}$ zamiast oligo$(dT)_{20}$. Obie zmiany razem przyczyniły się do zwiększenia swoistości jednostronnej reakcji PCR i znacząco zmniejszyły heterogeniczność końcowego produktu. Nie było zatem konieczności dodatkowych kroków, np. drugiej amplifikacji z zagnieżdżonymi starterami, ekstrakcji DNA z żelu i reamplifikacji, zwykle wymaganych w one-sided PCR do uzyskania pożądanej sekwencji i odczytów o wystarczającej jakości. Profil termiczny zastosowany w reakcji był zbliżony do uniwersalnych warunków przedstawionych w Tabeli 1, za wyjątkiem czasu denaturacji wstępnej (95°C przez 180 s) oraz elongacji końcowej (72°C przez 480 s). Do reakcji wykorzystano startery o stężeniu końcowym 0,6 µM każdy oraz resztę odczynników jak opisano w rozdziale poprzednim. Produkty pięciu reakcji one-sided PCR odpowiadające pięciu próbkom cDNA (E1-E5) zostały zsekwencjonowane w jednym kierunku (Genomed, Warszawa) za pomocą startera Amy993F. Na podstawie uzyskanych odczytów z powtórzeń biologicznych możliwe było ustalenie odcinka konsensusowego końca 3' sekwencji kodującej o długości około 500 par zasad. Na podstawie uzyskanej sekwencji konsensusowej zaprojektowano starter AmyEndR, który obejmował ostatnie 23 nukleotydy (tutaj corrigenda dla Gajda i in., 2024a), w tym 7 ostatnich kodonów sekwencji kodującej *Amy I* (Figura 4). Nowo zaprojektowany starter AmyEndR został zwalidowany za pomocą kontrolnych PCR z innymi starterami, w tym z Amy993F.

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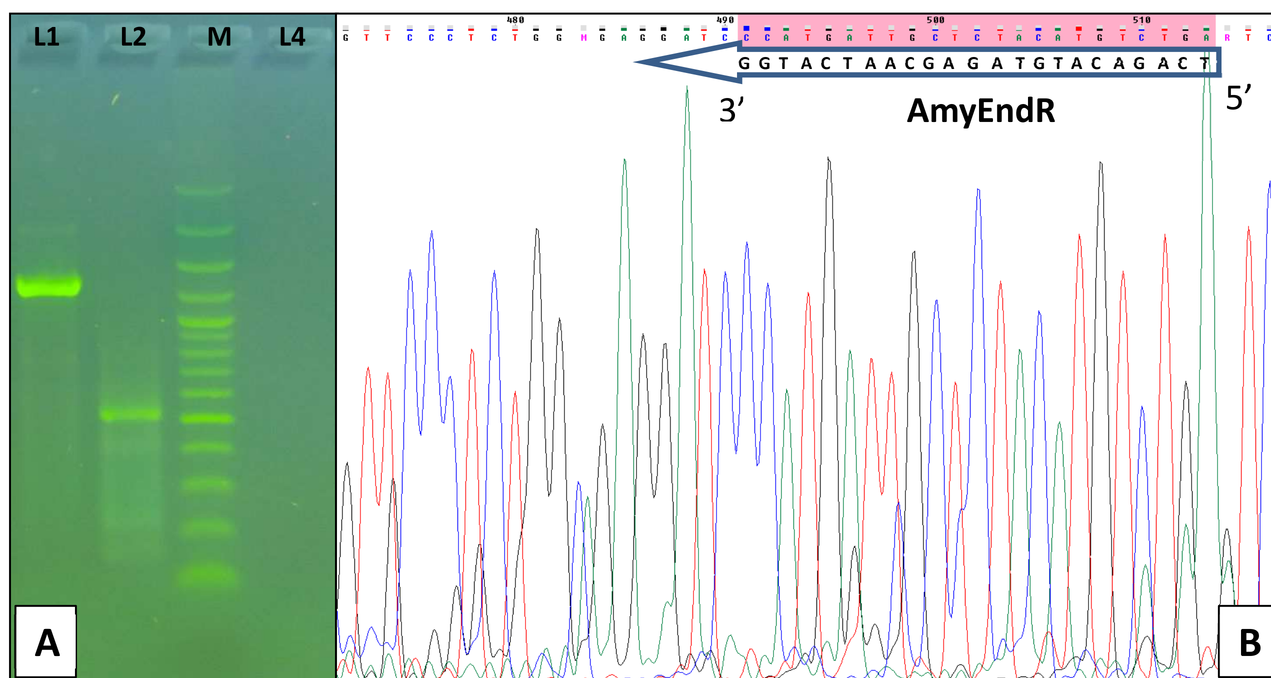

Figura 4. Identyfikacja nieznanego końca 3’ sekwencji kodującej α-amylazy I. A) Test reakcji one-sided PCR przeprowadzonej według modyfikacji autora rozprawy. Przedstawiono wynik rozdziału elektroforetycznego produktów PCR. Studzienka L2 zawierała mieszaninę poreakcyjną uzyskaną za pomocą jednostronnej reakcji PCR przy użyciu startera Amy993F oraz startera oligo(dT)$_{30}$. Studzienka L1 zawierała kontrolę pozytywną reakcji PCR (Y8AmyF + agAmyR), L4 zawierała ślepą próbę (reakcja bez matrycy cDNA), a M zawierała marker wielkości DNA GPB3000bp. B) Fragment fluorogramu z sekwencjonowania końca 3’ sekwencji *Amy I* próbki E2. Kolorem różowym zaznaczono obszar sekwencji zawierający prawie 8 ostatnich kodonów otwartej ramki odczytu (ORF), na podstawie którego zaprojektowano starter AmyEndR. Sekwencję startera zamieszczono w strzale, poniżej sekwencji komplementarnej.

3.5.2. Identyfikacja końca 5’ cDNA za pomocą trzyetapowej reakcji 5’ cRACE

Koniec 5’ cDNA *Amy I* zawierający 5’ UTR (rejon niepodlegający translacji) wraz z nieznanym fragmentem CDS został powielony za pomocą techniki zwanej trzyetapową cyrkularną szybką amplifikacją końców 5’ cDNA (ang. three-step circular rapid amplification of 5’ cDNA ends; three-step 5’ cRACE). Technikę opracował Dallmeier i Neyts (2013) i stanowi ona wariant klasycznej 5’ RACE oraz uproszczoną wersję czteroetapowej cRACE (Mandl i in., 1991). Procedurę zastosowaną w niniejszej rozprawie oparto o zmodyfikowany protokół utworzony na podstawie protokołów opisanych w pracach Dallmeier i Neyts (2013) oraz Vandecraen i inni (2016) (zob. Figura 5). Pół mikrograma RNA z *E. albidus* transkrybowano za pomocą NG dART RT (EURx) zgodnie z instrukcją producenta, z genowo-specyficznym 5'-ufosforylowanym starterem Amy520R (PAmy520R) w całkowitej objętości 20 µl w temperaturze 50 °C. Reakcję zakończono poprzez inkubację w temperaturze 85°C przez 15 minut. Z uzyskanej próbki cDNA przeniesiono 12,5 µl do nowej probówki i zmieszano z trzema objętościami buforu TE (10 mM Tris-HCl i 1 mM EDTA, pH 8.0) z dodatkiem RNazy A (4

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μg/ml). Następnie, dwie piąte tak przygotowanej mieszaniny, zawierającej cDNA, zostało poddane cyrkularyzacyjnej ligacji za pomocą 20 U ligazy T4 RNA w obecności 5 μl buforu reakcyjnego (10×) (Thermo Scientific) i 15% (w/v) glikolu polietylenowego 4000 (Thermo Scientific) i wody wolnej od nukleaz w całkowitej objętości 50 μl w temperaturze 37 °C przez 60 minut. Aby usunąć z mieszaniny pozostałości startera PAmy520R oraz niezligowane cząsteczki cDNA, do próbki dodano 1,5 U polimerazy Pfu (EURx) i inkubowano w temperaturze 37 °C przez 30 minut wykorzystując aktywność egzonukleazową 3'-5' enzymu. Następnie, 5 μl z otrzymanej mieszaniny zostało bezpośrednio użyte jako matryca do reakcji PCR z odwróconą parą starterów (ang. inverted primer pair) Amy364R oraz Amy499F. Reakcję PCR przeprowadzono z pozostałymi reagentami i w warunkach tak jak opisano poprzednio. Ponieważ uzyskany produkt był widoczny po rozdziale elektroforetycznym jako słaby prążek, amplikon wycięto z żelu i oczyszczono za pomocą zestawu Gel Purification GPB Mini Kit (GenoPlast Biochemicals) zgodnie z instrukcją wytwórcy. Z odzyskanego produkt PCR wykonano szereg rozcieńczeń (10^0 do 10^{-5}), który wykorzystano do reamplifikacji (Figura 6). Uzyskane amplikony dla rozcieńczeń 10^{-4} oraz 10^{-5}, którym nie towarzyszył rozmaz produktu (tzw. smear) zostały zsekwencjonowane (Genomed, Warszawa) za pomocą pary starterów Amy364R oraz Amy499F. Na podstawie uzyskanych odczytów ustalono sekwencję 5’ UTR wraz z nieznanym fragmentem CDS. Zaprojektowano również starter AmyStrF obejmujący pierwsze 25 nukleotydów sekwencji kodującej *Amy I*, który wraz ze starterem AmyEndR umożliwiał amplifikację kompletnej sekwencji kodującej α-amylazy I.

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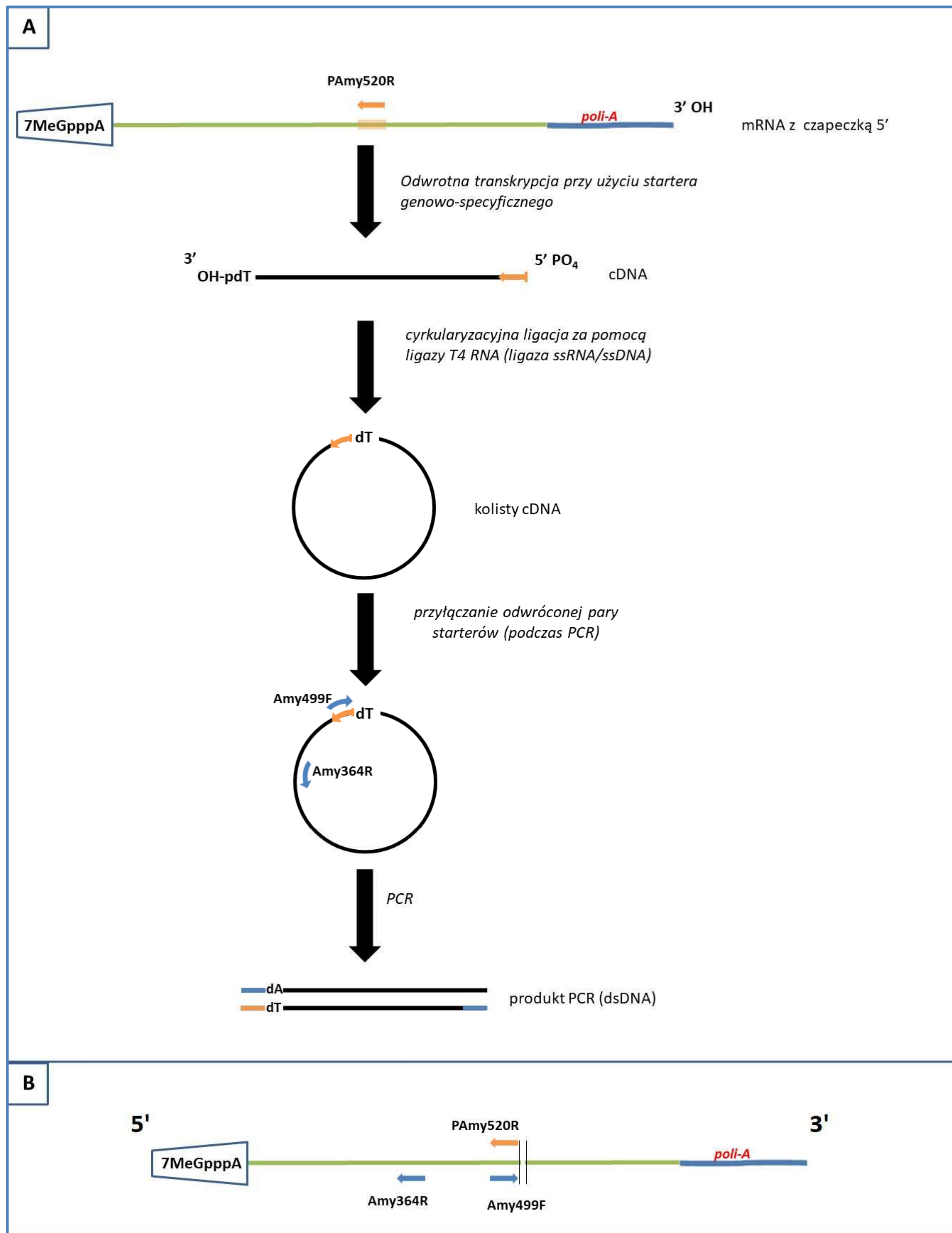

Figura 5. Schemat przebiegu trzyetapowej cyrkularnej szybkiej amplifikacji końców 5' cDNA (three-step cRACE). A) Schemat przebiegu cRACE z uwzględnieniem starterów zastosowanych w niniejszej rozprawie. Na schemacie przyjęto hipotetyczną sekwencję czapeczki 5'. Strzałki na linii oznaczają zainkorporowane startery. B) Poglądowe pozycje przyłączania starterów i ich orientacja względem cząsteczki RNA *Amy I*.

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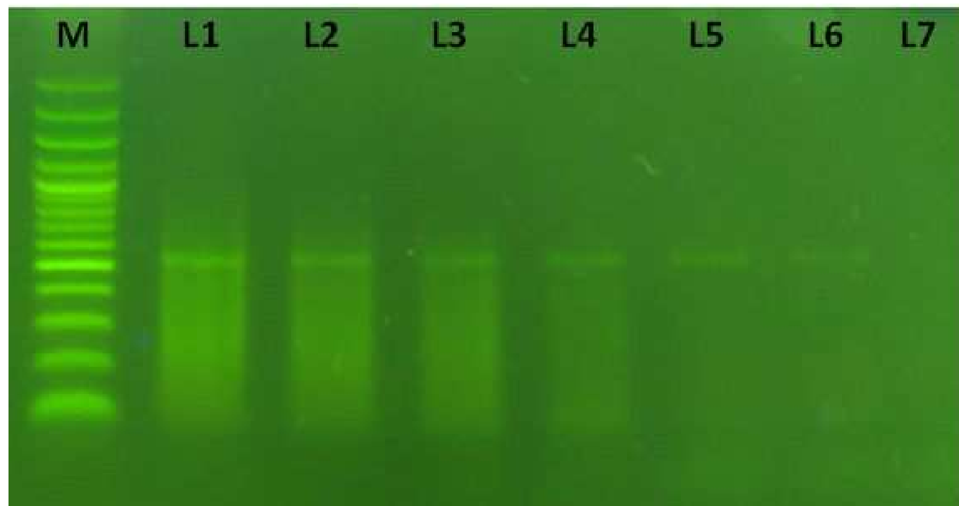

Figura 6. Reamplifikacja fragmentu 5' cDNA *Amy I* uzyskanego za pomocą trzyetapowej cyrkularnej szybkiej amplifikacji końców 5' cDNA (three-step cRACE). Przedstawiono wynik rozdziału elektroforetycznego produktów PCR. Studzienki L1-L6 zawierały produkt uzyskany z matrycy w serii rozcieńczeń (odpowiednio od 10^0 do 10^{-5}). Studzienka L7 zawierała ślepą próbę (bez dodatku matrycy) a studzienka M marker wielkości DNA GPB3000bp.

3.6. Klonowanie

Kompletna sekwencja kodująca α-amylazy I została zamplifikowana przy użyciu pary starterów AmyStrF i AmyEndR. Produkt PCR został wklonowany do wektora pGEM-T Easy przy użyciu zestawu pGEM-T Easy Vector System (Promega). Uzyskany konstrukt posłużył do transformacji komórek kompetentnych *Escherichia coli* JM109 zgodnie z protokołem producenta (Promega). Uzyskane kolonie zostały poddane selekcji blue/white (ang. blue-white screening). Siedem wybranych klonów zostało zsekwencjonowanych w obu kierunkach (Genomed, Warszawa) z użyciem starterów do selekcji klonów (T7long: TAATAC GACTCA CTATAG GGCGA; SP6: ATTTAG GTGACA CTATAG) oraz genowo-specyficznych (Amy660F: AGATCT GGAGGC GTTGTA TGG; Amy838R: CATCAC CGAGAT TCTTTC CGTG) (Figura 7).

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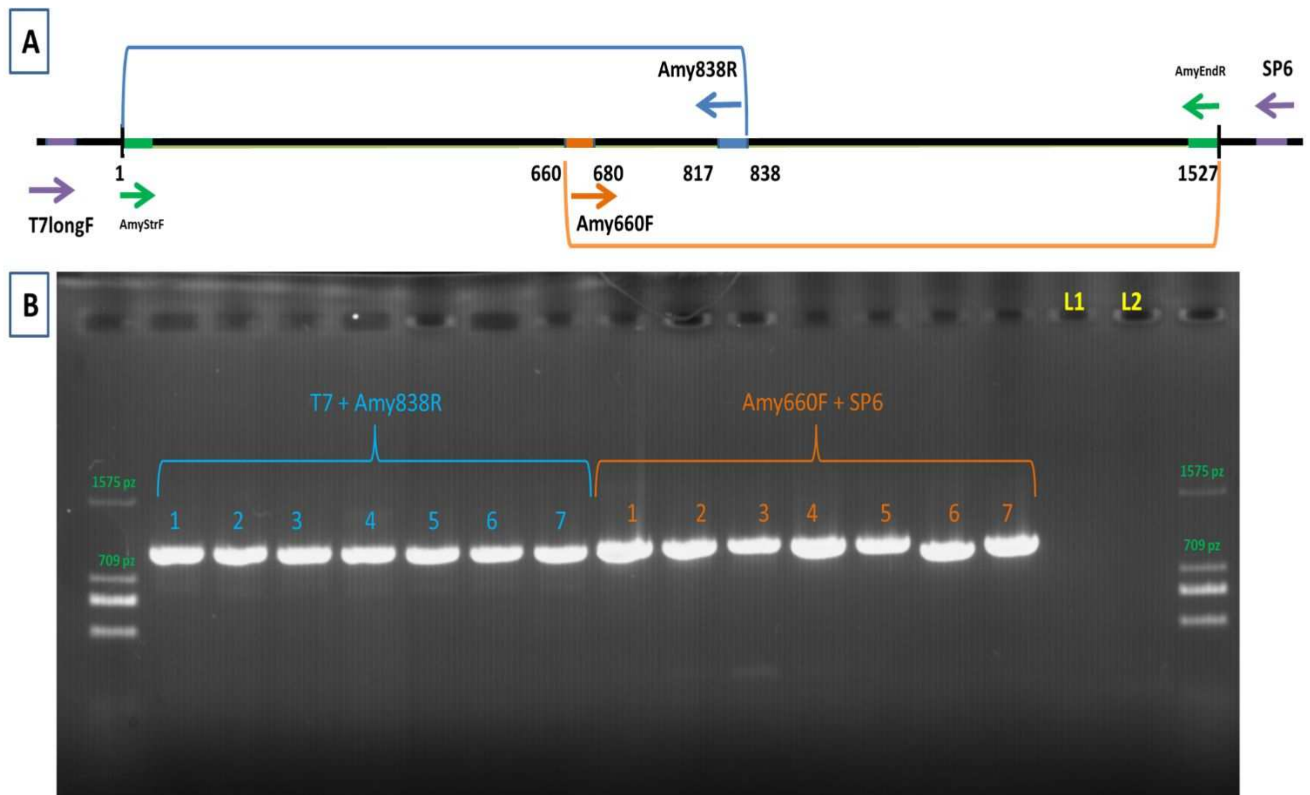

Figura 7. Strategia sekwencjonowania klonów. A) Schemat przedstawiający pozycje przyłączenia się starterów. B) Test starterów użytych do sekwencjonowania klonów C1-C7 jako dwóch, częściowo nakładających się fragmentów *CDS Amy I*. Przedstawiono wynik rozdziału elektroforetycznego produktów PCR. Studzienki L1 i L2 zawierały ślepe próby dla odpowiednich reakcji.

3.7. RNA-Seq

W trakcie wstępnych badań ustalono eksperymentalnie liczbę osobników *E. albidus* wymaganą do uzyskania ilości RNA odpowiedniej do przygotowania bibliotek RNA-Seq. W tym celu wykonano izolację RNA z jednego do pięciu dorosłych osobników na próbkę, przy użyciu zestawu GeneMATRIX Universal RNA Purification Kit (EURx, Gdańsk, Polska), zgodnie z protokołem producenta. Stężenie i jakość wyizolowanego RNA zostały ocenione przy użyciu mikrospektrofotometru NanoDrop 2000.

Właściwą izolację RNA na potrzeby RNA-Seq oraz sekwencjonowanie transkryptomu zlecono podmiotom zewnętrznym. Dorosłe osobniki *E. albidus* PL-A zostały wysłane w formie żywej na płytce agarozowej do A&A Biotechnology (Gdańsk, Polska) w celu ekstrakcji RNA. Procedura izolacji obejmowała użycie zestawu Total RNA Mini Kit (A&A Biotechnology) z oczyszczaniem DNazą (zestaw Clean-Up RNA Concentrator, A&A Biotechnology) i została przeprowadzona na próbce składającej się z czterech osobników. Jakość oraz stężenie wyizolowanego RNA przeanalizowano za pomocą elektroforezy w żelu agarozowym oraz

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spektrofotometru NanoDrop 2000. Porównanie wyników izolacji RNA uzyskanych z różnych procedur przez autora rozprawy i podmiot zewnętrzny przedstawiono w Tabeli 3.

Aby przeprowadzić sekwencjonowanie RNA i wygenerować odczyty RNA-Seq, próbka RNA została za pośrednictwem A&A Biotechnology wysłana do Macrogen Europe (Amsterdam, Holandia). Stężenie RNA próbki, która trafiła do tego podmiotu została oszacowana na miejscu na 236 ng/µl. Bibliotekę cDNA przygotowano przy użyciu zestawu TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA, USA). Sekwencjonowanie z dwóch stron (ang. pair-end) przeprowadzono na platformie Illumina (NovaSeq 6000; odczyty 2 × 151 pz) (zob. Figura 8).

Tabela 3. Porównanie rezultatów izolacji RNA uzyskanych przez autora rozprawy oraz A&A Biotechnology dzięki różnym procedurom i zestawom do ekstrakcji kwasów rybonukleinowych.

Nazwa Próbki	Liczba osobników użytych do izolacji RNA	Wykonawca	Zestaw	Stężenie kwasu nukleinowego [ng/µl]	A260/280	A260/230
E1	1	Autor rozprawy	GeneMATRIX Universal RNA Purification Kit (bez trawienia DNazą)	69,4	2,15	2,08
E2	2	Autor rozprawy	GeneMATRIX Universal RNA Purification Kit (bez trawienia DNazą)	163,9	2,17	2,26
E3	3	Autor rozprawy	GeneMATRIX Universal RNA Purification Kit (bez trawienia DNazą)	297,4	2,08	2,16
E4	4	Autor rozprawy	GeneMATRIX Universal RNA Purification Kit (bez trawienia DNazą)	393,7	1,99	2,04
E5	5	Autor rozprawy	GeneMATRIX Universal RNA Purification Kit (bez trawienia DNazą)	399,3	1,97	2,03
Sample 001	4	A&A Biotechnology	Total RNA Mini kit; Clean-Up RNA Concentrator Kit	295,1	2,24	2,13
Sample 002	4	A&A Biotechnology	Total RNA Mini Kit	263,5	2,21	2,18

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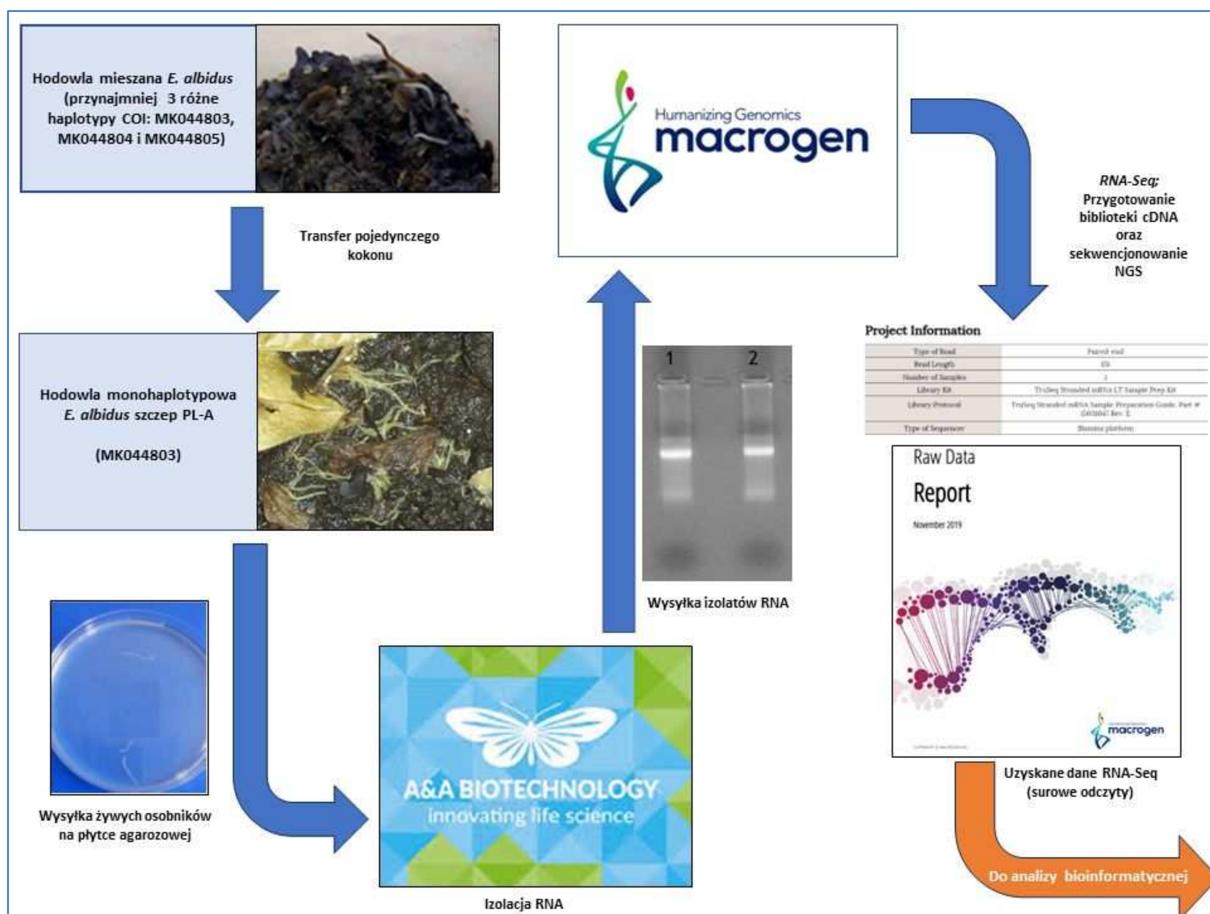

Figura 8. Ogólny schemat postępowania w celu uzyskania surowych danych RNA-Seq z *E. albidus* PL-A zastosowany w niniejszej rozprawie.

3.8. Asemblacja transkryptomu metodą *de novo*

Asemblacja transkryptomu metodą *de novo* polega na odtworzeniu oryginalnej sekwencji transkryptomu, czyli zestawu cząsteczek RNA, bez konieczności wykorzystania genomu referencyjnego jako szablonu (*E. albidus* nie posiada zsekwencjonowanego genomu). Jest to proces składania krótkich odczytów sekwencji RNA uzyskanych z sekwencjonowania nowej generacji w dłuższe fragmenty reprezentujące poszczególne transkrypty. W ramach niniejszej rozprawy opracowano własny potok składający się z serii programów, które w relatywnie łatwy sposób umożliwiają złożenie i adnotację danych RNA-Seq w systemie Windows, niezależnie od Linux i na niespecjalistycznej, standardowej platformie sprzętowej (PC klasy domowej z dostępem do Internetu).

Kontrola jakości surowych i przyciętych odczytów została przeprowadzona przy użyciu FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/ [dostęp 13.05.2024]). Usunięcie adapterów i przycięcie sekwencji o niskiej jakości odczytu zostało wykonane za pomocą wtyczki BBDuk w platformie oprogramowania Geneious Prime z następującymi

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ustawieniami: *trim adapters*, *default settings*; *trim partial adapters from ends with kmer length **11***; *trim low quality both ends*, *minimum quality **20***; *trim adapters based on paired read overhangs: minimum overlap **24***. Tak przygotowane dane RNA-Seq posłużyły dalej jako pliki wsadowe dla odpowiedniego asemblera sekwencji genetycznych. Transkryptom został złożony przy pomocy asemblera Trinity zintegrowanego w platformie oprogramowania OmicsBox (BioBam). Analiza została uruchomiona z następującymi ustawieniami: *K-mer Size: **25***; *Strand Specificity: **Strand Specific Reverse***; Minimum Contig Length: **200**; Pairs Distance: **500**. Uzyskany transkryptom został przetworzony przy użyciu programu TransDecoder (http://transdecoder.github.io [dostęp 13.05.2024]), również zintegrowanego w OmicsBox, z ustawieniami domyślnymi w celu wykrycia regionów kodujących. Zidentyfikowane w programie TransDecoder otwarte ramki odczytu (ORF) zostały przetłumaczone dalej na sekwencje białkowe o długości co najmniej 100 aminokwasów. Uzyskany zbiór sekwencji białkowych wywodzący się z transkryptomu został zadnotowany funkcjonalnie za pomocą narzędzi zaimplementowanych na serwerze GhostKOALA (Kanehisa i in., 2016) (http://www.kegg.jp/ghostkoala/ [dostęp 13.05.2024]), służących do automatycznej adnotacji funkcjonalnej i klasyfikacji sekwencji z użyciem bazy danych KEGG (Kyoto Encyclopedia of Genes and Genomes). Zadnotowany zestaw danych został następnie poddany dekontaminacji poprzez usunięcie sekwencji zidentyfikowanych na podstawie KEGG jako niepochodzące od zwierząt. Sekwencje te zostały usunięte za pomocą skryptu QIIME filter fasta (Caporaso i in., 2010), dostępnego na platformie Galaxy. Z kolei sekwencje, których nie udało się zadnotować inicjalnie za pomocą GhostKOALA-KEGG zostały wydzielone z pliku fasta i zadnotowane za pomocą serwera do automatycznej adnotacji funkcjonalnej sekwencji białkowych PANNZER2 (Törönen i Holm, 2022) (zob. Figura 9). Biorąc pod uwagę, że większość enzymów trawiennych występujących u zwierząt to enzymy hydrolityczne [z nielicznymi wyjątkami, takimi jak np. liaza pektynowa (Chen i in., 2021)], uzyskane dane przeszukano pod kątem hydrolaz. Szczególną uwagę zwrócono na glikozydazy, proteazy oraz lipazy.

Ustanowiony potok umożliwił również złożenie transkryptomów z publicznie dostępnych, surowych odczytów RNA-Seq dla genetycznie zróżnicowanych szczepów *E. albidus* tolerujących zamarzanie (de Boer i in., 2018) (stanowiły „referencje krzyżowe" dla szczepu PL-A) oraz dla innych przedstawicieli pierścienic.

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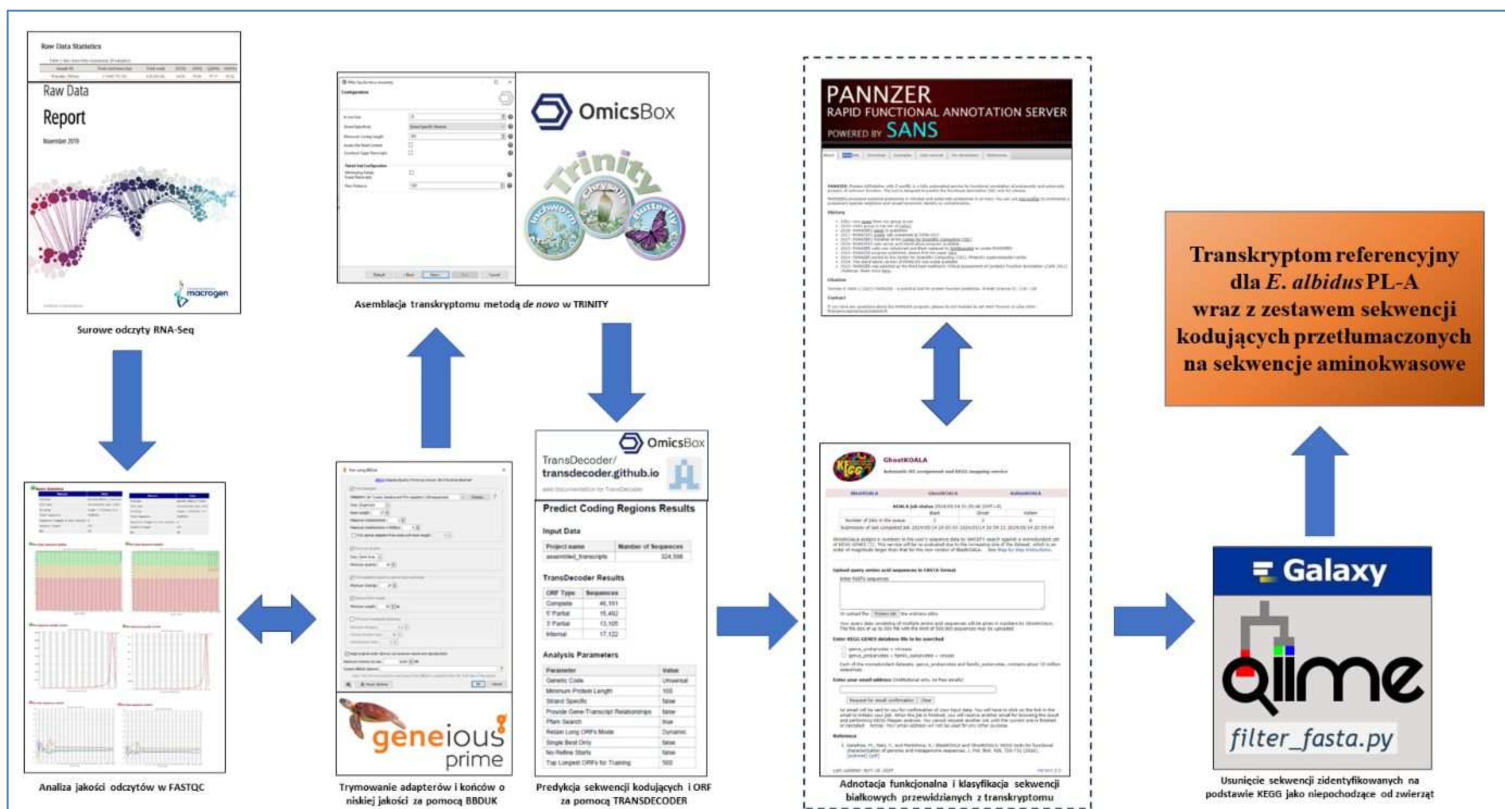

Figura 9. Ogólny schemat potoku opracowanego w celu złożenia transkryptomu *E. albidus* PL-A i jego adnotacji funkcjonalnej.

3.9. Analiza filogenetyczna

Dostępność sekwencji genetycznych w publicznych bazach danych, w tym sekwencji kodujących enzymy trawienne, jest bardzo ograniczona nie tylko dla wazonkowców czy siodełkowców, ale również dla całego typu Annelida. Zebrane dane, w tym sekwencje pochodzące z transkryptomów innych przedstawicieli pierścienic, umożliwiły przeprowadzenie analiz filogenetycznych wybranych enzymów trawiennych. Na potrzeby tych analiz złożono *de novo* 93 transkryptomy dla innych gatunków pierścienic niż *E. albidus*, w tym dla 6 gatunków wazonkowców. Transkryptomy pochodziły z odczytów RNA-Seq o zróżnicowanej jakości i głębokości sekwencjonowania, a tym samym wykazywały rożny stopień kompletności. Dodatkowo, ze względu na specyfikę enzymów trawiennych, odczyty musiały pochodzić z próbek RNA wyizolowanych z całych osobników albo przynajmniej z tkanek układu pokarmowego. Głównym źródłem wykorzystanych odczytów było repozytorium SRA (Sequence Read Archive).

Dojrzałe sekwencje białkowe (bez sekwencji peptydu sygnalnego), w zależności od zestawu danych, zostały przyrównane za pomocą programu MAFFT7 (Katoh i in., 2019) z

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automatycznym wyborem strategii przyrównania lub za pomocą programu MUSCLE (Edgar, 2004). Poprawność przyrównania (alignmentu) oceniano wizualnie dla małych zestawów danych (kilkanaście sekwencji) lub za pomocą programu GUIDANCE2 (Sela i in., 2015), który szacuje rzetelność przyrównania poszczególnych aminokwasów, kiedy zestaw danych był duży (kilkadziesiąt sekwencji). W pojedynczym przypadku (poboczna analiza dywergentnych amylaz; Gajda i in., 2024a), gdzie analizowano sekwencje o relatywnie niskim podobieństwie, regiony sporne przyrówniania usunięto z wykorzystaniem automatycznego filtrowania programem BMGE (Block Mapping and Gathering with Entropy) (Criscuolo i Gribaldo, 2010) korzystając z serwera NGPhylogeny.fr (Lemoine i in., 2019). We wszystkich przypadkach analiza filogenetyczna została przeprowadzona za pomocą serwerowej wersji IQ-TREE (Trifinopoulos i in., 2016). Program został użyty do wyboru najlepiej dopasowanego modelu substytucji aminokwasów, a następnie do skonstruowania drzewa metodą największej wiarygodności (ang. maximum likelihood). Wiarygodność otrzymanego drzewa szacowano za pomocą UFBoot (ang. ultrafast bootstrap approximation) oraz SH-like aLRT (ang. Shimodaira–Hasegawa-like approximate likelihood ratio test) w IQ-TREE (Simmons i Norton, 2014; Trifinopoulos i in., 2016). Wygenerowane drzewa zostały ukorzenione zgodnie z aktualnie postulowanymi hipotezami filogenetycznymi dla Annelida i Clitellata (Weigert i in., 2014; Struck i in., 2015; Erséus i in., 2020) oraz zwizualizowane za pomocą narzędzia iTol (Letunic i Bork, 2007).

3.10. Pozostałe analizy bioinformatyczne

Dane uzyskane z różnych źródeł, w tym zadnotowane dane transkryptomiczne dla *E. albidus* i sekwencje kodujące enzymy trawienne, zostały przeanalizowane przy użyciu szeregu narzędzi bioinformatycznych, w zależności od potrzeb (szczegóły w Gajda i in., 2024a, b). Poniżej, w Tabeli 4 przedstawiono alfabetyczną listę tych narzędzi wraz z krótkim objaśnieniem ich zastosowania.

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Tabela 4. Zestawienie narzędzi bioinformatycznych zastosowanych w prezentowanej rozprawie. W nawiasach kwadratowych podano docelowo wykorzystaną platformę lub bazę danych, w których dane narzędzie jest zintegrowane.

Nazwa narzędzia	Przeznaczenie
AlphaFold2/DeepMindv0.2 [Superbio.ai] https://www.superbio.ai/	Do automatycznego przewidywania struktury białek w oparciu o sztuczną inteligencję. Służy do modelowania struktury przestrzennej białek na podstawie ich sekwencji.
Augustus [OmicsBox] https://www.biobam.com/omicsbox/	Do przewidywania genów w sekwencjach genomowych organizmów eukariotycznych.
BLAST (Basic Local Alignment Search Tool) [NCBI] https://blast.ncbi.nlm.nih.gov/Blast.cgi	Do porównywania sekwencji nukleotydów lub białek z bazą danych, aby znaleźć sekwencje o podobnej strukturze lub funkcji.
BUSCA (Bologna Unified Subcellular Component Annotator) https://busca.biocomp.unibo.it/	Do przewidywania lokalizacji subkomórkowej białek na podstawie ich sekwencji.
BUSCO (Benchmarking Universal Single-Copy Orthologs) [OmicsBox] https://www.biobam.com/omicsbox/	Do oceny kompletności zestawów genów obecnych w genomach i transkryptomach poprzez porównywanie z zestawem genów ortologicznych.
codeML in PAML 4.9 package [Galaxy Europe] https://usegalaxy.eu/	Do analizy ewolucji sekwencji genetycznych, które pozwala na modelowanie zmian selekcyjnych i ocenę stopnia ewolucyjnej presji na sekwencje.
Compute pI/MW [ExPASy] https://web.expasy.org/compute_pi/	Do obliczania punktu izoelektrycznego (pI) i masy molekularnej (MW) białek na podstawie ich sekwencji aminokwasowych.
dbCAN3 https://bcb.unl.edu/dbCAN2/blast.php	Do identyfikacji genów kodujących enzymy związane z rozkładem węglowodanów i ich klasyfikacji EC.
DeepLoc 2.0 https://services.healthtech.dtu.dk/services/DeepLoc-2.0/	Do przewidywania lokalizacji subkomórkowej białek na podstawie ich sekwencji.
DeepTMHMM https://dtu.biolib.com/DeepTMHMM	Do przewidywania obecności transbłonowych helis w białkach na podstawie ich sekwencji.
Disulfide by Design 2.0 http://cptweb.cpt.wayne.edu/DbD2/	Do projektowania stabilnych połączeń dwusiarczkowych w białkach i analizy ich parametrów fizykochemicznych.
ESPript/ENDscript 2.0 https://endscript.ibcp.fr/ESPript/ENDscript/	Do analizy i wizualizacji struktury białek na podstawie ich sekwencji.
FirstGlance in Jmol https://bioinformatics.org/firstglance/fgij/	Do wizualizacji struktury przestrzennej białek i innych biomolekuł w przeglądarce internetowej.
GeneFisher2 https://bibiserv.cebitec.uni-bielefeld.de/genefisher2	Do projektowania zdegenerowanych starterów PCR na podstawie sekwencji konsensusowej DNA.
InterProScan https://www.ebi.ac.uk/interpro/search/sequence/	Do identyfikacji domen białkowych, motywów i innych cech funkcjonalnych w sekwencjach białkowych.
Jalview https://www.jalview.org/	Do analizy i wizualizacji sekwencji biologicznych, w tym białek i kwasów nukleinowych.
KofamKOALA https://www.genome.jp/tools/kofamkoala/	Do przypisywania funkcji metabolicznych białkom na podstawie podobieństwa sekwencji do białek w bazie KEGG Orthology.
MEGA7 https://www.megasoftware.net/home	Do analizy sekwencji DNA i białek, w tym budowy drzew filogenetycznych, kalkulacji dystansów genetycznych oraz tworzenia przyrównań sekwencji.
NetGPI 1.1 https://services.healthtech.dtu.dk/services/NetGPI-1.1/	Do przewidywania obecności kotwic lipidowych (GPI) w sekwencjach białek.
OligoAnalyzer [IDT] https://www.idtdna.com/pages/tools/oligoanalyzer	Do analizy i projektowania oligonukleotydów oraz analizy ich parametrów.
PANTHER Sequence Search [PANTHER 18.0] https://www.pantherdb.org/tools/sequenceSearchForm.jsp	Do porównywania sekwencji białek z bazą danych PANTHER w celu przypisania funkcji biologicznych.
Sequence Manipulation Suite: Version 2 https://www.bioinformatics.org/sms2/	Do manipulacji i analizy sekwencji nukleotydowych i białkowych.
SignalP 6.0 https://services.healthtech.dtu.dk/services/SignalP-6.0/	Do przewidywania peptydów sygnalnych w sekwencjach białkowych.
SMART (a Simple Modular Architecture Research Tool) http://smart.embl-heidelberg.de/	Do identyfikacji i analizy domen białkowych oraz ich struktury modularnej.
SWISS-MODEL https://swissmodel.expasy.org/	Do modelowania struktury przestrzennej białek na podstawie homologii z dostępnymi matrycami.
Translate Tool [ExPASy] https://web.expasy.org/translate/	Do tłumaczenia sekwencji nukleotydowych na sekwencje aminokwasowe.
UCSF ChimeraX https://www.cgl.ucsf.edu/chimerax/	Do wizualizacji i analizy struktury białek i innych biomolekuł w przestrzeni trójwymiarowej.

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4. Omówienie wyników i wniosków

Niniejsza rozprawa doktorska oparta jest na 3 opublikowanych pracach: pracy przeglądowej poświęconej preferencjom pokarmowy wazonkowców (Gajda i in., 2017) oraz dwóch pracach oryginalnych, w których analizowano odpowiednio, najpierw szczegółowo α-amylazy (Gajda i in., 2024a), a następnie cały profil enzymatyczny ujawniony przez dane transkryptomiczne (Gajda i in., 2024b). Warto zaznaczyć, że w pracy przeglądowej przeprowadzono oprócz szerokiej syntezy i analizy literatury, również dwa proste eksperymenty obserwacyjno-behawioralne z *E. albidus.* W pierwszym obserwowano sposób oddziaływania grupy głodzonych osobników wazonkowca białego na eksplantat (tj. wycięty fragment) pleszanki (*Pellia* sp.) na płytce agarowej. W drugim obserwowano rozkład suszonego liścia bzu czarnego (*Sambucus nigra*) podczas obecności i nieobecności wazonkowca. **W opublikowanej pracy przeglądowej wysunięto szereg wniosków wstępnych:**

1) Wazonkowce wykazują zróżnicowane strategie odżywiania. W zależności od gatunku, wazonkowce można zaklasyfikować jako saprofagi pierwszorzędowe lub saprofagi drugorzędowe. Przedstawiciele rodzaju *Enchytraeus*, w tym *E. albidus*, zostali wstępnie zaklasyfikowani do saprofagów drugorzędowych.
2) Wazonkowce nie są pasożytami roślin, ale często błędnie uważa się, że uszkadzają rośliny wyższe. Przeprowadzony eksperyment dotyczący kohabitacji głodzonych wazonkowców z eksplantatem pleszanki wykazał, że zwierzęta nie wpłynęły negatywnie na kondycję rośliny (doszło do regeneracji plechy).
3) Wazonkowce mają albo niewielkie zdolności celulolityczne, albo nie produkują celulaz wcale, a opisywana aktywność celulolityczna może być przypisana mikroorganizmom, gdyż nie badano pochodzenia wykrytych enzymów. Przeprowadzony eksperyment z liściem bzu czarnego i *E. albidus*, który stanowił niejako powtórzenie eksperymentu Reicherta i innych (1996) z gatunkiem pokrewnym (*Enchytraeus coronatus*) ujawnił, że nie można wykluczyć udziału mikroorganizmów w obserwowanym procesie dekompozycji materiału roślinnego. Liść na płytce kontrolnej (bez zwierząt) na której obserwowano wzrost mikroorganizmów, uległ podobnie jak na szalce ze zwierzętami wyraźnej maceracji (zmiękczeniu), aczkolwiek jego integralność na płytce kontrolnej została zachowana w okresie prowadzenia obserwacji.
4) Przypuszczalnie, hydrolazy mureiny mogą być enzymami uczestniczącymi w procesie trawienia bakterii w jelicie wazonkowców. Doniesienie na temat aktywności β-N-acetyloglukozaminidazy (β-NAGaza) w nabłonku jelitowym wazonkowca *Lumbricillus*

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lineatus (Gelder, 1984) sugerowało potencjalną rolę β-NAGazy w rozkładzie ścian komórkowych bakterii w przewodzie pokarmowym wazonkowców, jednakże hipoteza ta wymaga weryfikacji.

5) Wymagane są dalsze badania nad ekologią troficzną poszczególnych gatunków wazonkowców, a pomocne przy tym mogą być techniki biologii molekularnej, w tym RT-PCR. Technika ta wydaje się być szczególnie interesująca, gdyż potencjalnie umożliwia ona detekcję i analizę endogennej ekspresji genów enzymów trawiennych, w tym celulaz.

Na podstawie pierwszego etapu prac badawczych zwieńczonych publikacją wyżej wspomnianej pracy przeglądowej (Gajda i in., 2017), zostały sformułowane hipotezy badawcze. W drugim etapie prac badawczych skoncentrowano wysiłki na amplifikacji CDS wybranych genów enzymów trawiennych metodą RT-PCR z zastosowaniem podejścia „gene fishing", czyli za pomocą tzw. łowienia genów przy użyciu wysoce zdegenerowanych starterów. Znikoma dostępność sekwencji genetycznych dla enzymów trawiennych pierścienic w publicznych bazach danych utrudniała zaprojektowanie skutecznych starterów. Analiza za pomocą klasycznych technik biologii molekularnej okazała się wymagającym zadaniem i zakończyła się tylko częściowym sukcesem. W rezultacie udało się powielić i scharakteryzować pełną sekwencję kodującą α-amylazy I. Zaistniałe trudności skłoniły do poszukiwania bardziej wydajnych metod umożliwiających uzyskanie intersujących danych o ekspresji genów bez wcześniejszej znajomości bliskich sekwencji homologicznych bądź posiadania danych genomowych dla badanego gatunku. W związku z tym podjęto próbę wdrożenia w projekcie badawczym techniki RNA-Seq (tj. trzeci etap badawczy) opartej na sekwencjonowaniu nowej generacji. RNA-Seq umożliwia identyfikację i analizę ekspresji genów na szeroką skalę, bez posiadania wiedzy *a priori* na temat ich sekwencji.

Uzyskanie danych RNA-Seq dla *E. albidus* oraz przysposobienie potoku do złożenia transkryptomów metodą *de novo* i adnotacji funkcjonalnej pozwoliło na przeprowadzenie szeroko zakrojonych analiz porównawczych i filogenetycznych dla wybranych enzymów trawiennych. Ponadto, rozszerzono wyniki drugiego etapu badań o dane pochodzące z analizy transkryptomów wielu różnych gatunków pierścienic, w tym również transkryptomów innych szczepów *E. albidus* (szczepy G i N tolerujące zamarzanie, de Boer i in., 2018), dostępnych w publicznym repozytorium danych sekwencjonowania wysokoprzepustowego (SRA, Sequence Read Archive). Dzięki zebranym licznym sekwencjom α-amylaz w pierwszej pracy oryginalnej (Gajda i in., 2024a) zaproponowano wskazówki dotyczące ewolucyjnej i adaptacyjnej historii

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tego ważnego enzymu trawiennego w typie Annelida. **Na podstawie wykonanych analiz wysunięto szereg wniosków:**

1) Ujawniono ekspresję drugiego, paralogicznego genu kodującego α-amylazę u *E. albidus*. Gatunek ten posiada zatem dwa geny α-amylaz (*Amy I* i *Amy II*), które są homologiczne do genów α-amylaz dżdżownicy *Eisenia fetida* (Ef-Amy I i Ef-Amy II). Tworzą one grupę ortologiczną (tj. zestaw ortologów i paralogów posiadających ostatniego wspólnego przodka; Heller i in., 2019) u Annelida, wyróżnioną jako amylaza typu *Enchytraeus*-*Eisenia*.
2) Geny *Amy* wydają się być jedynym występującym typem α-amylaz u przedstawicieli *Enchytraeus*, w przeciwieństwie do wazonkowców z rodzaju *Mesenchytraeus*, które posiadają również α-amylazy innego typu (typ *Lingula-Platynereis)*.
3) Amy I wydaje się być genem wysoce polimorficznym i wielokopijnym, w przeciwieństwie do Amy II. Różne szczepy *E. albidus* posiadają unikalne allele amylaz z charakterystycznymi dla danego szczepu SNPs.
4) Architektura domen białkowych Amy I/Amy II jest zgodna z typowym schematem „ABC" budowy innych α-amylaz zwierząt.
5) Amy II nie posiada motywu aminokwasowego Gly-His-Gly-Ala (GHGA) w regionie zwanym pętlą elastyczną, podobnie jak niektóre α-amylazy owadów. Amylazy pozbawione tego motywu często wykazują dodatkową aktywność 4-α-glukanotrasferazy (EC 2.4.1.25). Adaptacyjne znaczenie Amy II u pierścienic może być związane ze skutecznym wykorzystaniem skrobi i podobnych polisacharydów z szerszego zakresu źródeł pokarmu.
6) Ostatni wspólny przodek siodełkowców posiadał amylazy typu Amy I i Amy II. Według zaproponowanej hipotezy, delecja motywu GHGA w pętli elastycznej była związana z tranzycją pierścienic ze środowiska morskiego do słodkowodnego i lądowego.
7) Utrata motywu GHGA w α-amylazie Amy jest cechą pierwotną dla Clitellata, choć modyfikacje lub nawet niezależne delecje w regionie pętli elastycznej miały miejsce również u niektórych przedstawicieli odległych ewolucyjnie linii wieloszczetów osiadłych (Sedentaria) [objaśnienie *ad marginem*: siodełkowce (Clitellata), a więc skąposzczety (Oligochaeta) z pijawkami (Hirudinea), tworzą takson zagnieżdżony w obrębie wieloszczetów osiadłych (Sedentaria)].

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8) Homologi α-amylazy typu *Enchytraeus-Eisenia* zostały zidentyfikowane w głównych grupach Annelida: bazalnych liniach rozwojowych pierścienic (ang. basal branching annelids), Errantia, Sedentaria i Oligochaeta, co wskazuje na ancestralne pochodzenie Amy w obrębie typu.
9) Wywnioskowany przebieg filogenezy oparty na sekwencjach białkowych genów Amy odbiegał istotnie w kilku kwestiach od ogólnie akceptowanych hipotez filogenetycznych pierścienic; jednakże wykazał zaskakującą zgodność z wynikami niedawnych dużych analiz filotranskryptomicznych (Erséus i in., 2020) i filogenomicznych (Martín-Durán i in., 2020) w odniesieniu do pozycji i relacji kilku wysoce problematycznych taksonów (*Dimorphilus*, *Hrabeiella* i *Aeolosoma*).

Analizując powyższe, należy zwrócić uwagę na adaptacyjne znaczenie obecności u zwierząt zróżnicowanych kopii genów tych samych enzymów trawiennych, takich jak np. amylazy Amy I i Amy II u *E. albidus*. Enzymy katalizujące ten sam typ reakcji (i posiadające ten sam numer w klasyfikacji EC) mogą wykazywać zróżnicowaną specyficzność substratową. Mogą więc różnić się w aktywności względem różnych frakcji danego polisacharydu, czy też względem aktywności na substraty o różnym pochodzeniu (i wynikającej z tego nieco odmiennej strukturze, np. różne typy skrobi, takie jak skrobia ziemniaczana i ryżowa) (Tsukamoto i in., 2021). Konkludując, rozważany powyżej aspekt adaptacyjny dotyczący tej samej grupy enzymów o subtelnie zróżnicowanych właściwościach biochemicznych może stanowić jeden z mechanizmów kształtujący bioróżnorodność w glebowej sieci troficznej zgodnie z hipotezą Andersona (1975) o nieodkrytych różnicach w wykorzystaniu zasobów pokarmowych.

Aby określić pozycję troficzną Enchytraeidae w glebowej sieci troficznej, niezbędna jest szczegółowa wiedza na temat preferencji pokarmowych poszczególnych gatunków wazonkowców. Zdecydowana większość wazonkowców [z wyjątkiem dwóch gatunków (Coates, 1990)] nie wykazuje istotnych różnic morfologicznych, ani też w budowie układu pokarmowego, które mogłyby wskazywać na odmienne preferencje troficzne. Różnice w preferencjach pokarmowych można jednak wyjaśnić zróżnicowaną aktywnością enzymatyczną wśród różnych gatunków wazonkowców. W drugiej opublikowanej pracy oryginalnej (Gajda i in., 2024b) wchodzącej w skład niniejszej rozprawy, autor analizował profil enzymatyczny *E. albidus* ujawniony przez dane transkryptomiczne dla szczepu PL-A. Analiza została wsparta, w celach porównawczych, danymi ze szczepów G i N tolerujących zamarzanie oraz danymi z innych siodełkowców, w tym innych gatunków wazonkowców. **W oparciu o przeprowadzane badania wysunięto następujące wnioski:**

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1) Na podstawie danych RNA-Seq dla *E. albidus* udało się zidentyfikować co najmniej 30 glikozydaz, 4 proteazy serynowe, 10 karboksypeptydaz A/B, 3 aminopeptydazy N, 4 lipazy i 1 fosfolipazę A2.
2) Wśród zadnotowanych genów ulegających ekspresji u *E. albidus* zidentyfikowano enzymy celulolityczne (endo-β-1,4-glukanazy; EC 3.2.1.4) oraz enzymy zaangażowane w trawienie mikroorganizmów (Ealb-iLys, trawienny lizozym typu i; EC 3.2.1.17 oraz dwie chitynazy, EC 3.2.1.14). *Enchytraeus albidus* łączy cechy zarówno saprofagów pierwszorzędowych, jak i saprofagów drugorzędowych. W kontekście pozycji troficznej gatunek ten został więc określony jako saprofag typu pośredniego (saprofag pośredni).
3) Na podstawie analiz filogenetycznych i bioinformatycznych ustalono, że endo-β-1,4-glukanazy występujące u *E. albidus* (Ealb-Eg I i Ealb-Eg II) są homologami kilku wcześniej opisanych celulaz pochodzących z dżdżownic *Eisenia fetida*, *Eisenia andrei* i *Metaphire hilgendorfi*. Te celulazy należą do rodziny hydrolaz glikozydowych GH9. Analiza danych transkryptomicznych wykazała ekspresję homologicznych genów celulaz u kilkunastu innych gatunków skąposzczetów, głównie dżdżownic i wazonkowców. Ponadto, homologiczna celulaza GH9 została zidentyfikowana u enigmatycznego gatunku glebowego wieloszczeta, *Hrabeiella periglandulata*.
4) Na podstawie analiz bioinformatycznych, w tym dzięki modelowaniu struktury przestrzennej, ustalono, że u *E. albidu*s multifunkcjonalny lizozym typu i (Ealb-iLys) należący do rodziny hydrolaz glikozydowych GH22i posiada domenę typu destabilaza-lizozym, charakteryzującą się podwójną aktywnością: lizozymu (muramidazy, EC 3.2.1.17) oraz destabilazy (izopeptydazy endo-ε-(γ-Glu)-Lys, EC 3.5.1.44), co potwierdzają przeprowadzone analizy strukturalne *in silico*. Ponadto Ealb-iLys zawiera dodatkowo domenę SH3b (ang. bacterial Src Homology domain 3 homologue), która odpowiada prawdopodobnie za rozpoznanie peptydoglikanu i wiązanie się enzymu ze ścianą komórkową bakterii. Hipoteza ta wymaga jednak dalszej weryfikacji eksperymentalnej, ponieważ tego typu destabilaza-lizozym z domeną SH3b była opisywana jedynie sporadycznie i to w ograniczonym zakresie. Jak dotąd, wysoce podobny i homologiczny enzym Ea-iLys u Annelida został opisany częściowo (oryginalni autorzy nie zidentyfikowali domeny SH3b) u jednego gatunku dżdżownicy (tj. *Eisenia andrei*) (Yu i in., 2019). Gen kodujący Ea-iLys charakteryzował się ekspresją w obrębie układu pokarmowego, co sugeruje

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udział tego typu lizozymów (liozyzmy typu *Enchytraeus–Eisenia*) w troficznej lizie bakterii.

5) Homologi destabilazy-lizozymu typu *Enchytraeus–Eisenia* z domeną SH3b zostały zidentyfikowane również w danych transkryptomicznych innych skąposzczetów. Ekspresja bliskich ortologów lizozymu typu *Enchytraeus–Eisenia* z domeną SH3b może być potencjalnym molekularnym markerem bakteriożerności u Clitellata.
6) Endo-β-NAGaza jest wewnątrzkomórkowym enzymem zlokalizowanym w cytoplazmie, który nie ulega sekrecji do światła układu pokarmowego. Nie jest więc enzymem trawiennym zaangażowanym w lizę troficzną bakterii u *E. albidus*.

Jak zademonstrowano w niniejszej rozprawie, metody molekularne, zwłaszcza RNA-Seq, w połączeniu z szerokim wachlarzem metod bioinformatycznych, mogą być wysoce przydatne w badaniach pozycji troficznej zwierząt niemodelowych. Sekwencjonowanie RNA o odpowiedniej głębokości, nawet dla pojedynczej próbki, aczkolwiek ściśle zdefiniowanej pod względem taksonomicznym, może być potężnym narzędziem, dostarczającym istotnych informacji o ekspresji kluczowych enzymów w kontekście ekologii troficznej. Co zaskakujące, potencjał RNA-Seq nie jest praktycznie wykorzystywany przez ekologów badających sieci pokarmowe do ustalania pozycji troficznej zwierząt glebowych.

Do czasu opublikowania drugiej pracy oryginalnej (Gajda i in., 2024b), pozycja troficzna wazonkowców badana metodą stabilnych izotopów ^{13}C i ^{15}N pozostawała bez wypracowanego konsensusu. Na podstawie eksperymentów ze stabilnymi izotopami, Scheu i Falca (2000) oraz Schmidt i in. (2004) zaklasyfikowali wazonkowce jako saprofagi drugorzędowe, podczas gdy Crotty i in. (2011) jako pierwszorzędowe. Dopiero niedawno wydana praca Korobushkina i współpracowników (2024), w której przeanalizowano 16 gatunków wazonkowców za pomocą metody stabilnych izotopów, okazała się niejako przełomowa w tej kwestii. Praca ta została opublikowana w czasie, gdy druga praca oryginalna dotycząca niniejszej rozprawy wróciła z pierwszej rundy recenzji. Szczęśliwy zbieg okoliczności sprawił, że możliwe było odniesienie się bezpośrednio do wyników pracy Korobushkina i współpracowników (2024) w manuskrypcie na etapie rewizji.

Korobushkin i współpracownicy (2024) zaklasyfikowali *E. albidus* wraz z innymi wazonkowcami epigeicznymi do saprofagów pierwszorzędowych, jednakże uzyskane wartości $\Delta^{15}N$ sugerowały również trawienie mikroorganizmów. W związku z tym wspomniany zespół wyraził opinię, że klasyfikacja indywidualnych gatunków wazonkowców powinna

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inkorporować dodatkowe metody umożliwiające wieloparametrową ocenę pozycji troficznej, zamiast bazować wyłącznie na sygnaturach stabilnych izotopów. Wyniki dotyczące *E. albidus* przedstawione przez Korobushkina i współpracowników (2024) okazały się zgodne z wnioskiem prezentowanym w niniejszej rozprawie tj. wnioskiem o pozycji pośredniej badanego gatunku pomiędzy pierwszorzędowymi i drugorzędowymi saprofagami (Gajda i in., 2024b). Przykład ten pokazuje, że metody molekularne, zwłaszcza RNA-Seq, mogą doskonale uzupełniać badania oparte na analizie stabilnych izotopów.

36:5527934250

5. Literatura

Anderson, J. M. (1975). The enigma of soil animal species diversity. W J. Vaněk (Red.), *Progress in Soil Zoology* (s. 51–58). Springer Netherlands. https://doi.org/10.1007/978-94-010-1933-0_5

Bardgett, R. D., & Van Der Putten, W. H. (2014). Belowground biodiversity and ecosystem functioning. *Nature*, *515*(7528), 505–511. https://doi.org/10.1038/nature13855

Boros, G. (2010). Enchytraeids (Oligochaeta, Enchytraeidae) from potting compost purchasable in the Hungarian retail trade. *Opuscula Zoologica (Budapest)*, *41*, 237–240.

Briones, M. J. I. (2014). Soil fauna and soil functions: A jigsaw puzzle. *Frontiers in Environmental Science*, *2*. https://doi.org/10.3389/fenvs.2014.00007

Cabrol, J., Winkler, G., & Tremblay, R. (2015). Physiological condition and differential feeding behaviour in the cryptic species complex *Eurytemora affinis* in the St Lawrence estuary. *Journal of Plankton Research*, *37*(2), 372–387. https://doi.org/10.1093/plankt/fbu111

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Peña, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., McDonald, D., Muegge, B. D., Pirrung, M., … Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, *7*(5), 335–336. https://doi.org/10.1038/nmeth.f.303

Chebanov, M. S., & Galich, E. V. (2013). Sturgeon hatchery manual. *FAO fisheries and aquaculture technical paper*, *558*, pp. 137–138.

Chen, J., Li, Z., Lin, B., Liao, J., & Zhuo, K. (2021). A *Meloidogyne graminicola* pectate lyase is involved in virulence and activation of host defense responses. *Frontiers in Plant Science*, *12*, 651627. https://doi.org/10.3389/fpls.2021.651627

Cima, F. (2017). Enzyme Histochemistry for Functional Histology in Invertebrates. W C. Pellicciari & M. Biggiogera (Ed.), *Histochemistry of Single Molecules* (Vol. 1560, pp. 69–90). Springer New York. https://doi.org/10.1007/978-1-4939-6788-9_5

Coates, K. A. (1990). Redescriptions of *Aspidodrilus* and *Pelmatodrilus* , enchytraeids (Annelida, Oligochaeta) ectocommensal on earthworms. *Canadian Journal of Zoology*, *68*(3), 498–505. https://doi.org/10.1139/z90-073

Cornelius, C. E. (1985). Hepatic ontogenesis. *Hepatology*, *5*(6), 1213–1221. https://doi.org/10.1002/hep.1840050625

Criscuolo, A., & Gribaldo, S. (2010). BMGE (Block Mapping and Gathering with Entropy): A new software for selection of phylogenetic informative regions from multiple sequence alignments. *BMC Evolutionary Biology*, *10*(1), 210. https://doi.org/10.1186/1471-2148-10-210

Crotty, F. V., Blackshaw, R. P., & Murray, P. J. (2011). Tracking the flow of bacterially derived ^{13}C and ^{15}N through soil faunal feeding channels. *Rapid Communications in Mass Spectrometry*, *25*(11), 1503–1513. https://doi.org/10.1002/rcm.4945

37:3837989502

Dai, W., Slotsbo, S., & Holmstrup, M. (2021). Thermal optimum for mass production of the live feed organism *Enchytraeus albidus*. *Journal of Thermal Biology*, *97*, 102865. https://doi.org/10.1016/j.jtherbio.2021.102865

Dallmeier, K., & Neyts, J. (2013). Simple and inexpensive three-step rapid amplification of cDNA 5′ ends using 5′ phosphorylated primers. *Analytical Biochemistry*, *434*(1), 1–3. https://doi.org/10.1016/j.ab.2012.10.031

de Boer, T. E., Roelofs, D., Vooijs, R., Holmstrup, M., & Amorim, M. J. (2018). Population-specific transcriptional differences associated with freeze tolerance in a terrestrial worm. *Ecology and Evolution*, *8*(7), 3774–3786.

Didden, W. A. M. (1990). Involvement of Enchytraeidae (Oligochaeta) in soil structure evolution in agricultural fields. *Biology and Fertility of Soils*, *9*(2), 152–158. https://doi.org/10.1007/BF00335799

Dorit, R. L., & Ohara, O. (1992). cDNA Amplification Using One-Sided (Anchored) PCR. *Current Protocols in Molecular Biology*, *17*(1), 15.6.1-15.6.10. https://doi.org/10.1002/0471142727.mb1506s17

Dózsa-Farkas, K. (1978). Die ōkologische Bedeutung des Mikrohabitates für das Vorkommen einiger Enchytraeiden-Arten. *Pedobiologia*, *18*(5–6), 366–372.

Edgar, R. C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, *32*(5), 1792–1797. https://doi.org/10.1093/nar/gkh340

Erséus, C., & Gustafsson, D. (2009). Cryptic speciation in clitellate model organisms. *Annelids in modern biology*, 31–46.

Erséus, C., Klinth, M. J., Rota, E., De Wit, P., Gustafsson, D. R., & Martinsson, S. (2019). The popular model annelid *Enchytraeus albidus* is only one species in a complex of seashore white worms (Clitellata, Enchytraeidae). *Organisms Diversity & Evolution*, *19*(2), 105–133. https://doi.org/10.1007/s13127-019-00402-6

Erséus, C., Williams, B. W., Horn, K. M., Halanych, K. M., Santos, S. R., James, S. W., Creuzé des Châtelliers, M., & Anderson, F. E. (2020). Phylogenomic analyses reveal a Palaeozoic radiation and support a freshwater origin for clitellate annelids. *Zoologica Scripta*, *49*(5), 614–640. https://doi.org/10.1111/zsc.12426

Fairchild, E. A., Bergman, A. M., & Trushenski, J. T. (2017). Production and nutritional composition of white worms *Enchytraeus albidus* fed different low-cost feeds. *Aquaculture*, *481*, 16–24. https://doi.org/10.1016/j.aquaculture.2017.08.019

Gelder, S. R. (1984). Diet and histophysiology of the alimentary canal of *Lumbricillus lineatus* (Oligochaeta, Enchytraeidae). *Hydrobiologia*, *115*, 71–81.

Giere, O. (1975). Population structure, food relations and ecological role of marine oligochaetes, with special reference to meiobenthic species. *Marine Biology*, *31*(2), 139–156.

Hebert, P. D. N., Cywinska, A., Ball, S. L., & deWaard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *270*(1512), 313–321. https://doi.org/10.1098/rspb.2002.2218

38:1891429197

Herrera, L. M., García-Laviña, C. X., Marizcurrena, J. J., Volonterio, O., De León, R. P., & Castro-Sowinski, S. (2017). Hydrolytic enzyme-producing microbes in the Antarctic oligochaete *Grania* sp. (Annelida). *Polar Biology*, *40*(4), 947–953. https://doi.org/10.1007/s00300-016-2012-0

Holmstrup, M. E., Gadeberg, S. F., Engell-Sørensen, K., Slotsbo, S., & Holmstrup, M. (2022). A new strategy in rearing of European flounder: Using live *Enchytraeus albidus* to enhance juvenile growth. *Journal of Insects as Food and Feed*, *8*(11), 1333–1341.

Illig, J., Langel, R., Norton, R. A., Scheu, S., & Maraun, M. (2005). Where are the decomposers? Uncovering the soil food web of a tropical montane rain forest in southern Ecuador using stable isotopes (^{15}N). *Journal of Tropical Ecology*, *21*(5), 589–593. https://doi.org/10.1017/S0266467405002646

Kanehisa, M., Sato, Y., & Morishima, K. (2016). BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences. *Journal of Molecular Biology*, *428*(4), 726–731. https://doi.org/10.1016/j.jmb.2015.11.006

Kasprzak, K. (1986). Skąposzczety wodne i glebowe. 2, Rodzina: Wazonkowce (Enchytraeidae). Polska Akademia Nauk Instytut Zoologii, PWN, Warszawa, 366 pp.

Katoh, K., Rozewicki, J., & Yamada, K. D. (2019). MAFFT online service: Multiple sequence alignment, interactive sequence choice and visualization. *Briefings in Bioinformatics*, *20*(4), 1160–1166. https://doi.org/10.1093/bib/bbx108

Korobushkin, D. I., Guseva, P. A., Gongalsky, K. B., Saifutdinov, R. A., Zaitsev, A. S., & Degtyarev, M. I. (2024). Are there different trophic niches of enchytraeids? A stable isotopic ($\delta^{13}C$, $\delta^{15}N$) evidence. *Soil Biology and Biochemistry*, *194*, 109422. https://doi.org/10.1016/j.soilbio.2024.109422

Kovačević, M., Stjepanović, N., Hackenberger, D. K., Lončarić, Ž., & Hackenberger, B. K. (2022). Toxicity of fungicide azoxystrobin to *Enchytraeus albidus*: Differences between the active ingredient and formulated product. *Pesticide Biochemistry and Physiology*, *187*, 105198. https://doi.org/10.1016/j.pestbp.2022.105198

Krištůfek, V., Fischer, S., Bührmann, J., Zeltins, A., & Schrempf, H. (1999). In situ monitoring of chitin degradation by *Streptomyces lividans* pCHIO12 within *Enchytraeus crypticus* (Oligochaeta) feeding on *Aspergillus proliferans*. *FEMS Microbiology Ecology*, *28*(1), 41–48. https://doi.org/10.1111/j.1574-6941.1999.tb00559.x

Lee, J., Klinth, M. J., & Jung, J. (2019). Two species of *Lumbricillus* (Enchytraeidae, Annelida) new to Antarctica. *Polar Research*.

Lemoine, F., Correia, D., Lefort, V., Doppelt-Azeroual, O., Mareuil, F., Cohen-Boulakia, S., & Gascuel, O. (2019). NGPhylogeny.fr: New generation phylogenetic services for non-specialists. *Nucleic Acids Research*, *47*(W1), W260–W265. https://doi.org/10.1093/nar/gkz303

Letunic, I., & Bork, P. (2007). Interactive Tree Of Life (iTOL): An online tool for phylogenetic tree display and annotation. *Bioinformatics (Oxford, England)*, *23*(1), 127–128. https://doi.org/10.1093/bioinformatics/btl529

Łuczak, J. (1956). Zagadnienie konkurencji międzygatunkowej w badaniach kierunku eksperymentalnego w ekologii. *Ekologia Polska. Seria B*, *2*(3), 183–197.

39:9056752368

Mandl, C. W., Heinz, F. X., Puchhammer-Stöckl, E., & Kunz, C. (1991). Sequencing the termini of capped viral RNA by 5’-3’ligation and PCR. *Biotechniques*, *10*(4), 484, 486–484, 486.

Maraun, M., Thomas, T., Fast, E., Treibert, N., Caruso, T., Schaefer, I., Lu, J.-Z., & Scheu, S. (2023). New perspectives on soil animal trophic ecology through the lens of C and N stable isotope ratios of oribatid mites. *Soil Biology and Biochemistry*, *177*, 108890. https://doi.org/10.1016/j.soilbio.2022.108890

Martín-Durán, J. M., Vellutini, B. C., Marlétaz, F., Cetrangolo, V., Cvetesic, N., Thiel, D., Henriet, S., Grau-Bové, X., Carrillo-Baltodano, A. M., Gu, W., Kerbl, A., Marquez, Y., Bekkouche, N., Chourrout, D., Gómez-Skarmeta, J. L., Irimia, M., Lenhard, B., Worsaae, K., & Hejnol, A. (2020). Conservative route to genome compaction in a miniature annelid. *Nature Ecology & Evolution*, *5*(2), 231–242. https://doi.org/10.1038/s41559-020-01327-6

Martinsson, S., & Erséus, C. (2014). Cryptic diversity in the well-studied terrestrial worm *Cognettia sphagnetorum* (Clitellata: Enchytraeidae). *Pedobiologia*, *57*(1), 27–35. https://doi.org/10.1016/j.pedobi.2013.09.006

Mothes-Wagner, U., Reichert, A., & Seitz, K. A. (1996). Functional histology of the enchytraeid *Enchytraeus coronatus* (Oligochaeta) digestive epithelium. *Pedobiologia*, *40*(4), 328–341. https://doi.org/10.1016/S0031-4056(24)00338-X

Nagy, H., Dózsa-Farkas, K., & Felföldi, T. (2023). New insights into the *Enchytraeus albidus* complex (Annelida, Enchytraeidae), with the description of three new species from seashores in Italy and Croatia. *European Journal of Taxonomy*, *870*. https://doi.org/10.5852/ejt.2023.870.2123

Nielsen, U. N., Osler, G. H. R., Campbell, C. D., Neilson, R., Burslem, D. F. R. P., & Van Der Wal, R. (2010). The enigma of soil animal species diversity revisited: The role of small-scale heterogeneity. *PLoS ONE*, *5*(7), e11567. https://doi.org/10.1371/journal.pone.0011567

Ohara, O., Dorit, R. L., & Gilbert, W. (1989). One-sided polymerase chain reaction: The amplification of cDNA. *Proceedings of the National Academy of Sciences*, *86*(15), 5673–5677. https://doi.org/10.1073/pnas.86.15.5673

Persson, L., Diehl, S., Johansson, L., Andersson, G., & Hamrin, S. F. (1992). Trophic interactions in temperate lake ecosystems: A test of food chain theory. *The American Naturalist*, *140*(1), 59–84. https://doi.org/10.1086/285403

Potapov, A. M., Beaulieu, F., Birkhofer, K., Bluhm, S. L., Degtyarev, M. I., Devetter, M., Goncharov, A. A., Gongalsky, K. B., Klarner, B., Korobushkin, D. I., Liebke, D. F., Maraun, M., Mc Donnell, R. J., Pollierer, M. M., Schaefer, I., Shrubovych, J., Semenyuk, I. I., Sendra, A., Tuma, J., … Scheu, S. (2022). Feeding habits and multifunctional classification of soil-associated consumers from protists to vertebrates. *Biological Reviews*, *97*(3), 1057–1117. https://doi.org/10.1111/brv.12832

Potapov, A. M., Pollierer, M. M., Salmon, S., Šustr, V., & Chen, T. (2021). Multidimensional trophic niche revealed by complementary approaches: Gut content, digestive enzymes, fatty acids and stable isotopes in Collembola. *Journal of Animal Ecology*, *90*(8), 1919–1933. https://doi.org/10.1111/1365-2656.13511

40:7665696969

Prantoni, A., Lana, P. C., & Erséus, C. (2017). Global checklist of species of *Grania* (Clitellata: Enchytraeidae) with remarks on their geographic distribution. *European Journal of Taxonomy*, *391*. https://doi.org/10.5852/ejt.2017.391

Puppe, D., Schrader, S., Giesemann, A., & Gebauer, G. (2012). Isotopic labelling of enchytraeids under FACE conditions: A possible way to analyse the residue-enchytraeidsoil system considering elevated atmospheric CO_2 concentrations. Landbauforschung − vTI Agriculture and Forestry Research 357, 21–26.

Purschke, G. (2003). Is *Hrabeiella periglandulata* (Annelida, „Polychaeta") the sister group of Clitellata? Evidence from an ultrastructural analysis of the dorsal pharynx in *H. periglandulata* and *Enchytraeus minutus* (Annelida, Clitellata). *Zoomorphology*, *122*(2), 55–66. https://doi.org/10.1007/s00435-002-0069-5

Rakus, K., Wiegertjes, G., Adamek, M., Bekh, V., Stet, R., & Irnazarow, I. (2008). Application of PCR-RF-SSCP to study major histocompatibility class II B polymorphism in common carp (*Cyprinus carpio* L.). *Fish & Shellfish Immunology*, *24*(6), 734–744. https://doi.org/10.1016/j.fsi.2007.11.015

Räty, M., & Huhta, V. (2003). Earthworms and pH affect communities of nematodes and enchytraeids in forest soil. *Biology and Fertility of Soils*, *38*(1), 52–58. https://doi.org/10.1007/s00374-003-0614-5

Reichert, A., Mothes-Wagner, U., & Seitz, K.-A. (1996). Ecohistological investigation of the feeding behaviour of the enchytraeid *Enchytraeus coronatus* (Annelida, Oligochaeta). *Pedobiologia*, *40*(2), 118–133. https://doi.org/10.1016/S0031-4056(24)00346-9

Scheu, S. (2002). The soil food web: Structure and perspectives. *European Journal of Soil Biology*, *38*(1), 11–20. https://doi.org/10.1016/S1164-5563(01)01117-7

Scheu, S., & Falca, M. (2000). The soil food web of two beech forests (*Fagus sylvatica*) of contrasting humus type: Stable isotope analysis of a macro- and a mesofauna-dominated community. *Oecologia*, *123*(2), 285–296. https://doi.org/10.1007/s004420051015

Schmelz, R. M., & Collado, R. (2010). A guide to European terrestrial and freshwater species of Enchytraeidae (Oligochaeta). *Soil organisms*, *82*(1), 1-176-1–176.

Schmelz, R. M., & Collado, R. (2013). „Cejkaian tubules" in the posterior midgut of terrestrial Enchytraeidae (Oligochaeta). *Soil Organisms*, *85*(2), 113-122-113–122.

Schmelz, R. M., & Collado, R. (2015). Checklist of taxa of Enchytraeidae (Oligochaeta): An update. *Soil Organisms*, *87*(2), 149-152-149–152.

Schmelz, R. M., Niva, C. C., Römbke, J., & Collado, R. (2013). Diversity of terrestrial Enchytraeidae (Oligochaeta) in Latin America: Current knowledge and future research potential. *Applied Soil Ecology*, *69*, 13–20. https://doi.org/10.1016/j.apsoil.2012.12.012

Schmelz, R. M., & Westheide, W. (2000). Ultrastructure of oesophageal appendages ("peptonephridia") in enchytraeids (Annelida: Clitellata). *Invertebrate Biology*, *119*(1), 94–103. https://doi.org/10.1111/j.1744-7410.2000.tb00177.x

Schmidt, O., Curry, J. P., Dyckmans, J., Rota, E., & Scrimgeour, C. M. (2004). Dual stable isotope analysis ($\delta^{13}C$ and $\delta^{15}N$) of soil invertebrates and their food sources. *Pedobiologia*, *48*(2), 171–180. https://doi.org/10.1016/j.pedobi.2003.12.003

41:9921142223

Sela, I., Ashkenazy, H., Katoh, K., & Pupko, T. (2015). GUIDANCE2: Accurate detection of unreliable alignment regions accounting for the uncertainty of multiple parameters. *Nucleic Acids Research*, *43*, W7–W14. https://doi.org/10.1093/nar/gkv318

Shain, D. H., Mason, T. A., Farrell, A. H., & Michalewicz, L. A. (2001). Distribution and behavior of ice worms (*Mesenchytraeus solifugus*) in south-central Alaska. *Canadian Journal of Zoology*, *79*(10), 1813–1821. https://doi.org/10.1139/z01-143

Simmons, M. P., & Norton, A. P. (2014). Divergent maximum-likelihood-branch-support values for polytomies. *Molecular Phylogenetics and Evolution*, *73*, 87–96. https://doi.org/10.1016/j.ympev.2014.01.018

Springett, J. A. (1967). *An ecological study of moorland Enchytraeidae*. Durham University. Durham (Ph.D. thesis)..

Struck, T. H., Golombek, A., Weigert, A., Franke, F. A., Westheide, W., Purschke, G., Bleidorn, C., & Halanych, K. M. (2015). The evolution of annelids reveals two adaptive routes to the interstitial Realm. *Current Biology*, *25*(15), 1993–1999. https://doi.org/10.1016/j.cub.2015.06.007

Topoliantz, S. (2000). Earthworm and enchytraeid activity under different arable farming systems, as exemplified by biogenic structures. *Plant and Soil*, *225*(1/2), 39–51. https://doi.org/10.1023/A:1026537632468

Torii, T. (2015). Descriptions of two new and one newly recorded enchytraeid species (Clitellata, Enchytraeidae) from the Ozegahara Mire, a heavy snowfall highmoor in Central Japan. *Zootaxa*, *4000*(4), 473. https://doi.org/10.11646/zootaxa.4000.4.6

Törönen, P., & Holm, L. (2022). PANNZER — A practical tool for protein function prediction. *Protein Science*, *31*(1), 118–128. https://doi.org/10.1002/pro.4193

Trifinopoulos, J., Nguyen, L.-T., von Haeseler, A., & Minh, B. Q. (2016). W-IQ-TREE: A fast online phylogenetic tool for maximum likelihood analysis. *Nucleic Acids Research*, *44*(W1), W232–W235. https://doi.org/10.1093/nar/gkw256

Ude, J. (1975). Elektronenmikroskopisch-enzymhistochemische Untersuchungen zum Sekretionsprozeß in den Septaldrüsenzellen von *Pachydrilus lineatus*. *Acta Histochemica, Supplementband*, *15*: 177–186.

Udekem, J. d'. (1855). Nouvelles classification des Annélides sétigères abranches. *Bulletins de l'Académie royale des sciences, des lettres et des beaux-arts de Belgique*, 22(2): 533–557.

Urbisz, A. Z., Chajec, Ł., Brąszewska-Zalewska, A., Kubrakiewicz, J., & Świątek, P. (2017). Ovaries of the white worm (*Enchytraeus albidus*, Annelida, Clitellata) are composed of 16-celled meroistic germ-line cysts. *Developmental Biology*, *426*(1), 28–42. https://doi.org/10.1016/j.ydbio.2017.04.009

Urbisz, A. Z., Chajec, Ł., Małota, K., Student, S., Sawadro, M. K., Śliwińska, M. A., & Świątek, P. (2022). All for one: Changes in mitochondrial morphology and activity during syncytial oogenesis. Biology of Reproduction, 106(6), 1232–1253. https://doi.org/10.1093/biolre/ioac035

Vandecraen, J., Monsieurs, P., Mergeay, M., Leys, N., Aertsen, A., & Van Houdt, R. (2016). Zinc-induced transposition of insertion sequence elements contributes to increased adaptability of *Cupriavidus metallidurans*. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.00359

42:7804419122

Vander Zanden, M. J., Shuter, B. J., Lester, N., & Rasmussen, J. B. (1999). Patterns of food chain length in lakes: a stable isotope study. *The American Naturalist*, *154*(4), 406–416. https://doi.org/10.1086/303250

Varute, A. T., & More, N. K. (1973). Lysosomal acid hydrolases in the chloragogen cells of earthworms. *Comparative Biochemistry and Physiology Part A: Physiology*, *45*(2), 607–635. https://doi.org/10.1016/0300-9629(73)90470-2

Walsh, M. L., Fairchild, E. A., Rennels, N., Howell, W. H., Mercaldo-Allen, R., & Kuropat, C. (2015). The effects of live and artificial diets on feeding performance of cultured winter flounder, *Pseudopleuronectes americanus*, in the wild: survival, feeding, growth, and nucleic acid analyses. *Journal of the World Aquaculture Society*, *46*(5), 461–474. https://doi.org/10.1111/jwas.12210

Weigert, A., Helm, C., Meyer, M., Nickel, B., Arendt, D., Hausdorf, B., Santos, S. R., Halanych, K. M., Purschke, G., Bleidorn, C., & Struck, T. H. (2014). Illuminating the base of the annelid tree using transcriptomics. *Molecular Biology and Evolution*, *31*(6), 1391–1401. https://doi.org/10.1093/molbev/msu080

Wolters, V. (1988). Effects of *Mesenchytraeus glandulosus* (Oligochaeta, Enchytraeidae) on decomposition processes. *Pedobiologia*, *32*(5/6), 387–398. https://doi.org/10.1016/S0031-4056(23)00257-3

Yu, Y.-S., Lee, J.-Y., Woo, J.-W., Kim, J.-S., Bae, Y.-H., Cho, S.-J., & Park, S. C. (2019). Identification and expression pattern of a new digestive invertebrate-type lysozyme from the earthworm. *Genes & Genomics*, *41*(3), 367–371. https://doi.org/10.1007/s13258-018-0776-z

43:8872949073

II. PUBLIKACJE WCHODZĄCE W SKŁAD ROZPRAWY

[1] Gajda, Ł.; Gorgoń, S.; Urbisz, A.Z. 2017. Food Preferences of Enchytraeids. Pedobiologia, 63, 19–36. https://doi.org/10.1016/j.pedobi.2017.06.002

[2] Gajda, Ł.; Daszkowska-Golec, A.; Świątek, P. 2024a. Discovery and characterization of the α-amylases cDNAs from *Enchytraeus albidus* shed light on the Evolution of "Enchytraeus-Eisenia type" Amy homologs in Annelida. Biochimie, 221, 38–59. https://doi.org/10.1016/j.biochi.2024.01.008

[3] Gajda, Ł.; Daszkowska-Golec, A.; Świątek, P. 2024b. Trophic position of the white worm (*Enchytraeus albidus*) in the Context of Digestive Enzyme Genes Revealed by Transcriptomics Analysis. Int. J. Mol. Sci., 25, 4685. https://doi.org/10.3390/ijms25094685

44:4382760483

Pedobiologia - Journal of Soil Ecology 63 (2017) 19–36

Contents lists available at ScienceDirect

Pedobiologia - Journal of Soil Ecology

journal homepage: www.elsevier.com/locate/pedobi

Review

Food preferences of enchytraeids

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A R T I C L E I N F O

Keywords:
Potworms
Primary decomposers
Secondary decomposers
Nutrient source
Cellulase
Plant pests

A B S T R A C T

The aim of this review is to evaluate the food sources for enchytraeids and to contribute to the current knowledge of their feeding preferences. Several food sources have been described in detail: (1) plant material, (2) macroalgae, (3) animal remains, (4) feces of invertebrates, (5) bacteria, (6) fungi, (7) microalgae, (8) nematodes, and (9) locust eggs. The present article considers selective ingestion, digestion, and factors affecting enchytraeid food selection. We also provide basic information on enzymatic activity, particularly cellulolytic capability. Recent findings on *Cognettia sphagnetorum*, which have shown that several cryptic species were hidden under this single species name, shed new light on the somewhat inconsistent data presented in the literature over a long time. Apart from that, the recurrent issue of enchytraeids as plant pests is re-discussed here. This unsupported assumption is still the subject of general agricultural textbooks and requires further clarification. Contrary to old hypotheses, potworms are not plant parasites, and they have little or no cellulolytic capability. Enchytraeids can be divided into two groups: (1) primary decomposers and (2) secondary decomposers/sapro-microphytophages. There is also some evidence, albeit weak, that some species prey on nematodes. So far, the trophic preferences of only a few enchytraeid species have been studied in detail, but it is evident that several feeding strategies exist within the family. These studies include direct observation of feeding behavior, gut content analyses, enzymatic analyses, cultivation on different nutrient sources, choice tests, various types of soil microcosms, litter bags, and isotopic techniques. Molecular methods have been used only occasionally, although they could largely support further necessary research on potworm feeding ecology.

1. Introduction

Enchytraeids, which are also known as potworms, are a widely distributed group of small- to medium-sized earthworm-like animals (Annelida: Clitellata) (Erséus et al., 2010). Their distribution ranges from the tropics to the polar regions (Didden, 1993). The family Enchytraeidae includes almost 700 species, most of which are terrestrial (Erséus et al., 2010). Enchytraeids occupy a variety of microhabitats, such as soil, compost, mosses, decayed wood, intertidal sands along the seashores, and fine sediments in the deep sea (Boros, 2010; Erséus et al., 2010). They can also be found in snow environments (Shain et al., 2001; Torii, 2015). The extremely specialized ice worms *Mesenchytraeus solifugus* and *Sinenchytraeus glacialis* are the only known annelids that spend their entire life cycle in the glacier ice and snow (Hartzell and Shain, 2009). However, in general, enchytraeids prefer sites rich in organic matter (Wolters, 1988). Their abundance in some soil habitats can be high, reaching more than 300 000 individuals per square meter in samples (Bardgett and van der Putten, 2014), which implies that they play an important role in soil processes (Didden, 1993; Briones et al., 1998). Despite this, detailed and reliable studies about the food preferences of these animals, especially for the aquatic species, are scarce. So far, there are only a few reviews containing some sections dedicated to the food preferences of Enchytraeidae. Stephenson (1930) discussing the trophic biology of annelids, evaluated the reports of enchytraeids as plant parasites. O'Connor (1967) summarized the feeding habits of the enchytraeids, but many more studies have been performed since then. Giere and Pfannkuche (1982) compiled the scattered data on the biology and ecology of marine clitellates and very briefly discussed the trophic spectrum of selected groups, including potworms. However, due to the lack of records, marine and brackish-water species were only marginally characterized. Dash (1983), in his “Biology of Enchytraeidae”, briefly recounted what was known about feeding ecology, but his scope was too broad to include many details on trophic biology. Didden (1993) wrote a comprehensive review on the ecology of terrestrial Enchytraeidae. He summarized the results of several authors and discussed feeding, conclusively pointing to microorganisms as an important part of enchytraeid diet. However, he did not discuss the nutritional contribution of animal remains, nematodes, or macroalgae,

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http://dx.doi.org/10.1016/j.pedobi.2017.06.002
Received 17 January 2017; Received in revised form 7 April 2017; Accepted 16 June 2017
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and investigators still lack in-depth insight into the fundamental traits of enchytraeids. Over the past 20 years, the development of new techniques and tools and the conduction of further studies allowed a better understanding of this issue. However, much of the relevant information is still scattered in the literature, including rather old and hard-to-reach papers. Some of the observations from more than 50 years ago remain questionable and a part of the information appears to be contradictory, possibly because of the existence of previously unrecognized species complexes within Enchytraeidae. For example, *Cognettia sphagnetorum* was a widely used model organism in soil biology until a recent molecular study revealed that, in fact, *C. sphagnetorum* includes at least five cryptic species (Martinsson and Erséus, 2014). Also, a nomenclatorial problem (rules of priority) regarding the genus *Cognettia* has been noticed by Schmelz and Collado (2010). After taxonomic revision, *Cognettia sphagnetorum* has been transferred to the *Chamaedrilus sphagnetorum* complex (Martinsson et al., 2015). However, the authors of the present article use the old binomial name due to the impossibility to verify the species used in the cited papers.

Enchytraeids are considered to be mainly saprophagous (Schlaghamerský and Krawczynski, 2015) or, more rarely, microphytophagous (i.e. microbivorous) (Didden, 1993). Enchytraeids cannot separate the nutritious components from the soil matrix, therefore, they ingest food along with mineral particles (O'Connor, 1967; Gelder, 1984; Reichert et al., 1996; Haimi and Siira-Pietikäinen, 2003) (Fig. 1). It is widely held that potworms digest a mixture of partially decomposed plant residues (Dash, 1983) and therefore play an important role in the turnover of soil nutrients (Maraldo et al., 2011). However, this issue is more complex than it seems because different representatives of Enchytraeidae have different food requirements (Dash, 1983). Some species prefer fungi in their diet (Hedlund and Augustsson, 1995) and others feed on bacteria (Krištůfek et al., 1995). The determination of the trophic position of enchytraeid species is problematic (Briones and Ineson, 2002) and without knowing the relative contributions of each food type to enchytraeid diet, we will not be able to define the correct trophic position of this group.

This paper reviews the current state of knowledge of enchytraeid feeding preferences in the context of potential food sources. Because much of the relevant information is scattered throughout the literature and difficult to obtain, we included a large number of details, sometimes even in an extensive manner. To address the important question of potworm classification within the decomposer system (i.e., primary or secondary decomposers), we divided the discussed food sources into two groups: dead organic matter and live material. We believe that this will help to systematize the knowledge; however, a clear-cut distinction between these two groups was sometimes impossible and solely based on literature indications.

2. Dead organic matter

2.1. Plant material - feeding selectivity in relation to plant remains

Enchytraeids are an invertebrate group which plays a prominent role in the decomposition of plant material (Ponge, 1991). In several cases, gut content analysis of Enchytraeidae revealed the presence of dead plant matter in various stages of decay (O'Connor, 1967; Dash and Cragg, 1972; Anderson, 1975; Springett and Latter, 1977; Standen and Latter, 1977; Standen, 1978; Latter and Howson, 1978; Toutain et al., 1982; Ponge, 1991, 2010; Haimi and Siira-Pietikäinen, 2003; Vaçulik et al., 2004). Several studies have assessed the feeding behavior of enchytraeids on leaf litter (Dózsa-Farkas, 1976, 1978b, 1982; Standen and Latter, 1977; Toutain et al., 1982). Based on these results, potworms prefer older leaves over freshly fallen leaves (Dózsa-Farkas, 1978b; Standen and Latter, 1977; Ponge, 1991). Some types of leaves can be suitable for Enchytraeidae shortly after falling, while others must pass the winter to become an adequate food source (Dózsa-Farkas, 1976, 1978b). Different species of Enchytraeidae may have different preferences regarding the origin of leaves (Dózsa-Farkas, 1976, 1982). Interestingly, these differences can affect not only specimens belonging to distinct genera (e.g. *Henlea*, *Fridericia*) (Dózsa-Farkas, 1976), but also different species within the same genus (e.g. *Fridericia galba*, *F. ratzeli*) living in the same environment (Dózsa-Farkas, 1982). An important question, that remains to be addressed, is precisely what factors makes a leaf attractive for Enchytraeidae (Dózsa-Farkas, 1976). Is it the leaf itself or the microorganisms that colonize its surface? Standen and Latter (1977) suggested that the initial attack by microorganisms is an essential part in the conditioning of some plant material as food for enchytraeids. In a feeding experiment, *Rubus* leaves had to be colonized by the fungus *Marasmius* before they were favored (Standen and Latter, 1977). Ponge (1991, 2010) found that *Cognettia* spp. ignored dead but uncolonized moss leaves in the L_2 layer, but leaves in the F_1 layer colonized by fungi were consumed. As the litter ages, enchytraeids ingest an increasing proportion of leaf material (Anderson, 1975), which could be related to physicochemical changes in the decomposing leaf or the presence of attractive microorganisms. Standen and Latter (1977) found that *C. sphagnetorum* individuals were absent on the surface of fresh litter (with the exception of a high number on the upper layers of some *Sphagnum* samples) and present in higher numbers at lower depths as well as in the later stages of decomposing *Rubus*, *Eriophorum*, and *Calluna*. They were absent on six-month-old litter but appeared and increased in numbers between two and four years in *Rubus*, two to five years in *Eriophorum*, and three to five years in *Calluna*. According to Standen and Latter (1977), the absence of enchytraeid worms on the

Fig. 1. *Enchytraeus albidus* (live specimen). Light micrograph of the middle part of the body. Most of the space within the body cavity is occupied by the gut (G). Soil particles (arrowheads) and plant residue (P) are visible in its lumen. BW − body wall.

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surface of examined samples was presumably due to physical characteristics of the litter and low moisture content, because individuals grew on these types of litter in the feeding tests when the food was chopped and kept moist. Potworms prefer soft, moist plant material with a high nitrogen content, a relatively low ratio of carbon to nitrogen, as well as lower concentrations of humic acids, tannins, and lignins (Dózsa-Farkas, 1976; Latter and Howson, 1978; Cochran et al., 1994). Toutain et al. (1982) demonstrated that *Fridericia striata* feeding on the fallen aspen leaves avoided the veins (which contained fewer microbes, although this may have been related to the hardness of the material) and ingested only pieces of the epidermis and parenchyma. Leaves of *Sambucus nigra*, which have an extremely rapid decomposition rate (Atkinson and Atkinson, 2002), were preferred by *Fridericia hegemon* (Dózsa-Farkas, 1976). In a similar study by Reichert et al. (1996), *Enchytraeus coronatus* used air-dried leaves of *S. nigra* collected in late summer. The authors observed that small pieces (1 cm^2) of these leaves showed signs of damage to the margins within five days. After seven days, only the remains of the veins were visible and after nine days, the tissue had been almost completely consumed by the enchytraeids. Residues of undigested *S. nigra* leaf material have rarely been found in the gut and those that could be traced were partially or almost completely digested. Reichert et al. (1996) suggested that before it was ingested by *E. coronatus*, leaf tissue was externally partially digested. For these reasons, the above mentioned authors postulated significant cellulolytic activity of *E. coronatus*. However, there are serious disagreements to this interpretation (Gajda and Gorgoń, unpublished data, see Fig. 2). For example, Kühnelt (1961) claimed that enchytraeids attack plant remains by releasing a strongly alkaline secretion from the salivary glands (septal glands?). As most of the cellulases originating from animals have optimal activity at acid to neutral pH values (Sami et al., 2011; Rahman et al., 2014), based on the observation of Kühnelt (1961) and the assumption of preoral digestion, the cellulolytic activity of the secretion in an experiment performed by Reichert et al. (1996) could be disputed. Ultrastructural studies on *Fridericia striata* (Toutain et al., 1982) showed that ingested aspen leaf litter material had not changed markedly during passage through the gut, apart from some changes in the microfibrillar arrangement in cell walls that were initiated before the ingestion by the activity of soil microorganisms. Bacteria and fungi present on the surface of ingested material were digested. Similarly, Ponge (1991) did not observe any significant change in the appearance of plant cell walls, although the crushing of ingested material was pronounced in *Cognettia sphagnetorum*. Latter and Howson (1978), observed that *C. sphagnetorum* thrived on leaf litter (*Rubus*, *Eriophorum*, or *Calluna*). The plant tissues were consumed and finely comminuted, but in contrast to the observations by Ponge (1991), the cells appeared crushed and sponge-like in the feces. It has been demonstrated that large enchytraeid species can shred fallen leaves (Dózsa-Farkas, 1976, 1978a, 1978b, 1982; Zimmermann, 1976; Mellin, 1990). Microscopic observation by Mellin (1990) revealed that *Mesenchytraeus glandulosus* was able to break leaves into pieces through actions of the prostomium, the peristomium, and the pharynx as well as the corresponding muscles; similar behavior was found for *Enchytraeus albidus* (Zimmermann, 1976) (Fig. 3). It seems that mechanical properties of the plant material can determine its nutritive value and palatability for Enchytraeidae. Brockmeyer et al. (1990) reported that *Enchytraeus christenseni* (syn. *Enchytraeus minutus*) and *Enchytraeus* cf. *globuliferus* assimilated nutrients to a small degree from fresh spinach after cells were burst through freezing and thawing. Therefore, physical processes such as freezing or drying can affect the cell wall integrity of plant material and have indirect impacts on nutrient uptake. On the other hand, Lindfeld et al. (2011) could not keep *E. albidus* on finely ground senescent wheat leaves without 33% supplementation of the oat flake powder, an easily assimilable source of food. Also, Puppe et al. (2012) recorded the reduction of the initial density of enchytraeids fed with air-dried ground barley straw in all treatments at the end of the experiment. Thus, both the mechanical properties, and the origin of plant-derived food are important factors in enchytraeid feeding; a fact that should be considered in feeding studies.

Enchytraeids are selective in the consumption of plant remains and their preferences can vary among species (Dózsa-Farkas, 1976, 1978b, 1982; Standen and Latter, 1977; Standen, 1978; Latter and Howson, 1978). However, under natural conditions enchytraeids appear to be supposedly non-discriminatory feeders (see Ponge, 1991), because they ingest a wide variety of food. The attractiveness of dead plant material to enchytraeids is influenced by both biotic and abiotic factors. The most explicit effect in conditioning of plant remains can be attributed to bacteria and fungi. Microorganisms act as external softeners of the refractory material, which makes it acceptable food for enchytraeids.

2.2. Plant material – direct or indirect food sources

It is difficult to estimate to what extent plant remains are direct food sources for Enchytraeidae. In ecological studies, some authors (Persson et al., 1980; Lagerlöf et al., 1989) have considered enchytraeids to be 50% saprophagous and 50% microphytophagous, while others (Whitfield, 1977; MacLean, 1980) have considered them to be only 20% saprophagous and 80% microphytophagous. Didden (1993) favored the latter view. In a litter-bag study conducted to estimate the impact of potworms on the decomposition of plant matter, Lagerlöf and Andrén (1985) calculated that enchytraeid respiration is responsible for the loss of 0.8% of carbon from barley straw within two years. In a similar study, Lagerlöf et al. (1989) estimated that Enchytraeidae ingested 3–12% organic matter input, which corresponded to 16–34 g of dry mass m^{-2} $year^{-1}$. Mellin (1990) demonstrated that *Mesenchytraeus glandulosus* can ingest a leaf mass equal to 10% of the individual body dry weight per day, while Wolters (1988) found that the effect of this species on litter decomposition depends upon the density, temperature, and growth conditions of the microflora. Some studies show a positive correlation between the number of enchytraeids and plowing (Hendrix et al., 1986; Lagerlöf et al., 1989; House and Parmelee, 1985; Manetti et al., 2010; Severon et al., 2012). Additionally, increasing the amount of plant remains had no effect on the number of enchytraeids in unplowed fields, while the number of enchytraeids increased in plowed fields (Severon et al., 2012). The positive effect of plowing on enchytraeids could be the result of increased comminution of plant residues and the enhanced colonization and conditioning of the material by bacteria (Hendrix et al., 1986). In contrast, there are also studies indicating no changes or even decreases in the number of enchytraeids in plowed fields (Röhrig et al., 1998; Parmelee et al., 1990; Vavoulidou et al., 1999; van Capelle et al., 2012). The differences could be a consequence of the different time scale of studies (but see Manetti et al., 2010) or the food-web shifts caused by environmental factors such as climate or soil type (Beylich et al., 2015). For example, Cole et al. (2002) showed that soil warming reduced the functional role of enchytraeids with respect to carbon mineralization. It should be mentioned that Bengtsson et al. (1997) observed that doubling the amount of forest logging residues in a Scots pine (*Pinus sylvestris*) stand did not affect the number of enchytraeids, although it is possible that enchytraeids cannot effectively decompose this type of plant residues, even with the support of microorganisms. Alternatively, microorganisms not heavily engaged in decomposition of this kind of material, or other plant remains are the main food sources (see Nieminen, 2009).

The experiment using labeled plant material (^{13}C and ^{15}N) showed that *E. crypticus* and *E. buchholzi* partly fed on air-dried ground barley straw (Puppe et al., 2012). Most studies using stable isotope techniques to evaluate the role of invertebrates in plant decomposition are unable to ascertain whether an intermediary microbial step is involved (Crotty et al., 2011). The analysis of the isotopic signature of microbial PLFAs (phospholipid fatty acids) can provide evidence that microorganisms decompose and assimilate compounds derived from specific plant matter, which potentially allows the construction of a food-web using lipid biomarkers (Watzinger, 2015). It has been suggested that

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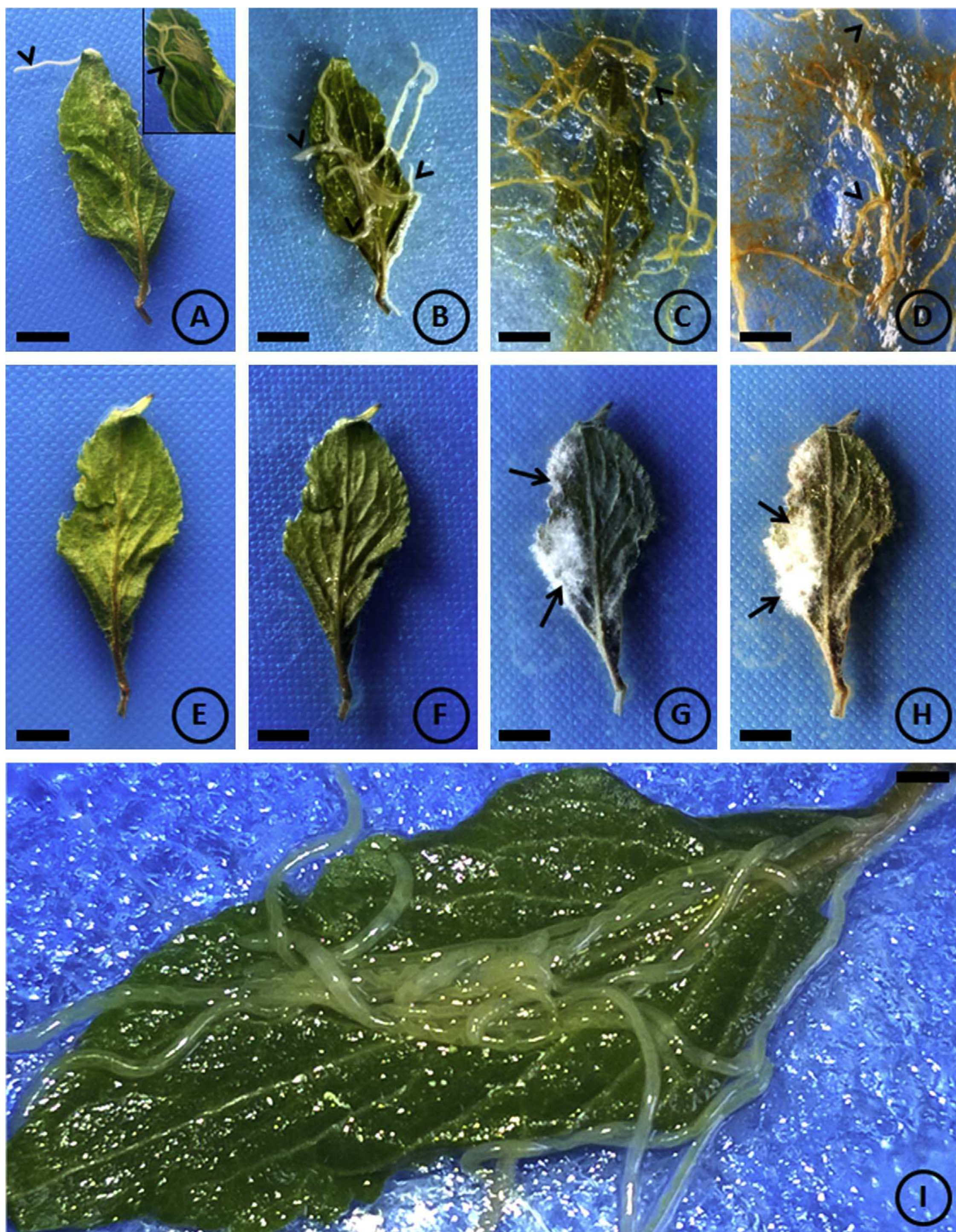

Fig. 2. *Enchytraeus albidus* with *Sambucus nigra* leaf in 0.8% non-nutrient agar in tap water. **Experimental plate:** 15 individuals were starved for two weeks, rinsed three times in distilled water, transferred to a new plate with a freshly-dried *S. nigra* leaf (40 °C, 25 min), and kept at room temperature. The leaf was attractive in the short time (day 0) (A) to enchytraeids, which densely aggregated beneath the material (small box in A: bottom view). After one day (B), individuals were seen crawling on the leaf surface (B, I). In the subsequent two days, the leaf showed signs of damage (not shown). No evident microbial growth was noticed. After four days (C), more apparent damage to the leaf lamina was observed. After five days (D), almost the whole leaf was disintegrated, with the exception of the petiole and the colorless midrib (which is difficult to distinguish and was identified using a preparation needle). Animal guts filled with the material were macroscopically noticeable. **Control plate:** A freshly-dried *S. nigra* leaf was placed in the center of the plate and kept without animals under the same conditions as described for the experimental plate (day 0–1, E-F). In the subsequent two days, unidentified fungal hyphae grew on the leaf (not shown), which were more apparent (arrows) after four days (G). After five days (H) plant tissue, including petiole, was softened, although the integrity of the leaf was maintained. The contribution of fungal activity to the maceration of the plant material on the experimental plate cannot be excluded. Bar = 5.5 mm (A-H); Bar = 1.5 mm (I).

microorganisms, apart from acting directly as food source, can affect Enchytraeidae feeding indirectly by releasing nutrients from debris, changing physicochemical properties of plant remains and deactivating harmful substances (Latter and Howson, 1978). Recently, Larsen et al. (2016a,b) have found with the use of stable isotope fingerprints that enchytraeids from arctic peatlands derived more than 80% of their essential amino acids from symbiotic bacteria.

Analysis of the current state of knowledge shows that it is difficult to generalize plant matter feeding in enchytraeids; there are significant differences even between species of the same genus. According to Latter

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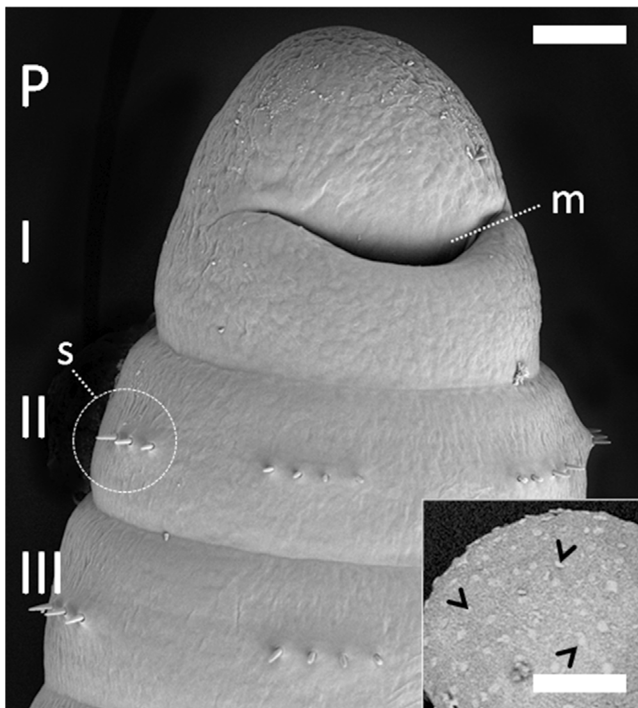

Fig. 3. Ventral view of anterior end of *Enchytraeus albidus.* Prostomium (P), and peristomium (first segment) (I) which contains the mouth opening (m), are visible. Displayed segments II and III are equipped with bundles of chaetae; a single chaetal bundle was marked (S). Scanning electron microscopy (SEM); bar = 50 μm; small box: magnification of prostomium region of other specimen with visible sensory structures (arrowheads). SEM. Bar = 40 μm.

and co-workers (1977–1978), *C. sphagnetorum*, relies heavily on plant remains, while other studies (Ponge, 1991, 2010; Cole et al., 2002; Haimi and Siira-Pietikäinen, 2003) find that it also feeds on live microorganisms, particularly fungi. However, it is not clear which of the cryptic species of the *C. sphagnetorum* complex were studied. Some enchytraeid species, such as *Mesenchytraeus glandulosus*, which is considered to be greatly engaged in litter decomposition, can also digest microbes (Wolters, 1988; Mellin, 1990). On the other hand, some litter-ingesting species, for example, *Fridericia galba* are rather "pseudosaprophages" than primary decomposers (Toutain et al., 1982). Dual stable isotope analysis (δ^{13}C and δ^{15}N) has shown that isotopic patterns of *Fridericia christeri* and *F. galba* from an arable field correspond with the geophagous group (secondary decomposers), but not with typical litter-feeding species (Schmidt et al., 2004).

Dead plant material may play a different role in the diet of various enchytraeid species. For some of them, it is the direct food (Latter, 1977; Springett and Latter, 1977; Latter and Howson, 1978), while for the others it is more of a carrier of microorganisms, which are the primary food (Toutain et al., 1982). Therefore, more than one feeding strategy exists among potworm species in relation to plant remains.

2.3. Cellulolytic capability of enchytraeids

Enchytraeids have no organs to attack living plant tissue (Schmelz et al., 2013). They ingest dead plant material; however, they have little or no cellulolytic capability. Nielsen (1962) performed a qualitative analysis of carbohydrase enzymes in 34 soil invertebrates, including four species of enchytraeids (*C. sphagnetorum*, *E. albidus*, *M. glandulosus*, and *F. hegemon*), and found no cellulolytic activity in the enchytraeids. Dash et al. (1981) studied digestive enzymes (protease, amylase, invertase, cellulase, and urease) in three tropical enchytraeid species and reported that *Hemienchytraeus khallikotosus* showed maximum enzymatic activities, and *Fridericia kalinga* and *Enchytraeus berhampurosus* showed moderate to minimum activities. According to the authors, the differences in the rate of activities in those species were probably related to the food type and rate of feeding. The worms that were investigated showed moderate cellulase activity. However, Dash et al. (1981) used carboxymethyl cellulose (CMC) in their study, but naturally occurring plant-derived cellulose is usually encrusted with hemicellulose, lignin, pectin, and a variety of other compounds. Microorganisms can break linkages within and between some of these complex substances, thus releasing the intermediates that can then be used by the soil microfauna (Dash, 1983). Because of that, the protozoans, fungi, and bacteria that are ingested along with the soil and litter must be considered an important part of the enchytraeid diet (Adl, 2008). Moreover, the homogenates of entire worms were used to investigate enzymatic activity, so it cannot be determined whether the enzymes that were active in the experiment were of animal origin only or if they had been synthesized by the intestinal microflora (Šustr and Chalupský, 1996). Urbášek and Chalupský (1991) reported a low activity of Cx-cellulase (*endo*-1,4-β-D-glucanase) and the cellulase complex (a mixture of exo- and *endo*-1,4-β-D-glucanases) in whole body homogenate of *C. sphagnetorum*, while similar activity was not found by Šustr and Chalupský (1996). The contribution of microbial cellulases cannot be excluded because the activity of the microflora can conceal the actual enzymatic activity of enchytraeids. Urbášek and Chalupský (1992) suggested that the increase of the cellulase and amylase activity in *Fridericia* sp. after soil acidification was caused by enhanced growth of amylolytic and cellulolytic microorganisms that had been ingested along with soil particles and plant debris. Liming of acidified soils decreased cellulase activity to the same level as in the control group (Urbášek and Chalupský, 1992). In contrast, Šustr et al., 1997 found that acidification of soil decreased amylolytic activity in *C. sphagnetorum*, and that raising the pH of acidified plots, by liming, was associated with increased amylolytic activity. These differences are not easy to explain in terms of the invertebrate-microbial interactions, and may be related to different changes in the pH of the substrate and the feeding preferences between the two species (Šustr et al., 1997). Seasonal variations of *C. sphagnetorum* digestive enzymes, including amylase, have been reported (Urbášek and Chalupský, 1991). Axenic cultivation of *C. sphagnetorum* in sterile leaf litter revealed that live microorganisms do not form an essential part of its diet (Latter, 1977), which suggests that at least some species in the *C. sphagnetorum* complex are able to produce cellulases themselves. However, it should be noted that the 1–2-year-old *Calluna* used in Latter's experiment was pretreated with enzymes (cellulase and pectinase, for details see: Latter, 1977; Latter and Howson, 1978), in contrast to 2–5-year-old plant material, which was naturally exposed to microbial activity longer period. The capacity to produce endogenous and functional cellulase in the gut has been shown in the earthworm *Pheretima hilgendorfi* (Nozaki et al., 2009). In the case of Enchytraeidae, further research is needed at the molecular level.

Enchytraeids are limited in their ability to use cellulose as food but are able to use some other polysaccharides, disaccharides, monosaccharides, and proteins (Nielsen, 1962; Dash et al., 1981; Urbášek and Chalupský, 1991, 1992; Šustr and Chalupský, 1996; Šustr et al., 1997). Probably most of the cellulolytic activity reported in enchytraeids is derived primarily from microflora. This conclusion can be supported by the fact that the presence of Cytophagales, a group of bacteria whose representatives are known cellulose degraders (Reichenbach, 2006), has recently been demonstrated in the enchytraeid gut with the use of molecular methods (Larsen et al., 2016b).

2.4. Macroalgae

Enchytraeids common in the seashore not only feed on decaying seagrasses, but also on thalloid algae. *Lumbricillus lineatus*, *Lumbricillus reynoldsoni*, and *Enchytraeus albidus* can be found in large numbers among decaying seaweed in shoreline tidal debris called "wrack beds" (Giere, 1980). Members of these two genera are dominant in decaying

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seaweed in the littoral zone along the sea (Christensen and Glenner, 2010). Enchytraeids are attracted to decayed algal material (Tynen, 1969; Schöne, 1971; Giere and Hauschildt, 1979; Giere and Pfannkuche, 1982), and *L. lineatus* and *E. albidus* have been observed to penetrate decomposing *Fucus* spp. (Giere, 1975). Pieces of *Ascophyllum nodosum* were ingested by *L. reynoldsoni* and algal fragments have been observed in their gut (Tynen, 1969). Gut content analysis of *Lumbricillus rivalis* from the North Sea coast of Germany revealed the preferential uptake of *Ulva* (syn. *Enteromorpha*) sp. thalli (Giere, 1975). Distinguishing fresh from decaying algal material can be difficult. Some field observations have shown that, at least occasionally, enchytraeids can ingest fresh algae (Giere, 1975; Giere and Pfannkuche, 1982). However, fresh seaweed washed up on the shore are poorly colonized and used only by some larger enchytraeids (Giere, 1975). Experiments with *L. reynoldsoni* and *Ascophyllum nodosum* showed that potworms prefer decaying algal material over fresh algae (Tynen, 1969). In an attraction test, *L. lineatus* sensed layers of decaying *Fucus* inserted into sterile sand columns from a distance of 20 cm and after 48 h, enchytraeids were densely aggregated there (Giere and Hauschildt, 1979). Researchers suggest that microorganisms play a substantial role in making the wrack beds more suitable for colonization by enchytraeids (Tynen, 1969; Giere, 1975). Microorganisms could enhance the attractiveness of the wrack and release nutrients available to the worms (Tynen, 1969), or be a direct food (Giere, 1975; Giere and Pfannkuche, 1982). However, Giere and Hauschildt (1979) successfully cultivated *L. lineatus* in cultures with rotten *Fucus* in near sterile conditions with the addition of tetracycline hydrochloride, which indicates that the main nutritive basis is the algal material and not the bacterial film on the decaying thalli. The study on *L. lineatus* also demonstrated that the attractiveness and nutritive value of algal debris depend on the decomposition state of the algal cells, the algal species, and probably its mechanical properties (Giere and Hauschildt, 1979). Backlund (1945) failed to maintain *E. albidus* fed with sterile *Fucus*; the culture died in less than one month, while it lived and reproduced in non-sterile material derived from wrack beds. Fresh thalli must partially decompose before they are accepted by this species (Schöne, 1971). Backlund (1945) reported that *E. albidus* reared on small portions of seaweed, in contrast to *Tipula* (Diptera) larvae, changed algal material into a slimy mass with high bacterial activity. Similarly, Giere and Pfannkuche (1982) found that algae such as *Fucus vesiculosus*, *Ulva* spp., and *Cladophora* sp. were converted by *L. rivalis* in laboratory cultures within a few days into a brownish, amorphous mud. Many enchytraeid species prefer *Fucus*, most probably due to its optimal microbial degradability (Giere and Hauschildt, 1979). A few experiments have demonstrated the feeding selectivity of enchytraeids for specific groups of algae (Schöne, 1971; Giere and Hauschildt, 1979; Giere and Pfannkuche, 1982). Brown algae are widely preferred, followed by green algae and the least attractive red algae, which may be refused. Schöne (1971) found that the amount of digested food, rate of reproduction, and life span were largest when *E. albidus* were fed on *Fucus* and respectively decreased on green algae (*Ulva* sp. and *Monostroma* sp.), *Zostera marina* (a vascular plant), and *Delesseria* (a red algae) diet. Similar findings were shown with *L. lineatus* (Giere and Hauschildt, 1979). It should be clarified that drifted seaweed, referred to as 'wrack' by some authors (e.g. Backlund, 1945), often include not only brown algae such as *Fucus*, *Laminaria*, or *Ascophyllum*, but many other algae and even the sea-grass *Zostera* (a vascular plant). In many older papers, there is no distinction between algal and plant material from wrack, which makes it difficult to draw conclusions, because cell walls of some macroalgae differ from cell walls of land plants (Mabeau and Kloareg, 1987). For example, brown algae contain cellulose in their cell walls, but these crystalline fibers account for only a small proportion of the cell wall, between 1 and 8% of the dry weight of the thallus (Michel et al., 2010). A study on the brown algae *Pelvetia canaliculata* and *Ascophyllum nodosum* revealed that the cell walls are composed mainly of alginates and sulphated fucans (Mabeau and Kloareg, 1987). Some green algae of the Bryopsidales group (e.g., *Bryopsis*, *Codium*, *Derbesia*) lack cellulose as the major structural cell wall polysaccharide and contain mannan instead (Fernández et al., 2012). Therefore, contrary to opinions voiced by some authors (Tynen, 1969; Palka and Spaul, 1970; Giere and Pfannkuche, 1982), enzymes other than cellulases may be mainly involved in the digestion of macroalgae by enchytraeids.

Decomposing macroalgae from wrack beds are an important source of food for enchytraeid species that occupy the intertidal zone. The nutritional value of this material differed among algal species. However, the results from experimental feeding (Schöne, 1971; Giere and Hauschildt, 1979; Giere and Pfannkuche, 1982) showed that the most attractive are brown algae. Bacterial activity plays a great role in the conditioning of algal material, but it is not as explicit as in the case of plant remains, since large enchytraeid species occasionally ingest seaweed in the early stages of decomposition, and sometimes even fresh seaweed.

2.5. Animal remains

Enchytraeids are known to gather at places with concentrations of dead organic matter not only from plants, but also of animal origin (Schlaghamerský and Krawczynski, 2015). Shore enchytraeids are occasionally attracted by carcasses washed up by the waves (Stephenson, 1930). According to O'Connor (1967), *E. albidus* assembles in large numbers in and around the bodies of dead fish and marine birds. Stephenson (1922, 1930) reported that *Lumbricillus pagenstecheri* (syn. *Lumbricillus necrophagus*) fed on the corpse of a seal washed ashore. Giere and Pfannkuche (1982) regularly encountered *Lumbricillus* spp. in the dense layers of disintegrating *Hydrobia* mud snails. Similarly, Palka and Spaul (1970) demonstrated that *L. rivalis* has an apparent preference for protein-rich decomposing material, including that of animal origin. Briones and Ineson (2002) reported Mellin's observation (unpublished data) that enchytraeids feed on dead bodies of lumbricids and arthropods. Michaelsen (1927) experimentally demonstrated that a culture of *E. albidus* reduced the body of a small dead frog to the bones. When the body started to rot, *E. albidus* worms massively accumulated close to it. The corpse slightly liquefied from below and after about five to seven days, only the fleshless bones of the frog remained; the rest had apparently been absorbed by the enchytraeids that then dispersed (Michaelsen, 1927). Kühnelt (1961) claimed that potworms liquefy the flesh of dead animals and suck up the resulting pulp. However, O'Connor (1967) strongly disagreed with this statement, indicating that it is equally possible that they rely upon saprophytic bacteria to perform the preliminary softening of the body. It could also be assumed that bacteria themselves are the attractant and the "main target" for enchytraeids in this case. Unfortunately, the activity of potworms in the breakdown of animal remains has not been studied in detail.

Almost all reports on the enchytraeid involvement in the decomposition of animal remains took place where the carrion was flooded or touched by the waves. The biodegradation of animal bodies includes changes in pH, conductivity (Schlaghamerský and Krawczynski, 2015) and the production of potentially toxic chemical compounds (Forbes and Carter, 2016) that can prevent the colonization of the carcass by enchytraeids. However, diffusion on submerged carrion may partially offset the environmental disturbance, perhaps explaining why the decomposition of carrion by enchytraeids has not been observed in strictly terrestrial environments.

2.6. Feces of invertebrates

In addition to plant and animal remains, potworms have also been observed consuming the feces of some invertebrates. Enchytraeids consume earthworm droppings, creating channels that they then fill with their own feces (Babel, 1975). They also ingest excrements of litter-feeding springtails along with other loose particles (Zachariae, 1963). Feces of oribatid mites containing partly degraded fungi are also

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ingested (Ponge, 2010). Vaçulik et al. (2004) found potworms to consume mite droppings in biological crusts on tropical inselbergs. The examination of mite excrements within enchytraeid guts revealed only tannin-rich plant-derived material (Vaçulik et al., 2004). Double digestion, as highlighted by Ponge (2010), seems to be important in the use of some melanized fungi (Ponge, 2010) and refractory dead plant material (Vaçulik et al., 2004). The excrements of other invertebrates are potential sources of coprophilous streptomycetes for potworms (Krištůfek et al., 2001); those microorganisms could also contribute to the digestion of fungal and plant material due to their chitinolytic and cellulolytic activities (Seipke et al., 2012).

3. Live material

3.1. Use of live plant material

Fresh plant matter is generally resistant to enchytraeid digestion as enchytraeids have a poor cellulolytic capability and are therefore not able to break down cell walls (Brockmeyer et al., 1990; Gajda, unpublished data, see Fig. 4). Admittedly, the uptake of small, still green parts of plants, for example, leaves of mosses from the L_1 litter layer, has sometimes been observed, especially in the genus *Cognettia* (Ponge, 1991, 2010). However, the condition of *Cognettia sphagnetorum* experimentally fed with green leaves of *Sphagnum* moss was poor (Latter and Howson, 1978). Similarly, Standen (1978) reported weight losses of *C. sphagnetorum* individuals on *Sphagnum*-derived material.

The role of the live plant fragments in enchytraeid feeding is unknown and can only be vaguely interpreted. The cytoplasmic contents released from wounded tissue of small plant fragments could be mainly responsible for the attractiveness of this kind material (see also Ponge, 2010), thus promoting its ingestion.

3.2. Role of enchytraeids in plant diseases

When browsing through various guides or bulletins that deal with houseplant pests, one can come across claims that potworms are plant-parasitic animals. Moreover, some Paraguayan and Brazilian farmers blame enchytraeids for damage to soybean roots (Schmelz et al., 2013). The belief that enchytraeids are parasites of Plantae *sensu strictissimo* is false. It is the result of a lack of knowledge about the biology of enchytraeids and their superficial morphological similarity to the herbivorous nematodes. To date, no enchytraeid species is known to feed on the living tissue of healthy plants (Esser and Simpson, 1994; Didden et al., 1997; Schmelz et al., 2013). However, there are several papers of early naturalists in which they erroneous stated that potworms can be plant pests.

More than one hundred years ago, Harker (1889) conducted a pot experiment to investigate the noxious effect of *Enchytraeus buchholzi* on plants (one sunflower, one “geranium”, one “tradescantia”). He was the first to report, albeit without reliable evidence, that enchytraeids injure the roots of many vegetables and ornamental plants. Harker (1889) did not observe enchytraeids inside the living roots, only around them or within the decaying stem, but he assumed that they injure the plant by sucking the fine root-hairs. In addition, the soil used in his investigation had been sifted, but not sterilized. Therefore, the activity of other plant pathogens cannot be excluded. It is also worth noting that in Harker’s experiment (1889) only the sunflower died after two months. The two remaining plants (one “geranium”, one “tradescantia”) growing in the presence of *Enchytraeus buchholzi* were unaffected.

Eight years later, Stoklasa (1897) reported that potworms are serious parasites of sugar beets and claimed that they penetrated the epiderm, cortex, and vascular bundle of young plants by a stylet-like mouth structure in laboratory experiments. Based on this description, it can be concluded that Stoklasa (1897) used potworms mixed with plant-parasitic nematodes, probably from the order Tylenchida or Dorylaimida; protrusible spears are characteristic features of these herbivorous nematodes (Maggenti, 1981; Kornobis, 2008). Therefore, Stoklasa’s results (1897) are unreliable. However, the work by Vanha and Stoklasa (1896) shed more light on this case. The authors claimed that potworms possess a stylet-like mouth structure with two chitin needles that could be quickly everted and could cause plant damage (Fig. 5). However, Jegen (1920) reported that such an anatomical feature did not exist in enchytraeids. Zimmermann (1899) studying coffee tree found that enchytraeids, considered by many authors at that time as plant parasites, occurred only in more or less rotten roots together with herbivorous nematodes, but never in healthy or newly disease-affected coffee plants. Infestation trials with potworms failed repeatedly (Zimmermann, 1899). Soon afterward, and on the contrary, Friend (1902) and Carpenter (1903, 1905, 1906, 1907, 1913) claimed that some potworm species, including *E. albidus*, were plant pathogens, infesting celery, asters, tulips, fritillaries, cabbage, tomatoes, swedes, strawberry, spinach, carrot, parsnip, and onion. However, these authors misinterpreted the massive occurrence of potworms in decaying tissues. Friend (1916) recorded the results of a long series of experiments with asters and dragon flowers (*Antirrhinum* sp.) on at the Birmingham Botanical Gardens between 1914 and 1915, which showed that enchytraeids do not harm healthy plants, but act as scavengers when plants start to decompose.

More than 30 years later, Chitwood and Oteifa (1952), in their review about plant-parasitic nematodes, focused on annelids as dominant organisms in decaying vegetable matter. They suggested that potworms were commonly mistaken for nematodes by laymen and even by plant pathologists; they stated, based on available evidence, that soil clitellates, including potworms, do not feed on healthy plant tissues. However, this group of animals was the one most easily seen in diseased plant tissues, resulting in a natural tendency to attribute the plant

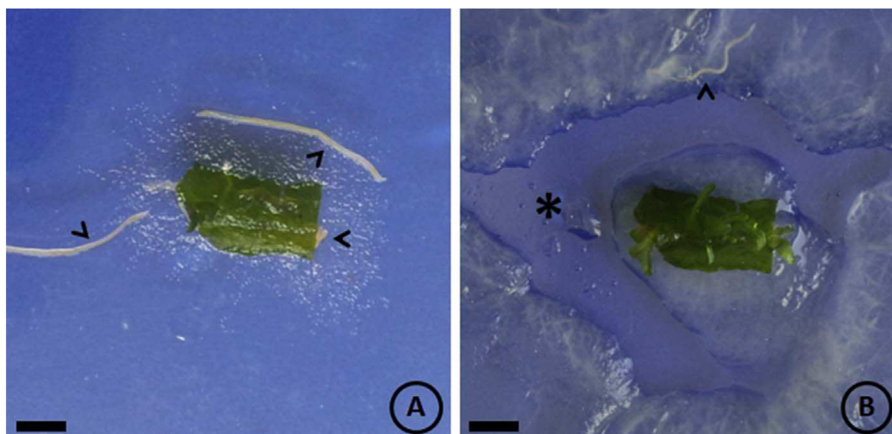

Fig. 4. *Enchytraeus albidus* (arrowheads) with bryophyte (*Pellia* sp.) in 1.2% non-nutrient agar in tap water. Ten individuals were starved for 14 days, rinsed three times in distilled water, transferred to a new agar plate with an excision-wounded piece of thallus, and kept at room temperature. The explant was attractive in short time (day 0) to enchytraeids, which have densely aggregated in the vicinity and beneath the material (A). Potworm activity did not harm the explant during the experimental period of two months and plant growth occurred (B). Extensive ingestion of agar medium was observed during the first month of cohabitation. Animal activity caused the agar to collapse locally and established a zone (asterisk) which desiccated over time (B). The same effect was observed in the replicate (not shown), but not in the control plates (without animals or without plant; not shown). We assume that the extensive ingestion occurred due to attractiveness of contents released from wounded plant tissue, because no microbial growth was observed. Bar = 3.5 mm.

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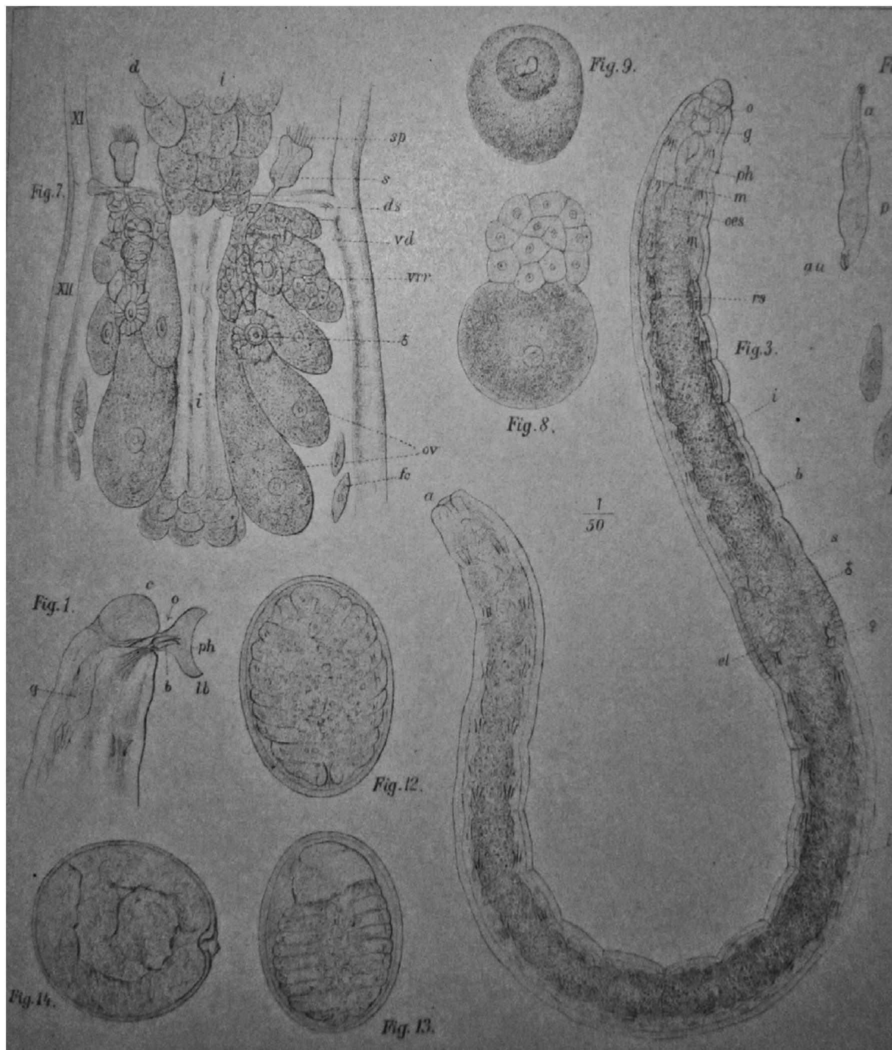

Fig. 5. The original drawings of the anatomy, morphology, and development of "*Enchytraeus Buchholzii*", published in: "Die Rüben-Nematoden (*Heterodera*, *Dorylaimus* und *Tylenchus*) mit Anhang über die Enchytraeiden" (Vanha and Stoklasa, 1896). Front part of the body with everted stylet-like mouth structure equipped with two chitin needles (b) is shown in Fig. 1.

Fig. 6. Enchytraeids found under the bark of a fallen Norway spruce tree (*Picea abies*) in Pohorje, Slovenia. Enchytraeids feeding on unidentified material, which presumably contains attractive microorganisms (A). The filled gut is visible in some specimens (B, C).

condition to them (Chitwood and Oteifa, 1952). Some horticulturists suspected that enchytraeids damage live plant roots. However, Bell (1958) did not find any authentic case of noxious activity from Enchytraeidae. The presence of enchytraeids on dead roots attested only to their action in the reduction of dead plant material (Bell, 1958).

Although most reports of enchytraeids as plant parasites were in regards to agricultural and ornamental plants, there are also available data on their supposed deleterious action to conifers. Hewitt (1908), based on observations of a high number of individuals around the main root and on the examination of cortical tissue injuries, assumed that *Fridericia bisetosa* was a pest of larch seedlings. Similarly, 56 years later, Kurir (1964) claimed that *Fridericia galba* from adjacent compost heaps

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had a detrimental effect on spruce saplings through decortications in the soil-surface zone. However, this author could not provide clear-cut evidence that this enchytraeid species is a plant pest, but accused *F. galba* of parasitism based on its abundant presence in the decaying cortex. Similarly, Springett (1967) assumed that in this case, it was not clear whether the infestation of plants by enchytraeid worms had taken place before or after microbial attacks. Plant cortical tissue disintegrates in conifer nurseries as a result of many diseases caused by various pests (for more details, see Hamm et al., 1990). In addition, Head (1968) demonstrated that enchytraeids and some soil arthropods feed on the degenerating cortical root tissue of apple trees without actually harming the trees, but leave the living central vascular cylinder exposed. Ponge (2010) noticed the behavioral tendency of enchytraeids to penetrate any kind of decaying plant material and observed several specimens between bark and wood in a decomposing pine branch (see also Fig. 6).

The presence of enchytraeids in the rhizosphere may be associated with many plant root diseases, such as bacteriosis, fungal infection, or nematode infestation (Zimmermann, 1899; Friend, 1916; Jegen, 1920; Schaerffenberg, 1950; Schaerffenberg and Tendl, 1951). However, the death of a plant is a consequence of the progress of an infestation and root necrosis and not from enchytraeid activity (Friend, 1916; Esser and Simpson, 1994). In the worst-case scenario, enchytraeids could accelerate the decomposition process, even though they are not the causative agent of the disease (Friend, 1916; Jegen, 1920; Schaerffenberg, 1950; Schaerffenberg and Tendl, 1951). In theory, these animals could be vectors of parasites or plant pests, but that has not been investigated (Schmelz et al., 2013).

3.3. Bacteria

Dougherty and Solberg (1960) succeeded in keeping *Enchytraeus fragmentosus*, which reproduces asexually via architomy (reproduction by fission along with the regeneration of a lost body part), under monoxenic conditions (in a culture in which one species is grown with only one other organism present) with the bacterium *Escherichia coli* growing on a nutrient agar medium. However, to sustain vigorous growth and reproduction of the animals, it was necessary to supplement their diet with a few grains of autoclaved rolled oats dropped onto the surface of the agar slant. In this case, the thin layer of *E. coli* on the medium surface provided minimal nutrition for the enchytraeid culture, but it did not enable optimal growth and indefinite cultivation of *E. fragmentosus* through successive generations in serial subcultures. Dougherty and Solberg (1961) also established an axenic culture (a culture of one species completely isolated from other living organisms) of *E. fragmentosus* on a nutrient agar medium supplemented with sterile lamb liver extract and concluded that *E. fragmentosus* is not an obligate bacterivore. These two experiments were the first successful attempts to cultivate annelids in axenic and gnotobiotic (monoxenic) conditions and provided basic information about how to keep enchytraeids on an agar medium under fully controlled laboratory conditions (Rodriguez et al., 2002). This technique facilitated the use of potworms in ecotoxicological tests (Westheide et al., 1991; Castro-Ferreira et al., 2012) as well as the further development of *in vitro* studies on enchytraeid feeding (O'Connor, 1967). Gotthold et al. (1967) and Gotthold and Koch (1974) developed an artificial medium for axenical cultivation of *E. fragmentosus*. Gotthold and Koch (1974) defined protein sources and vitamin requirements to maintain this species in the laboratory, while Springett (1964) developed a soil-agar culture method to keep *Marionina clavata*, *Chamaedrilus cognettii*, *Achaeta eiseni* and *Achaeta affinis* over long periods. In contrast, *C. sphagnetorum* could be kept only over short times (Springett, 1964). At present, at least 15 enchytraeid species can be cultivated on agar media (Westheide and Bethke-Beilfuss, 1991).

The consumption of bacteria by enchytraeids has been observed both in the laboratory and in the field. Reynoldson (1939) reported *Lumbricillus lineatus* (re-identified as *Lumbricillus rivalis* by Kirk, 1971) in sewage bacteria beds, feeding on cyanobacteria of the genus *Phormidium* (formerly classified as an alga) in the live state. Gelder (1984) found that substratum ingested by *L. lineatus* contained “blue-green algae” (cyanobacteria) and Vaçulik et al. (2004) reported that some potworm species ingest and transform cyanobacterial mass in the cyanobacterial crust of a tropical inselberg. The biofilm covering rocks contained several species of filamentous cyanobacteria, among which the genera *Stigonema*, *Scytonema*, and *Schizothrix* were most abundant (Vaçulik et al., 2004). Microscopic observations of the gut of *C. sphagnetorum* during digestion revealed that filamentous cyanobacteria are separated and then emptied, with the “cellulosic walls” remaining untouched (Ponge, 1991). Palka and Spaul (1970) found that gelatinous zooglea of bacteria and fungi taken from pebbles in sewage beds allowed normal growth in the laboratory of young *L. rivalis* (re-identified by Learner, 1972), while Haimi and Siira-Pietikäinen (2003) noticed that *C. sphagnetorum* was able to survive, although at low density, in mineral soil with extremely low organic matter content, by ingesting a significant number of bacteria and protozoans.

Reichert et al. (1996) investigated the influence of different diets on the behavior and fertility of *Enchytraeus coronatus*. They reported that enchytraeid worms fed with *Bacillus cereus* were in good condition, but their reproduction rate was lower than those fed with rolled oats, a common diet for the breeding of enchytraeids in the laboratory. The histological examination of *E. coronatus* revealed partly or totally digested bacteria inside the gut lumen and the presence of unaffected, unknown ciliates (Reichert et al., 1996). The use of *B. cereus* as a nutrient source for *Enchytraeus* species has also been demonstrated by Brockmeyer et al. (1990). In their experiment, bacteria and the yeast *Saccharomyces cerevisiae* were radiolabeled by the addition of ^{35}S-methionine to the growth medium. Mixtures of labeled microorganisms and unlabeled plant material (fresh and processed) were used as food sources for two enchytraeid worms, *Enchytraeus* cf. *globuliferus* and *Enchytraeus christenseni* (syn. *Enchytraeus minutus*). The incorporation of microbial proteins was directly shown by liquid scintillation counting. A recent study on potworms from temperate grasslands demonstrated that they received almost 50% of their essential amino acids from bacteria (Larsen et al., 2016a). However, not all bacteria are an adequate source of food for enchytraeids, particularly for some species in the *C. sphagnetorum* complex (Springett and Latter, 1977; Latter and Howson, 1978). Potworms can be selective in their bacterial feeding. One report described the selection of various *Streptomyces* species by *Enchytraeus crypticus* (Krištůfek et al., 1995). *Streptomyces*, which are Gram-positive bacteria, resemble microfungi in their morphology and have a complex life cycle, *inter alia*, they grow as branching hyphae that form a substrate (vegetative) mycelium, produce specialized reproductive structures known as aerial hyphae (aerial mycelium), and disperse through spores (Flärdh and Buttner, 2009). *E. crypticus* prefers *Streptomyces lividans* and *Streptomyces nogalater* in its diet over *Streptomyces reticuli*. Moreover, worms select only the living mycelia of attractive strains, while autoclaved mycelia of the same strains are refused, possibly because attractive metabolites are destroyed during the autoclaving process. It has been experimentally demonstrated that metabolites that adhere to mycelia and/or diffuse into the media can act as chemorepellents or chemoattractants for *E. crypticus* (Krištůfek et al., 1995). In addition, there is some evidence that cellular polyunsaturated fatty acids (PUFA) may affect the feeding preferences of *E. crypticus*. However, further research is needed to understand the role of PUFA-producing microorganisms in microbial-invertebrate interactions (Krištůfek et al., 2005). Suitable *Streptomyces* species can serve as the sole nutrient source for *E. crypticus* and can promote growth in the laboratory (Krištůfek et al., 1995). However, Dougherty and Solberg (1960) observed from the monoxenic culture of *E. fragmentosus* and *E. coli* that the bacterial layer was not a satisfactory source of food. Some bacteria of the genera *Pseudomonas* and *Rhizobium* remain active during passage through the gut and after egestion from worms such as *Enchytraeus bigeminus*, *E. albidus*, *Lumbricillus rivalis*, and *L. lineatus* (Ştefan,

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1990). Rashed et al. (1992) found that twenty-six *Pseudomonas* strains represent the predominant species of the gut microbiota of *Fridericia hegemon.* Therefore, *Pseudomonas* spp. seems to be adapted well to survive conditions inside the alimentary tract of enchytraeids. Crotty et al. (2011) used a ^{13}C and ^{15}N enriched bacteria (*Pseudomonas lurida*) to track the trophic transfer of bacterially derived ^{13}C and ^{15}N to soil invertebrates. They found that the level of isotopic enrichment obtained by the not further specified woodland enchytraeids was not significantly different from natural abundance levels. Based on these results, they classified potworms as primary decomposers (but see Scheu and Falca, 2000). However, when considering these findings, it is doubtful that *P. lurida* was a suitable food source. On the assumption that there are no significant qualitative differences in the enzyme spectrum of each *Enchytraeus* species, the distinct ability to use different bacterial strains as a nutrient source could be the result of differences in the cell wall structure of Gram-positive and Gram-negative bacteria or the presence of a capsule. Unfortunately, no detailed studies of the correlation between the bacterial cell envelope and bacteriolysis in the enchytraeid gut have been performed. However, such studies have been carried out on other annelids. For example, Plante and Shriver (1998) showed that not all bacteria respond similarly to digestion by gut fluids of a polychaete lugworm (*Arenicola marina*). They found that none of the tested Gram-positive strains were lysed, whereas almost one-third of the Gram-negative strains showed significant susceptibility. There was also no significant correlation between the production of capsules and the resistance of the strains (Plante and Shriver, 1998). However, it cannot be excluded that capsules could protect some but not all strains against lysis. Brinkhurst and Chua (1969) reported that in three sympatric tubificin Naididae, numerous species of bacteria were ingested, but only certain species were present in the alimentary tract after one week. However, it was uncertain whether digestion of some absent microorganisms, for example, *B. cereus,* had occurred (Brinkhurst and Chua, 1969). Whitley and Seng (1976) suggested that tubificin Naididae digest only some Gram-negative bacteria, while Gram-positive bacteria pass through the intestine undamaged. Some enchytraeids can use Gram-positive (Krištůfek et al., 1995; Brockmeyer et al., 1990; Reichert et al., 1996) and Gram-negative bacteria (Dougherty and Solberg, 1960; Lukešová and Frouz, 2007), suggesting that digestion of both groups occurs. Gelder (1984) reported the occurrence of β-N-acetylglucosaminidase in the intestinal epithelium of *L. lineatus*. Here, β-N-acetylglucosaminidase (β-GlcNAcase) cleaves the glycan component of the bacterial peptydoglycan on the reducing site of N-acetylglucosamine (Stark et al., 2010). Both exo- and endo-types of this enzyme can act on the bacterial cell envelope, but only the latter can open up the mucopeptide sacculus (Ortiz et al., 1973). Murein hydrolases, such as lysozyme or β-GlcNAcase (Vollmer et al., 2008; Allocati et al., 2015), are more effective for Gram-positive bacteria, since those prokaryotes have a fully exposed peptidoglycan cell wall, while in Gram-negative bacteria, the external membrane constrains the access of the lytic enzyme to the peptidoglycan (Barrera Rivas et al., 2015). It therefore seems that Gram-positive bacteria are more suitable food sources for enchytraeids than Gram-negative strains. However, because of the scarcity of data, further investigations are needed to evaluate this conclusion.

Although some older works (Dougherty and Solberg, 1960; Toutain et al., 1982; Brockmeyer et al., 1990; Ponge, 1991) cited in Didden's (1993) review showed that enchytraeids can potentially use bacteria as source of food, the importance of bacterial diet was not well understood at the time. Further studies on the enchytraeid bacterivory in the last two decades (e.g. Krištůfek et al., 1995; Reichert et al., 1996; Lukešová and Frouz, 2007) have revealed that suitable bacteria are a complete food, rich in some compounds like PUFA (Krištůfek et al., 2005) or specific amino acids that are essential for animal growth and development (Larsen et al., 2016a,b).

3.4. Fungi

In addition to bacteria, fungi are also considered to be an important part of the enchytraeid diet (Persson et al., 1980). Springett and Latter (1977) investigated the importance of microorganisms in the diet of moorland Enchytraeidae and reported that young *C. sphagnetorum* grew in cultures of sterile dark mycelium (probably *Mollisia* sp.) and basidiomycete mycelium in non-nutrient water agar in the laboratory. However, growth (here defined as an increase in the number of segments) of the worms was poor, and the mortality rate was extremely high. Furthermore, exudates from tested fungi, especially basidiomycete metabolites, were toxic to *C. sphagnetorum* (Springett and Latter, 1977). Therefore, these authors did not obtain a firm evidence that bacteria and fungi are good nutrient sources and concluded that microorganisms are not a part of the natural diet of the investigated potworm species. However, this conclusion is restricted to the microbial isolates from the blanket bog area used in their study. Latter and Howson (1978) carried out culture studies on the importance of microorganisms in the feeding of Enchytraeidae. They examined the growth and survival of *C. sphagnetorum* on various substrates, including different types of litter, fungi, and bacteria. Five fungal isolates (basidiomycete, sterile dark form, segmented form, *Penicillium spinulosum*, and *Mortierella* sp.) were tested. Growth was markedly poor or even absent with fungi or bacteria as the sole food source and mortality was extremely high. In contrast, organic matter, mainly older litter of *Calluna* and *Eriophorum* plants, was a suitable source of food (Latter and Howson, 1978). Therefore, *C. sphagnetorum* was considered to be a primary rather than a secondary decomposer (Briones and Ineson, 2002), although contrasting evidence exists. Hedlund and Augustsson (1995) demonstrated that hyphae of the fungus *Morteriella isabellina* were grazed and partly removed by *C. sphagnetorum* in a laboratory experiment. Cryptic lineages may differ in certain ecological and physiological properties (see Martinsson et al., 2015 for references). The recent studies on some invertebrates provide evidence that these differences can also be related to feeding behavior (Cabrol et al., 2015; Derycke et al., 2016). The conflicting results of experiments with *C. sphagnetorum* yielded by Latter and co-workers (1977, 1978) and Hedlund and Augustsson (1995) were probably caused by the fact that they used different cryptic species. This assumption can be supported by the presence of other contradictory findings in the literature (see Table 1). Therefore, at least one species in the *C. sphagnetorum* complex can use fungi as a food source. Another explanation could be that some specific fungi, such as *M. isabellina*, are suitable for potworms but other fungal species, even congeners, are not. However, this is rather unlikely, because fungi used by Latter and co-workers (1977, 1978) were representative types commonly isolated from the studied area, and preliminary field tests with agar baits gave no evidence of selection of other fungal species. Remén et al. (2010) found that the number of *C. sphagnetorum* individuals decreased or remained unchanged in the presence of ectomycorrhizal (EM) fungi during a soil microcosm experiment; they suggested that *C. sphagnetorum* may be sensitive to metabolites produced by actively growing EM fungi.

Fungal material is commonly found in the gut contents of many Enchytraeidae (O'Connor, 1967; Dash and Cragg, 1972; Standen and Latter, 1977; Dash et al., 1980; Toutain et al., 1982; Ponge, 1991; , 2010). Dash et al. (1980) examined the food preferences of three tropical enchytraeid worms via squash preparations, culturing of gut contents, and selective feeding on pure fungal cultures. They showed that tropical enchytraeids, such as *Fridericia kalinga*, *Hemienchytraeus khallikotosus*, and *Enchytraeus berhampurosus*, can commonly graze on two (*Rhizopus nigricans* and *Syncephalastrum racemosum*) of the eight microfungi species that were isolated from the gut contents of the animals. In contrast, common filamentous fungi, such as *Penicillium steckii* and *Aspergillus niger*, were not easily digested (Dash et al., 1980). This is in accordance with the results of an ecohistological study of the feeding behavior of *Enchytraeus coronatus* (Reichert et al., 1996) because the

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Table 1
Contradictory reports on the fungivory of *Cognettia sphagnetorum*.

References	Methods	Fungivorous behavior	Notes
Springett, 1964	Cultivation on agar plates	No	Maintained on agar plates, did not feed on yeast as a sole nutrient source.
Springett and Latter, 1977	Bait traps, cultivation on agar plates, microscopic analysis	No	Growth extremely poor or absent with fungi (some constitutes released from dead microorganisms could be used).
Latter and Howson, 1978	Cultivation on agar plates, microscopic analysis	No	Growth extremely poor or absent with fungi, including *Mortierella* sp. (some constitutes released from dead microorganisms could be used).
Ponge, 1991;	Microscopic analysis of the gut content (phase contrast microscopy)	Yes	Fed on hyaline fungi in Scots pine litter.
Hedlund and Augustsson, 1995	Microscopic analysis, microcosm study	Yes	Hyphae of the fungus *Mortierella isabellina* were grazed and partly removed.
Augustsson and Rundgren, 1998	Microcosm study	Yes	Growth in the presence of baker's yeast over a period of four weeks was better than in control soil. Low mortality when fed with *M. isabellina.*
Briones and Ineson, 2002	^{14}C isotope analysis	No	Predominantly uses 5–10-year-old material for carbon assimilation.
Cole et al., 2002	Microcosm study, PLFA/FAME analysis	Yes	Reduced fungal PLFA (i.e., fed selectively on fungal hyphae).
Nowak and Piotrowska-Seget, 2005	Microcosm study, PLFA analysis	Yes	Soil microcosm cultures were established twice; each time, increase or decrease of enchytraeid numbers differed and seemed to be unpredictable. However, there is evidence for a relationship between enchytraeids and fungi.
Nowak et al., 2005	Experimental plots, PLFA analysis	No	No relationship was found between animals and microorganism abundance. Numbers of individuals increased after fungicide application (possibly due to dead microorganisms).
Ponge, 2010[a]	Microscopic analysis of the gut content (phase contrast microscopy)	Yes	Fed on hyaline fungi in Scots pine litter.
Remén et al., 2010	Microcosm study, chitin analysis	No	Did not increase in abundance in any of the treatments with five different ectomycorrhizal fungi or with wood decomposer fungus (*Hypholoma capnoides*), but increased significantly its abundance in non-treated soils.

[a] English translation of three papers published in French by Ponge (1984, 1985 and 1988).

tested culture of *Penicillium* sp. showed no sign of digestion. Dash and Cragg (1972) reported that in some potworms, isolates prepared from the postclitellar part of the gut contents did not give rise to fungi (*Penicillium* sp. and *Cladosporium* sp.) as did those from the preclitellar part, but produced actinomycetes. Rashed et al. (1992) found that the intestinal microbiota of *Fridericia hegemon* also includes actinomycetes. In a similar study, Krištůfek et al. (1999) demonstrated that actinomycete bacteria can enhance the digestion of fungi within the potworm gut. In this way, the nutritional value of the fungus *Aspergillus proliferans* for *E. crypticus* was increased by a *Streptomyces lividans* strain overproducing exochitinase in a co-feeding experiment (Krištůfek et al., 1999). Therefore, the contribution of incorporated bacteria and their enzymatic machinery can be important for the fungal cell-wall lysis in enchytraeids.

Several studies performed by Krištůfek et al., 1995 have shown that some streptomycetes and microscopic fungi (e.g. *Aspergillus flavus, A. proliferans*, *Paecilomyces carneus*, and *Verticillium tenerum*) can serve as food for enchytraeids. Food selection experiments combined with reproduction tests in *E. crypticus* indicated that the nutritional value of individual microbial species can be either increased or decreased by the presence of other microbes (fungi or streptomycetes) in the mixture (Krištůfek et al., 2001). According to Krištůfek et al. (2001), the attractivness of microbes to *E. crypticus* depends not exclusively on the microbial species itself, but also on the cultivation medium used, conditions of cultivation, vitality (living or dead), and culture age. All these factors can affect the production of metabolites in fungi and actinomycetes (Calvo et al., 2002; Bundale et al., 2015), and those substances could potentially act as chemoattractants and chemorepellents for enchytraeids. Potworms, like earthworms, possess chemoreceptors (Laverack, 1960; Bicho et al., 2015) and can therefore express approach or avoidance behavior in response to certain substances (Bicho et al., 2015). A recent study has shown that earthworms use odor cues to locate and feed on fungi in the soil (Zirbes et al., 2011), and olfaction may also play a key role in potworm fungi foraging.

The last 20 years of laboratory tests (e.g. Krištůfek et al., 1995, 2005; Reichert et al., 1996) and recent investigations with stable isotope fingerprints (Larsen et al., 2016a,b) have refuted the suggestion that generally enchytraeids are fungivorous rather than bacterivorous (e.g. Maraun et al., 2003; Briones, 2006). Both, fungi and bacteria can be a high quality food for enchytraeids.

3.5. Microalgae

When considering the role of algae as food of potworms one encounters a number of problems arising from the different understanding of the informal term “algae” by different authors and at different times. The incorrect use of this term in many papers and the common lack of any detailed taxonomic identification make it extremely difficult to draw accurate conclusions. For example, over many decades research on enchytraeid diet (cf. Palka and Spaul, 1970) has considered cyanobacteria, also referred to as “blue-green algae”, as equivalent to unicellular, filamentous, or even thalloidal eukaryotic forms. Currently, it is widely known that “blue-green algae” are true bacteria. In contrast to many eukaryotic algae, the presence of cellulose in the cyanobacterial cell wall is unusual (de Winder et al., 1990; Zhao et al., 2015). However, cellulose-like homoglucan is sometimes present in extracellular sheaths produced by some cyanobacteria (Hoiczyk and Hansel, 2000; Nobles et al., 2001). The strong generalization about algal diet of Enchytraeidae in the literature seems to be inaccurate. Therefore, the term “algae” is used here refers only eukaryotic species.

Microscopic algae have often been found in the alimentary canal of enchytraeids (Palka and Spaul, 1970; Giere and Pfannkuche, 1982; Toutain et al., 1982; Gelder, 1984; Ponge, 1991, 2010; Murakami et al., 2015). Gut content analyses mainly revealed diatoms (Giere, 1975; Gelder, 1984; Healy and Walters, 1994) and unicellular Chlorophyceae *sensu lato* (Ponge, 2010; Murakami et al., 2015). Among the family Enchytraeidae, the ice worm (*Mesenchytraeus solifugus*) is probably the most explicit algal feeder. Certain snow and ice microalgae, including red-pigmented *Chlamydomonas nivalis* and *Chlamydomonas sanguinea*, constitute the primary source of food for this species (Goodman, 1971; Murakami et al., 2015). However, in this case, it is still unclear whether the suitable food sources are live algal cells or decomposing algal matter or both, depending on the enzymes acting in the digestive tract (Goodman, 1971). Some microscopic details of the digestion of

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unicellular algae in enchytraeid guts are available. Palka and Spaul (1970) noticed that cell walls of green algae passed through the alimentary canal of *L. rivalis* apparently unaffected, in contrast to the cell contents. This resembles the degradation mode of cyanobacteria described by Ponge (1991, 2010) in representatives of *Cognettia*. However, the digestion of *Chlorella*-like algae in *Cognettia* sp. and *C. sphagnetorum* occurs in a slightly different way with the support of the intestinal microflora. Initially, intact algal cells are coated with bacteria. After bacterial attack, the cells cytoplasm disappears and ghost cells collapse. However, the cell walls of algae are only partially digested. According to Ponge (1991, 2010), the digestion of microalgae seemed to be somewhat difficult and it is not certain whether cell wall degradation always occurs. However, the use of algal nutrients by *Enchytraeus albidus* from *Chlorocloster pachychlamys* and two strains of *Chlamydomonas reinhardtii* has been demonstrated using ^{15}N labels. (Shtina et al., 1981). They found that after feeding on algae marked with the ^{15}N isotope, enchytraeids incorporated about 4–5% of the algal cellś marked nitrogen. The contribution of gut microbiota to the digestion of refractory algal material in enchytraeids has been suggested by several authors (e.g. Palka and Spaul, 1970; Goodman, 1971; Murakami et al., 2015). Unfortunately, no detailed studies on the influence of the enchytraeid microflora on the nutritional value of microscopic algae have been performed. The cellulolytic enhancement by symbionts has been considered essential for Enchytraeidae (Palka and Spaul, 1970). However, the belief that cellulase activity is necessary for microalgae digestion could be partially true, depending on the algae species or even the strain that is ingested. Microalgal cell walls are complex, and their composition is still poorly understood (Gerken et al., 2013). For example, the cell walls of most *Chlamydomonas* do not contain cellulose, and their major constituents are crystalline glycoproteins (Harris, 2009; Domozych et al., 2012). Some strains of *Chlorella vulgaris* have little or no cellulose in their cell walls (Gerken et al., 2013). Therefore, in some cases, the digestion of algae may require enzymes other than cellulases. Gerken et al. (2013) found that in *Ch. vulgaris* strain (UTEX395) the lysozyme degraded an outer surface of the cell wall, including externally-oriented hair-like fibers. Treatment with lysozyme in combination with other enzymes, such as sulfatase, lyticase, or phospholipase A1, drastically increased algal cell permeability. In contrast, cellulase caused no systematic changes in the ultrastructure of rigid layers and had no significant effect on permeability. On the other hand, lysozyme alone did not have a marked impact on permeabilizing mature algal cells. However, due to the loss of the protective layer, other cell wall components become available as substrates to a wide range of different enzymes (Gerken et al., 2013).

A few studies have shown that the consumption of certain algal species can positively affect the growth rates of potworms. Feeding on the yellow-green alga *Botrydiopsis intercedens* stimulated the reproduction of enchytraeids in laboratory tests, while the cyanobacterium *Trichormus variabilis* was rejected as a food source and proved to be toxic (Krištůfek et al., 1997). Conversely, *Nostoc* cf. *calcicola* (Cyanobacteria) was preferably consumed by *Enchytraeus crypticus* (Lukešová and Frouz, 2007). Augustsson and Rundgren (1998) found that *C. sphagnetorum* fed with *Pleurococcus* spp. algae had better growth (number of segments added) than those fed with the fungus *M. isabellina* in copper-polluted soil. Microalgae can serve as either the main or a complementary food source for enchytraeids. On a daily basis, one enchytraeid individual can ingest $1.2 \times$ to 4.1×10^{-3} mg (in terms of dry weight) of algae or cyanobacteria according to specific microbial species (Nekrasova and Domracheva, 1972; Nekrasova et al., 1976). Assuming that enchytraeids from forest soil feed only upon algae and cyanobacteria, they can use a biomass of 131 to 149 kg $ha^{-1}year^{-1}$ (Nekrasova and Domracheva, 1972). Soil annelids, including potworms, can play an active role in controlling microalgal populations and in the transformation of their biomass (Shtina et al., 1981).

Microalgae are a heterogeneous polyphyletic group of microorganisms and their susceptibility to digestion depends on both the algal and the enchytraeid species. Microalgae containing cellulose-based cell walls are generally refractory for enchytraeids, and their use is possible only with the support of symbiotic bacteria (Toutain et al., 1982; Ponge, 1991, 2010). However, even with symbiotic bacteria, digestion is usually incomplete, as has been observed in many other groups of invertebrates (Lukešová and Frouz, 2007). On the other hand, microalgal species that do not contain large amounts of cellulose in their cell wall are rather a good source of food, at least for some enchytraeid species, and can have a positive effect on animal growth (Goodman, 1971; Shtina et al., 1981; Gelder, 1984; Krištůfek et al., 1997).

3.6. Nematodes

Apart from microorganisms, living or partially digested nematodes have been found in the guts of enchytraeids (Palka and Spaul, 1970; Dash and Cragg, 1972; Dash, 1973). Dash (1973) reported the interintestinal presence of free-living nematode females from the family Monhysteridae. However, according to Dash and Cragg (1972), it is unlikely that nematodes constitute an important source of food for potworms; experimental feeding of dead nematodes to *L. rivalis* yielded inconclusive results (Palka and Spaul, 1970). The presence of nematodes in the enchytraeid gut could be explained as a result of their accidental ingestion with plant residues or with other remains (Palka and Spaul, 1970; Dash and Cragg, 1972; Dash, 1983). However, some nematodes observed in the alimentary canal and coelom are most likely parasitic (see Dash and Cragg, 1972 or Dash, 1973). For example, one of the well-known parasites is *Soboliphyme baturini*, which has a complex life cycle involving enchytraeids as intermediate hosts (Koehler et al., 2007). On the other hand, there is some evidence that earthworms, which are closely related to potworms (Erséus, 2005), selectively feed on nematodes (Hyvönen et al., 1994). Additionally, Jegen's (1920) observations of large numbers of Enchytraeidae in and around the roots of nematode-infested strawberry plants indicate that there is some correlation in the occurrence of these animals. Jegen (1920) claimed that enchytraeids can kill root-feeding nematodes by gathering around them, converting them into a liquid mass, and then consuming the resulting pulp. He experimentally demonstrated that introducing enchytraeids (*Enchytraeus* spp., *Lumbricillus* spp., *Fridericia* spp.) to the soil early enough could stop nematode infestation of strawberry plants. If, however, this procedure was performed in an advanced stage of infestation, the enchytraeids accelerated the process of plant decomposition by opening the channels in the roots, which were then more easily colonized by saprophytic microflora (Jegen, 1920). Similar studies have also been conducted by Schaerffenberg (1950) and Schaerffenberg and Tendl (1951), who investigated the interactions between potworms and the rhizophagous nematode *Heterodera schachtii*. The addition of *Enchytraeus* spp. and *Fridericia* spp. to soil containing the nematode-infested sugar beets arrested the disease. However, complete plant recovery was only possible in early infestation stages before the appearance of adults of *H. schachtii*. The introduction of potworms to soil in an advanced stage of infestation, after the development of nematodes has been completed and mature individuals have massively left the roots, accelerated plant degeneration, which is in agreement with the results obtained by Jegen (1920). Schaerffenberg (1950) and Schaerffenberg and Tendl (1951) stated that only the populations of endoparasitic larvae in the roots were greatly reduced, but not the mature individuals of *H. schachtii*. They assumed rather than experimentally confirmed, that immature enchytraeid individuals penetrate the epidermis of roots of sugar beets and feed on nematode larvae. Though some root tissue sections were prepared by these authors, the presence of enchytraeids in low nematode-infested plants was neither shown nor clearly reported as observed. This makes their statement controversial and was questioned by Boosalis and Mankau (1965), who pointed out that (1) enchytraeids are normally saprophagous and they have no special organs for penetrating live roots, therefore the nematode location is probably unavailable to them; (2)

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Table 2
Trophic types of the enchytraeids of the most commonly studied genera in relation to food preferences and feeding behavior.

	Group	Potential food sources	References	Notes
Primary decomposers	*Cognettia sphagnetorum* complex (trophic type I)	plant material (P)	Springett, 1964 [P]; Latter, 1977 [P]; Springett and Latter, 1977 [P]; Standen and Latter, 1977 [P]; Standen, 1978 [P]; Latter and Howson, 1978 [P];	Some constitutes released from dead microorganisms, particularly fungi, could be used (Springett and Latter, 1977; Standen and Latter, 1977). Extensive development of bacteria on the nutrient agar plates negatively impacted the animal (Springett, 1964; Springett and Latter, 1977) as well as the metabolites produced by some fungi (Springett and Latter, 1977; Remén et al., 2010).
Secondary decomposers/sapro-microphytophages	*Cognettia sphagnetorum* complex (trophic type II)	plant material (P), microalgae (μA), fungi (F), bacteria (B), testate amoebae (T)	Hedlund and Augustsson, 1995 [F]; Augustsson and Rundgren, 1998 [FμA]; Ponge, 1991, 2010 [PμA,FBT]; Cole et al., 2002 [F]; Haimi & Siira-Pietikäinen, 2003 [PFB]	More fungivorous than bacterivorous (Cole et al., 2002). The fate of ingested bacteria in Ponge's (1991, 2010) studies was uncertain, with the exception of cyanobacteria, which were digested with the help of intestinal microflora. Bacteria can be a food source, at least to some extent, as the animal was able to survive, albeit in low numbers, feeding on bacteria and protozoans in resource-poor mineral soil (Haimi & Siira-Pietikäinen, 2003).
	Fridericia spp.	plant material (P), fungi (F), bacteria (B), nematodes (N)	Jegen, 1920 [N]; Schaerffenberg, 1950 [N]; Schaerffenberg and Tendl, 1951 [N]; Dózsa-Farkas, 1976, 1978a, 1978b, 1982 [P]; Dash et al., 1980 [F]; Toutain et al., 1982 [PFB]	The presence of microalgae in the gut of *Fridericia* spp. has been reported by Toutain et al. (1982), although no sign of digestion was observed.
	Lumbricillus spp.	plant material (P), macroalgae (A), microalgae (μA), bacteria (B), nematodes (N), animal remains (AR)	Jegen, 1920 [N]; Stephenson, 1922, 1930 [AR]; Reynoldson, 1939 [B]; Tynen, 1969 [A]; Palka and Spaul, 1970 [μA N]; Giere, 1975 [PAμA,B]; Giere and Hauschildt, 1979 [PA]; Giere and Pfannkuche, 1982 [PAμA,AR]; Gelder, 1984 [μA,B]	No reports on the contribution of fungi to the diet. Palka and Spaul (1970) found that gelatinous zooglea of bacteria and fungi could be used as food, but there was no attempt to cultivate animals on pure fungal cultures.
	Enchytraeus spp.	plant material (P), macroalgae (A), microalgae (μA), fungi (F), bacteria (B), nematodes (N), animal remains (AR)	Jegen, 1920 [N]; Michaelsen, 1927 [AR]; Backlund, 1945 [A]; Schaerffenberg, 1950 [N]; Schaerffenberg and Tendl, 1951 [N]; Dougherty and Solberg, 1960 [B]; O'Connor, 1967 [AR]; Schöne, 1971 [PA]; Dash and Cragg, 1972 [FN]; Dash et al., 1980 [F]; Shtina et al., 1981 [μA]; Giere and Pfannkuche, 1982 [PA]; Brockmeyer et al., 1990 [PFB]; Krištůfek et al., 1995 [B], 1999, 2001 [FB]; Reichert et al., 1996 [PB]; Jaffee et al., 1997 [F]; Schmidt et al., 2004 [μA]; Lukešová and Frouz, 2007 [B]; Puppe et al., 2012 [P]; Larsen et al., 2016b [FB]	The most explicit microphytophagous behavior has been reported for *E. crypticus* (Krištůfek et al., 1995, 1999, 2001; Jaffee et al., 1997; Lukešová and Frouz, 2007; Larsen et al., 2016b).

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Heterodera spp. require relatively sound plant tissue in order to accomplish their specialized host-parasite relationship, while enchytraeids can dislodge developing nematodes in highly decomposed roots. But such condition are also unfavorable for the endoparasitic larvae. Therefore, the explanation given by Schaerffenberg and Tendl (1951) on the antagonistic relationship between enchytraeids and rhizophagous nematode *H. schachtii* seems to be unreliable. This raises questions about whether enchytraeids interact with sedentary endoparasitic nematodes (i.e. plant-cyst or plant-root-knot nematodes) and if so, how do they interact?

The interrelationships between enchytraeids, fungal biological control agents, and plant-root-knot nematode (*Meloidogyne javanica*) were investigated by Jaffee et al. (1997). They found that immature *E. crypticus* did not affect the numbers of root-knot nematodes but reduced the population densities of the nematophagous fungi. As a result, *E. crypticus* had negative impact on biological control of *M. javanica* by fungi. However, unexpectedly, in another microcosm experiment exploring the interrelationships among rhizosphere community, *Meloidogyne incognita* and pepper (*Capsicum annuum*) seedlings, and unspecified enchytraeids, a pattern of abundance that was similar to that observed with predatory nematodes was seen, which was consistent with reduction in juvenile root-knot nematodes in soil (McSorley et al., 2006). These inconsistent findings might be explained in a several various ways (e.g. different species involved), however, data is limited and the questions remain.

Enchytraeids have been associated with a reduction in rhizophagous nematode populations under some circumstances (Jegen, 1920; Schaerffenberg, 1950; Schaerffenberg and Tendl, 1951; McSorley et al., 2006). Therefore it seems possible that they actively prey on some nematode species, however this has not been proven. It can be concluded that in the case of sedentary endoparasitic nematodes, juvenile forms are consumed (Schaerffenberg, 1950; Schaerffenberg and Tendl, 1951; McSorley et al., 2006), and this is possible until they enter the roots of host plant (Boosalis and Mankau, 1965). The external digestion of nematodes suggested by Jegen (1920) and assumed by Schaerffenberg and Tendl (1951) has not been confirmed and is contradicted by other authors (Palka and Spaul, 1970; Dash and Cragg, 1972; Dash, 1983). The importance of nematodes in enchytraeid diet remains unknown.

3.7. Locust eggs

Apart from nematodes, at least two incidents of the consumption of locust eggs by potworms have been recorded. Beddard (1905) described in detail an enchytraeid worm received from India, referred to *Henlea lefroyi*, that attacked and destroyed locust eggs in moist soil. According to Michaelsen, the position of this species within the genus *Henlea* was doubtful (see Beddard, 1912). Ackonor and Vajime (1995) studied environmental factors affecting egg development and survival in *Locusta migratoria migratorioides* in the Lake Chad basin outbreak area. They reported that an unknown enchytraeid species attacked eggs and caused the total or partial destruction of egg pods. The reports of Beddard (1905) and Ackonor and Vajime (1995) are controversial, as the ecology of non-European species is still very poorly understood. Curious cases of feeding habits of some exotic enchytraeids are nothing extraordinary in the literature. For example, *Aspidodrilus kelsalli* and *Pelmatodrilus planariformis*, which have a partially or pronouncedly flattened body, respectively, are ectocomensals living on large earthworms, probably feeding on surface mucus along with soil particles, bacteria, and organic debris trapped in it (Coates, 1990). Therefore, such reports, even though relatively old, should not be disregarded, albeit they should be treated with caution.

4. Conclusions and perspectives

Detailed studies on the food preferences of enchytraeids have only been carried out on a few species. The diet of Enchytraeidae consists mainly of partially degraded plant debris, bacteria, fungi, and microalgae. In addition, some species feed on rotting seaweed (thalloid algae), feces, and animal remains after the initial action of saprophytic microflora. There is a lack of information on the contribution of protozoans to the enchytraeid diet. A single case of completed lysis of "testate amoebae" in *Cognettia sphagnetorum* has been reported by Ponge (1991). Enchytraeids are not plant pests, but have often been wrongly implicated in the damage of higher plants. Unfortunately, this idea is still presented in some general textbooks and agriculture brochures. In contrast, there are indications that some species of the genera *Lumbricillus*, *Enchytraeus*, and possibly *Fridericia* can consume herbivorous nematodes, at least their juvenile forms. The present review reinforces the conclusion made by Didden (1993) that more than one feeding strategy exists within the family Enchytraeidae. Based on the available literature, we can distinguish several trophic types among potworms (Table 2). It is self-evident that due to the lack of reliable information on many aspects presented view is at least partly simplified. However, it generates at least a picture that can be further supplemented and corrected. Even cryptic species, that are very similar to each other in terms of morphology, may differ significantly in terms of food preferences. Enchytraeidae can be divided into two groups: the first one contains primary decomposers (at least one cryptic species within the *C. sphagnetorum* complex) using only dead organic matter, and the second one consists of species that are able to thrive on specific microorganisms, sometimes as their sole source of food. Under appropriate conditions, some species may act as microphytophages, therefore, dead plant material is probably less important in their diet than previously thought. However, some strains of bacteria (*Pseudomonas* spp.) and fungi (*Penicillium* spp.) are particularly resistant to lysis in enchytraeid guts and therefore, not suitable as food. Defining enchytraeids as intermediate type decomposers (Eisenhauer and Schädler, 2011) seems to be accurate for most of the studied species. Potworms are discriminatory feeders, but some have a wide food spectrum. Selective feeding by enchytraeids on microbes, seaweed, and plant material has been shown. Preferential ingestion of certain species of bacteria and fungi has been particularly well demonstrated in *Enchytraeus crypticus*. However, there is no general answer to the question whether potworms are more fungivorous or bacterivorous. Thus, it seems that *C. sphagnetorum* investigated by Ponge (1991) is rather fungivorous, while *E. crypticus* uses bacteria and fungi in approximately the same amounts. Apart from microorganisms, the selection of specific kinds of seaweed has been shown for *E. albidus* and *Lumbricillus* spp., while the choice between different leaf litters has been documented in *Henlea nasuta* and *Friderica* spp. Potworms prefer decaying plants and macroalgae over fresh material. They have different levels of certain digestive enzymes, probably depending on the rate of feeding, type, and source of the food that is ingested. Most enchytraeids are not capable of digesting cellulose or at least, degrading this polysaccharide with difficulty. Some cellulase activity has been detected in a few (mainly tropical) species of potworms. However, it has not been well clarified whether the origin of those cellulases are the potworms themselves or the microorganisms that were ingested with their food, or perhaps from symbiotic microflora.

Studies on the feeding behavior of Enchytraeidae are complicated by the small size of the animals and the presence of species complexes. Moreover, some potworms (e.g. *Cognettia* spp., *Lumbricillus* spp.) can starve for several weeks and even reproduce for a while during this period (Springett, 1964; Palka and Spaul, 1970; Reichart et al., 1996), which may complicate the drawing of reliable conclusions in feeding experiments. Molecular methods such as DNA barcoding (Hebert et al., 2003) could be applied to improve the identification of species (Schlegel et al., 1991; Erséus et al., 2010; Martinsson and Erséus, 2014; Vivien et al., 2015). Techniques such as RT-PCR (Reverse Transcription-Polymerase Chain Reaction) may also help to distinguish the origin of enzymes that are active in the worm gut (Nozaki et al., 2009).

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Additionally, some methods such as T-RFLP (Terminal Restriction Fragment Length Polymorphism) could be used to study the prokaryotic community structure in the digestive tract of annelids (Egert et al., 2004).

Many aspects of enchytraeids feeding require further study. Selective ingestion (e.g. the nature of attractants or repellents), digestion (external or internal?) and the role of microbial interaction in the consumption of different food types still need to be clarified. For example, there are no studies on the susceptibility of different bacterial groups to digestion in the enchytraeid gut. No definite conclusions have been made about the role of nematodes as a food source. The older papers, which provided some essential data on the feeding biology, were sometimes inconsistent; this was particularly evident for studies on *C. sphagnetorum*. The fact that this species turned out to be a complex composed of at least four well-separated lineages could explain differences obtained among several experiments on ecology and food preferences of enchytraeids. Therefore, there is still a need for further research on enchytraeid feeding ecology at the species level, as several authors have stressed before (e.g. Dózsa-Farkas, 1982; Didden, 1993; Schmidt et al., 2004).

Acknowledgements

The authors would like to thank Prof. Elżbieta Dumnicka for valuable and constructive comments that contributed to improving the quality of the paper. Special thanks go to Dr. Andrzej Rybak and Dr. Stanisław Rosadziński for their help in the taxonomic identification of the bryophyte material.

References

Ackonor, J.B., Vajime, C.K., 1995. Factors affecting *Locusta migratoria migratorioides* egg development and survival in the Lake Chad basin outbreak area. Int. J. Pest Manage. 41, 87–96.

Adl, S.M., 2008. Enchytraeids. In: Carter, M.R., Gregorich, E.G. (Eds.), Soil Sampling and Methods of Analysis, second edition. CRC Press, Boca Raton, pp. 445–453.

Allocati, N., Masulli, M., Di Ilio, C., de Laurenzi, V., 2015. Die for the community: an overview of programmed cell death in bacteria. Cell Death Dis. http://dx.doi.org/10.1038/cddis.2014.570.

Anderson, J.M., 1975. Succession, diversity and trophic relationships of some soil animals in decomposing leaf litter. J. Anim. Ecol. 44, 475–495.

Atkinson, M.D., Atkinson, E., 2002. *Sambucus nigra* L. J. Ecol. 90, 895–923.

Augustsson, A.K., Rundgren, S., 1998. The enchytraeid *Cognettia sphagnetorum* in risk assessment: advantages and disadvantages. Ambio 27, 62–69.

Babel, U., 1975. Micromorphology of Soil Organic Matter. In: Gieseking, J.E. (Ed.), Springer, Berlin, pp. 369–473.

Backlund, H.O., 1945. Wrack fauna of Sweden and Finland, Ecology and chorology. Opusc. Entomol. Suppl. 5, 1–236.

Bardgett, R.D., van der Putten, W.H., 2014. Belowground biodiversity and ecosystem functioning. Nature 515, 505–511.

Barrera Rivas, C.I., Cajero Juarez, M., Oviedo-Boyso, J., Nuñez-Anita, R.E., Kameyama Kawabe, L., et al., 2015. Advances in the use of endolysins: general remarks, structure, applications, genetic modifications and perspectives. In: In: Méndez-Vilas, A. (Ed.), The Battle Against Microbial Pathogens: Basic Science, Technological Advances and Educational Programs, Edition: Microbiology Series No 5, vol. 1. Formatex Research Center, Badajoz, pp. 259–268.

Beddard, F.E., 1905. On a new enchytraeid worm (*Henlea lefroyi* sp. n.) from India destructive to the eggs of a locust (*Acridium* sp.). Proc. Zool. Soc. Lond. 2, 558–561.

Beddard, F.E., 1912. Earthworms and their allies. The Cambridge Manuals of Science and Literature. Cambridge University Press, London.

Bell, A.W., 1958. The anatomy of the oligochaete *Enchytraeus albidus*, with a key to the species of the genus *Enchytraeus*. Am. Mus. Novit. 1902, 1–13.

Bengtsson, J., Persson, T., Lundkvist, H., 1997. Long-term effects of logging residue addition and removal on macroarthropods and enchytraeids. J. Appl. Ecol. 34, 1014–1022.

Beylich, A., Graefe, U., Elsner, D.C., 2015. Response of microannelids to tillage at soil-monitoring sites in Schleswig-Holstein, Germany. Soil Organ. 87, 121–135.

Bicho, R.C., Gomes, S.I.L., Soares, A.M.V.M., Amorim, M.J.B., 2015. Non-avoidance behaviour in enchytraeids to boric acid is related to the GABAergic mechanism. Environ. Sci. Pollut. Res. 22, 6898–6903.

Boosalis, M.G., Mankau, R., 1965. Parasitism and predation of soil microorganisms. In: Baker, K.F., Snyder, W.C. (Eds.), Ecology of Soil-borne Plant Pathogens: Prelude to Biological Control. University of California Press, Berkeley, pp. 374–389.

Boros, G., 2010. Enchytraeids (Oligochaeta, Enchytraeidae) from potting compost purchasable in the Hungarian retail trade. Opusc. Zool. Budapest 41, 237–240.

Brinkhurst, R.O., Chua, K.E., 1969. Preliminary investigation of the exploitation of some potential nutritional resources by three sympatric tubificid oligochaetes. J. Fish. Res. Bd. Can. 26, 2659–2667.

Briones, M.J.I., Ineson, P., 2002. Use of ^{14}C carbon dating to determine feeding behaviour of enchytraeids. Soil Biol. Biochem. 34, 881–884.

Briones, M.J.I., Carreira, J., Ineson, P., 1998. *Cognettia sphagnetorum* (Enchytraeidae) and nutrient cycling in organic soils: a microcosm experiment. Appl. Soil. Ecol. 9, 289–294.

Briones, M.J.I., 2006. Enchytraeidae. In: Second Edition. In: Lal, R. (Ed.), Encyclopedia of Soil Science Vol. 1. Taylor & Francis, pp. 514–518. http://dx.doi.org/10.1081/E-ESS-120042668.

Brockmeyer, V., Schmid, R., Westheide, W., 1990. Quantitative investigations of the food of two terrestrial enchytraeid species (Oligochaeta). Pedobiologia 34, 151–156.

Bundale, S., Begde, D., Nashikkar, N., Kadam, T., Upadhyay, A., 2015. Optimization of culture conditions for production of bioactive metabolites by *Streptomyces* spp. isolated from soil. Adv. Microbiol. 5, 441–451.

Cabrol, J., Winkler, G., Tremblay, R., 2015. Physiological condition and differential feeding behaviour in the cryptic species complex *Eurytemora affinis* in the St Lawrence estuary. J. Plankton Res. 37, 372–387.

Calvo, A.M., Wilson, R.A., Bok, J.W., Keller, N.P., 2002. Relationship between secondary metabolism and fungal development. Microbiol. Mol. Biol. Rev. 66, 447–459.

Carpenter, G.H., 1903. Injurious insects and other animals observed in Ireland during the year 1902. Econ. Proc. Royal Dublin Soc. 1, 195–222.

Carpenter, G.H., 1905. Injurious insects and other animals observed in Ireland during the year 1904. Econ. Proc. Royal Dublin Soc. 1, 281–305.

Carpenter, G.H., 1906. Injurious insects and other animals observed in Ireland during the year 1905. Econ. Proc. Royal Dublin Soc. 1, 321–344.

Carpenter, G.H., 1907. Injurious insects and other animals observed in Ireland during the year 1906. Econ. Proc. Royal Dublin Soc. 1, 421–452.

Carpenter, G.H., 1913. Injurious insects and other animals observed in Ireland during the year 1912. Econ. Proc. Royal Dublin Soc. 2, 79–104.

Castro-Ferreira, M.P., Roelofs, D., van Gestel, C.A.M., Verweij, R.A., Soares, A.M.V.M., et al., 2012. *Enchytraeus crypticus* as model species in soil ecotoxicology. Chemosphere 87, 1222–1227.

Chitwood, B.G., Oteifa, B.A., 1952. Nematodes parasitic on plants. Annu. Rev. Microbiol. 6, 151–184.

Christensen, B., Glenner, H., 2010. Molecular phylogeny of Enchytraeidae (Oligochaeta) indicates separate invasions of the terrestrial environment. J. Zool. Syst. Evol. Res. 48, 208–212.

Coates, K.A., 1990. Redescriptions of *Aspidodrilus* and *Pelmatodrilus*, enchytraeids (Annelida, Oligochaeta) ectocommensal on earthworms. Can. J. Zool. 68, 498–505.

Cochran, V.L., Sparrow, S.D., Sparrow, E.B., 1994. Residue effect on soil micro- and macroorganisms. In: Unger, P.W. (Ed.), Managing Agricultural Residues. CRC Press, Boca Raton, pp. 163–184.

Cole, L., Bardgett, R.D., Ineson, P., Hobbs, P.J., 2002. Enchytraeid worm (Oligochaeta) influences on microbial community structure, nutrient dynamics and plant growth in blanket peat subjected to warming. Soil Biol. Biochem. 34, 83–92.

Crotty, F.V., Blackshaw, R.P., Murray, P.J., 2011. Tracking the flow of bacterially derived ^{13}C and ^{15}N through soil faunal feeding channels. Rapid Commun. Mass Spectrom. 25, 1503–1513.

Dash, M.C., Cragg, J.B., 1972. Selection of microfungi by Enchytraeidae (Oligochaeta) and other members of the soil fauna. Pedobiologia 12, 282–286.

Dash, M.C., Nanda, B., Behera, N., 1980. Fungal feeding by Enchytraeidae (Oligochaeta) in a tropical woodland in Orissa, India. Oikos 34, 202–206.

Dash, M.C., Nanda, B., Mishra, P.C., 1981. Digestive enzymes in three species of Enchytraeidae (Oligochaeta). Oikos 36, 316–318.

Dash, M.C., 1973. Note on nematodes occurring in Enchytraeidae (Oligochaeta). Megadrilogica 1, 1–2.

Dash, M.C., 1983. The Biology of Enchytraeidae (Oligochaeta). International Book Distributors, Dehradun.

Derycke, S., de Meester, N., Rigaux, A., Creer, S., Bik, H., et al., 2016. Coexisting cryptic species of the *Litoditis marina* complex (Nematoda) show differential resource use and have distinct microbiomes with high intraspecific variability. Mol. Ecol. 25, 2093–2110.

de Winder, B., Stal, L.J., Mur, L.R., 1990. *Crinalium epipsammum* sp. nov.: a filamentous cyanobacterium with trichomes composed of elliptical cells and containing poly-β-(1,4) glucan (cellulose). J. Gen. Microbiol. 136, 1645–1653.

Didden, W.A.M., Fründ, H.C., Graefe, U., 1997. Enchytraeids. In: Benckiser, G. (Ed.), Fauna in Soil Ecosystems: Recycling Processes, Nutrient Fluxes and Agricultural Production. Marcel Dekker, Inc., New York, pp. 135–172.

Didden, W.A.M., 1993. Ecology of terrestrial Enchytraeidae. Pedobiologia 37, 2–29.

Domozych, D.S., Ciancia, M., Fangel, J.U., Mikkelsen, M.D., Ulvskov, P., et al., 2012. The cell walls of green algae: a journey through evolution and diversity. Front. Plant Sci. 3 (82), 1–7.

Dougherty, E.C., Solberg, B., 1960. Monoxenic cultivation of an enchytraeid annelid. Nature 186, 1067–1068.

Dougherty, E.C., Solberg, B., 1961. Axenic cultivation of an enchytraeid annelid. Nature 192, 184–185.

Dózsa-Farkas, K., 1976. Über die Nahrungswahl zweier Enchytraeiden-Arten (Oligochaeta: Enchytraeidae). Acta Zool. Acad. Hung. 22, 5–28.

Dózsa-Farkas, K., 1978a. Die Bedeutung zweier Enchytreiden-Arten bei der Zersetzung von Hainbuchenstreu in mesophilen Laubwäldern Ungarns. Acta Zool. Acad. Sci. Hung. 24, 321–330.

Dózsa-Farkas, K., 1978b. Nahrungswahluntersuchungen mit der Enchytraeiden-Art *Fridericia galba* (Hoffmeister, 1843)(Oligochaeta: Enchytraeidae). Opusc. Zool. Budapest 15, 75–82.

Dózsa-Farkas, K., 1982. Konsum verschiedener Laubarten durch Enchytraeiden.

59:2642076189

Pedobiologia 23, 251–255.
Egert, M., Marhan, S., Wagner, B., Scheu, S., Friedrich, M.W., 2004. Molecular profiling of 16S rRNA genes reveals diet-related differences of microbial communities in soil, gut, and casts of *Lumbricus terrestris* L. (Oligochaeta: Lumbricidae). FEMS Microbiol. Ecol. 48, 187–197.
Eisenhauer, N., Schädler, M., 2011. Inconsistent impacts of decomposer diversity on the stability of aboveground and belowground ecosystem functions. Oecologia 165, 403–415.
Erséus, C., Rota, E., Matamoros, L., De Wit, P., 2010. Molecular phylogeny of Enchytraeidae (Annelida, Clitellata). Mol. Phylogenet. Evol. 57, 849–858.
Erséus, C., 2005. Phylogeny of oligochaetous Clitellata. Hydrobiologia 535/536, 357–372.
Esser, R.P., Simpson, S.E., 1994. Enchytraeids. Fla. Dept. Agric. & Consumer Serv. Div. of Plant Industry. Nematology Cir. 207, 3.
Fernández, P.V., Estevez, J.M., Cerezo, A.S., Ciancia, M., 2012. Sulfated β-D-mannan from green seaweed *Codium vermilara*. Carbohydr. Polym. 87, 916–919.
Flärdh, K., Buttner, M.J., 2009. *Streptomyces* morphogenetics: dissecting differentiation in a filamentous bacterium. Nat. Rev. Microbiol. 7, 36–49.
Forbes, S.L., Carter, D.O., 2016. Processes and mechanisms of death and decomposition of vertebrate carrion. In: Benbow, M.E., Tomberlin, J.K., Taron, A. (Eds.), Carrion Ecology, Evolution, and Their Applications. CRC Press, Boca Raton, pp. 13–30.
Friend, H., 1902. Studies in Irish enchytraeids. Ir. Nat. J. 11, 110–115.
Friend, H., 1916. The action of enchytraeid worms. Ann. Appl. Biol. 3, 49–51.
Gelder, S.R., 1984. Diet and histophysiology of the alimentary canal of *Lumbricillus lineatus* (Oligochaeta, Enchytraeidae). Hydrobiologia 115, 71–81.
Gerken, H.G., Donohoe, B., Knoshaug, E.P., 2013. Enzymatic cell wall degradation of *Chlorella vulgaris* and other microalgae for biofuels production. Planta 237, 239–253.
Giere, O., Hauschildt, D., 1979. Experimental studies on the life cycle and production of the littoral oligochaete *Lumbricillus lineatus* and its response to oil pollution. In: Naylor, E., Hartnall, R.G. (Eds.), Cyclic Phenomena in Marine Plants and Animals. Pergamon, Oxford, pp. 113–122.
Giere, O., Pfannkuche, O., 1982. Biology and ecology of marine oligochaeta, a review. In: In: Barnes, M. (Ed.), Oceanography and Marine Biology Vol. 20. Aberdeen Univeristy Press, Aberdeen, pp. 173–308.
Giere, O., 1975. Population structure, food relations and ecological role of marine oligochaetes, with special reference to meiobenthic species. Mar. Biol. 31, 139–156.
Giere, O., 1980. Tolerance and preference reactions of marine Oligochaeta in relation to their distribution. In: Brinkhurst, R.O., Cook, D.G. (Eds.), Aquatic Oligochaete Biology. Plenum Press, New York, pp. 385–409.
Goodman, D., 1971. Ecological Investigations of Iceworms on Casement Glacier, Southeastern Alaska. Report No. 39. Institute of Polar Studies, Columbus: Ohio State University Research Foundation.
Gotthold, M.L., Koch, J., 1974. Further development of an artificial medium for the axenically cultured annelid *Enchytraeus fragmentosus*. Comp. Biochem. Physiol. 48, 307–314.
Gotthold, M.L., Brody, B., Stokstad, E., 1967. Axenic cultivation of the microannelid *Enchytraeus fragmentosus* (Bell, 1959). Comp. Biochem. Physiol. 21, 75–81.
Haimi, J., Siira-Pietikäinen, A., 2003. Activity and role of the enchytraeid worm *Cognettia sphagnetorum* (Vejd.) (Oligochaeta: Enchytraeidae) in organic and mineral forest soil. Pedobiologia 47, 303–310.
Hamm, P.B., Campbell, S.J., Hansen, E.M., 1990. Growing Healthy Seedlings: Identification and Management of Pests in Northwest Forest Nurseries. Special publication 19. Forest Research Laboratory, Oregon State University, Corvallis.
Harker, A., 1889. A new pest of farm crops. Nature 40, 11–12.
Harris, E.H., 2009. The *Chlamydomonas* Sourcebook: Introduction to *Chlamydomonas* and Its Laboratory Use, vol. 1 Academic Press, Oxford.
Hartzell, P.L., Shain, D.H., 2009. Glacier ice worms. In: Shain, D.H. (Ed.), Annelids in Modern Biology. Wiley-Blackwell, Hoboken, pp. 301–313.
Head, G.C., 1968. Studies of growing roots by time-lapse cinematography. In: Holmes, J.W. (Ed.), Trans. 9th Int. Congr. Soil Sci. Adelaide. pp. 751–758.
Healy, B., Walters, K., 1994. Oligochaeta in Spartina stems: the microdistribution of Enchytraeidae and Tubificidae in a salt marsh Sapelo Island, USA. Hydrobiologia 278, 111–123.
Hebert, P.D.N., Cywinska, A., Ball, S.L., de Waard, J.R., 2003. Biological identifications through DNA barcodes. Proc. Biol. Sci. 270, 313–321.
Hedlund, K., Augustsson, A., 1995. Effects of enchytraeid grazing on fungal growth and respiration. Soil Biol. Biochem. 27, 905–909.
Hendrix, P.F., Parmelee, R.W., Crossley Jr, D.A., Coleman, D.C., O'dum, E.P., et al., 1986. Detritus food webs in conventional and no-tillage agroecosystems. Bioscience 36, 374–380.
Hewitt, G.C., 1908. On an enchytraeid worm injurious to the seedlings of the larch. J. Econ. Biol. 3, 43–45.
Hoiczyk, E., Hansel, A., 2000. Cyanobacterial cell walls: news from an unusual prokaryotic envelope. J. Bacteriol. 182, 1191–1199.
House, G.J., Parmelee, R.W., 1985. Comparison of soil arthropods and earthworms from conventional and no-tillage agroecosystems. Soil Till. Res. 5, 351–360.
Hyvönen, R., Andersson, S., Clarholm, M., Persson, T., 1994. Effects of lumbricids and enchytraeids on nematodes in limed and unlimed coniferous mor humus. Biol. Fertil. Soils 17, 201–205.
Jaffee, B.A., Muldoon, A.E., Didden, W.A.M., 1997. Enchytraeids and nematophagous fungi in soil microcosms. Biol. Fertil. Soils 25, 382–388.
Jegen, G., 1920. Zur Biologie und Anatomie einiger Enchytraeiden. Vierteljahrschr. naturforsch. Ges. Zürich 65, 100–208.
Kirk, R.G., 1971. Reproduction of *Lumbricillus rivalis* (Levinsen) in laboratory cultures and in decaying seaweed. Ann. Appl. Biol. 67, 255–264.
Koehler, A.V.A., Hoberg, E.P., Dokuchaev, N.E., Cook, J.A., 2007. Geographic and host range of the nematode *Soboliphyme baturini* across Beringia. J. Parasitol. 93, 1070–1083.
Kornobis, F.W., 2008. Trophic types of the nematodes. Wiad. Parazytol. 54, 1–9.
Krištůfek, V., Hallmann, M., Westheide, W., Schrempf, H., 1995. Selection of various streptomyces species by *Enchytraeus crypticus* (Oligocheta). Pedobiologia 39, 547–554.
Krištůfek, V., Lukešová, A., Nováková, A., 1997. Soil microorganisms as source of food for enchytraeids (Annelida, Enchytraeidae). In: Ďugová, O. (Ed.), Life in Soil. Czechoslovak Society for Microbiology, Institute of Microbiology SAS, Bratislava, pp. 41–42.
Krištůfek, V., Fischer, S., Buhrmann, J., Zeltins, A., Schrempf, H., 1999. In situ monitoring of chitin degradation by *Streptomyces lividans* pCHIO12 within *Enchytraeus crypticus* (Oligochaeta) feeding on *Aspergillus proliferans*. FEMS Microbiol. Ecol. 28, 41–48.
Krištůfek, V., Nováková, A., Pižl, V., 2001. Coprophilous streptomycetes and fungi − food sources for enchytraeid worms (Enchytraeidae). Folia Microbiol. 46, 555–558.
Krištůfek, V., Elhottová, D., Šustr, V., Lasák, R., Kováč, L., et al., 2005. Does the cellular fatty acid and enzyme content of cave bacteria affect the feeding preferences of *Enchytraeus crypticus* (Oligochaeta, Enchytraeidae)? In: Tajovský, K., Schlaghamerský, J., Pižl, V. (Eds.), Proceedings of the 7th Central European Workshop on Soil Zoology. České Budějovice. pp. 71–75.
Kühnelt, W., 1961. Soil Biology. Faber and Faber, London.
Kurir, A., 1964. *Fridericia galba* (Enchytraeidae) als Fichtenschädling in einem Forstgarten. Pedobiologia 4, 269–280.
Lagerlöf, J., Andrén, O., 1985. Succession and activity of microarthropods and enchytraeids during barley straw decomposition. Pedobiologia 28, 343–357.
Lagerlöf, J., Andrén, O., Paustian, K., 1989. Dynamics and contribution to carbon flows of Enchytraeidae (Oligochaeta) under four cropping systems. J. Appl. Ecol. 26, 183–199.
Larsen, T., Pollierer, M.M., Holmstrup, M., D'Annibale, A., Maraldo, K., et al., 2016a. Substantial nutritional contribution of bacterial amino acids to earthworms and enchytraeids: a case study from organic grasslands. Soil Biol. Biochem. 99, 21–27.
Larsen, T., Ventura, M., Maraldo, K., Triadó-Margarit, X., Casamayor, E.O., et al., 2016b. The dominant detritus-feeding invertebrate in Arctic peat soils derives its essential amino acids from gut symbionts. J. Anim. Ecol. 85, 1275–1285.
Latter, P.M., Howson, G., 1978. Studies on the microfauna of blanket bog with particular reference to Enchytraeidae: II. Growth and survival of *Cognettia sphagnetorum* on various substrates. J. Anim. Ecol. 47, 425–448.
Latter, P.M., 1977. Axenic cultivation of an enchytraeid worm, *Cognettia sphagnetorum*. Oecologia 31, 251–254.
Laverack, M.S., 1960. Tactile and chemical perception in earthworms. 1. Responses to touch sodium chloride, quinine and sugars. Comp. Biochem. Physiol. 1, 155–163.
Learner, M.A., 1972. Laboratory studies on the life histories of four enchytraeid worms (Oligochaeta) which inhabit sewage percolating filters. Ann. Appl. Biol. 70, 251–266.
Lindfeld, A., Lang, C., Knop, E., Nentwig, W., 2011. Hard to digest or a piece of cake? Does GM wheat affect survival and reproduction of *Enchytraeus albidus* (Annelida: Enchytraeidae). Appl. Soil Ecol. 47, 51–58.
Lukešová, A., Frouz, J., 2007. Soil and freshwater micro-algae as a food source for invertebrates in extreme environments. In: Seckbach, J. (Ed.), Algae and Cyanobacteria in Extreme Environments. Springer, Dordrecht, pp. 265–284.
Mabeau, S., Kloareg, B., 1987. Isolation and analysis of the cell walls of brown algae: *Fucus spiralis*, *F. ceranoides*, *F. vesiculosus*, *F. serratus*, *Bifurcaria bifurcata* and *Laminaria digitata*. J. Exp. Bot. 38, 1573–1580.
MacLean Jr, S.F., 1980. The detritus-based trophic system. In: Brown, J., Miller, P.C., Tieszen, L.L., Bunnell, F.L. (Eds.), An Arctic Ecosystem: the Coastal Tundra at Barrow, Alaska. Stroudsburg: US/IBP Synthesis Series 12. Hutchinson and Ross, Dowden, pp. 411–457.
Maggenti, A.R., 1981. General Nematology. Springer-Verlag, New York.
Manetti, P.L., López, A.N., Clemente, N.L., Faberi, A.J., 2010. Tillage system does not affect soil macrofauna in southeastern Buenos Aires province, Argentina. Span. J. Agric. Res. 8, 377–384.
Maraldo, K., Christensen, B., Holmstrup, M., 2011. The excretion of ammonium by enchytraeids (*Cognettia sphagnetorum*). Soil Biol. Biochem. 43, 991–996.
Maraun, M., Martens, H., Migge, S., Theenhaus, A., Scheu, S., 2003. Adding to the enigma of soil animal diversity: fungal feeders and saprophagous soil invertebrates prefer similar food substrates. Eur. J. Soil 39, 85–95.
Martinsson, S., Erséus, C., 2014. Cryptic diversity in the well-studied terrestrial worm *Cognettia sphagnetorum* (Clitellata: Enchytraeidae). Pedobiologia 57, 27–35.
Martinsson, S., Rota, E., Erséus, C., 2015. Revision of *Cognettia* (Clitellata, Enchytraeidae): reestablishment of *Chamaedrilus* and description of cryptic species in the *sphagnetorum* complex. Syst. Biodiver. 13, 257–277.
McSorley, R., Wang, K.-H., Kokalis-Burelle, N., Church, G., 2006. Effects of soil type and steam on nematode biological control potential of the rhizosphere community. Nematropica 36, 197–214.
Mellin, A., 1990. Quantitative Frassleistung von *Mesenchytraeus glandulosus* (Annelida, Oligochaeta). Verh. Ges. Ökol. 19, 192–199.
Michaelsen, W., 1927. Oligochaeta. In: Grimpe, G., Wagler, E. (Eds.), Tierwelt der Nord- und Ostsee. VI (c1), 9. Akademische Verlagsgesellschaft mbH, Leipzig, pp. 1–44.
Michel, G., Tonon, T., Scornet, D., Cock, J.M., Kloareg, B., 2010. The cell wall polysaccharide metabolism of the brown alga *Ectocarpus siliculosus.* Insights into the evolution of extracellular matrix polysaccharides in Eukaryotes. New Phytol. 188, 82–97.
Murakami, T., Segawa, T., Bodington, D., Roman, D., Takeuchi, N., et al., 2015. Census of bacterial microbiota associated with the glacier ice worm *Mesenchytraeus solifugus*. FEMS Microbiol. Ecol. http://dx.doi.org/10.1093/femsec/fiv003.
Nekrasova, K.A., Domracheva, L.I., 1972. Importance of the investigation of soil invertebrates in the quantification of soil algae. Methods of Studying and Practical

Application of Soil Algae. Report Kirov Agric. Inst., Kirov, pp. 175–181.
Nekrasova, K.A., Kozlovskaya, L.S., Domracheva, L.I., Shtina, E.A., 1976. The influence of invertebrates on the development of algae. Pedobiologia 16, 286–297.
Nielsen, C.O., 1962. Carbohydrases in soil and litter invertebrates. Oikos 13, 200–215.
Nieminen, J.K., 2009. Are spruce boles hot spots for enchytraeids in clear-cut areas? Boreal Environ. Res 14, 382–388.
Nobles, D.R., Romanovicz, D.K., Brown, R.M., 2001. Cellulose in cyanobacteria. Origin of vascular plant cellulose synthase? Plant Physiol. 127, 529–542.
Nowak, E., Piotrowska-Seget, Z., 2005. Relationship between *Cognetia sphagnetorum* Vejd. (Oligochaeta: Enchytraeidae) and soil microorganisms: a microcosm experiment. Pol. J. Ecol. 53, 215–223.
Nowak, E., Piotrowska-Seget, Z., Chmielewski, K., 2005. Response of enchytraeid community (Oligochaeta, Enchytraeidae) to manipulation of microbial biomass. Pol. J. Ecol. 53, 53–63.
Nozaki, M., Miura, C., Tozawa, Y., Miura, T., 2009. The contribution of endogenous cellulase to the cellulose digestion in the gut of earthworm (*Pheretima hilgendorfi*: Megascolecidae). Soil Biol. Biochem. 41, 762–769.
O'Connor, F.B., 1967. The Enchytraeidae. In: Burges, A., Raw, F. (Eds.), Soil Biology. Academic Press, London, pp. 227–230.
Ortiz, J.M., Berkeley, R.C.W., Brewer, S.J., 1973. Production of exo-β-N-acetylglucosaminidase by *Bacillus subtilis* B. J. Gen. Microbiol. 77, 331–337.
Palka, J., Spaul, E.A., 1970. Studies on feeding and digestion in the enchytraeid worm *Lumbricillus lineatus* Müll. in relation to its activity in sewage bacteria beds. Proceedings of the Leeds Philosophical and Literary Society, Scientific Section 10 45–59.
Parmelee, R.W., Beare, M.H., Cheng, W., Hendrix, P.F., Crossley Jr, D.A., 1990. Earthworms and enchytraeids in conventional and no-tillage agroecosystems: a biocide approach to assess their role in organic matter breakdown. Biol. Fert. Soils 10, 1–10.
Persson, T., Bååth, E., Clarholm, M., Lundkvist, H., Söderström, B.E., et al., 1980. Trophic structure, biomass dynamics and carbon metabolism of soil organisms in a Scots pine forest. Ecol. Bull. 32, 419–459.
Plante, C.J., Shriver, A.G., 1998. Differential lysis of sedimentary bacteria by *Arenicola marina* L.: examination of cell wall structure and exopolymeric capsules as correlates. J. Exp. Mar. Biol. Ecol. 229, 35–52.
Ponge, J.F., 1991. Food resources and diets of soil animals in a small area of Scots pine litter. Geoderma 49, 33–62.
Ponge, J.F., 2010. The soil under the microscope: the optical examination of a small area of Scots pine litter (*Pinus sylvestris* L.). Museum National d'Histoire Naturelle, Paris HAL-00488081.
Puppe, D., Schrader, S., Giesemann, A., Gebauer, G., 2012. Isotopic labelling of enchytraeids under FACE conditions: a possible way to analyse the residue-enchytraeid-soil system considering elevated atmospheric CO_2 concentrations. Landbauforschung − vTI Agric. For. Res. 357, 21–26 Secial Issue.
Röhrig, R., Langmaack, M., Schrader, S., Larink, O., 1998. Tillage systems and soil compaction − their impact on abundance and vertical distribution of Enchytraeidae. Soil Till. Res. 46, 117–127.
Rahman, M.M., Inoue, A., Ojima, T., 2014. Characterization of a GHF45 cellulase, AkEG21, from the common sea hare *Aplysia kurodai*. Front. Chem. 2, 1–13.
Rashed, H.A., Szabó, I.M., Dózsa-Farkas, K., 1992. On the composition of the intestinal microbiota of *Fridericia hegemon* (Enchytraeidae). Soil Biol. Biochem. 24, 1291–1294.
Reichenbach, H., 2006. The order Cytophagales. In: In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E. (Eds.), The Prokaryotes: a Handbook on the Biology of Bacteria Vol. 7. Springer-Verlag, New York, pp. 549–590.
Reichert, A., Mothes-Wagner, U., Seitz, K.A., 1996. Ecohistological investigation of the feeding behaviour of the enchytraeid *Enchytraeus coronatus* (Annelida, Oligochaeta). Pedobiologia 40, 118–133.
Remén, C., Fransson, P., Persson, T., 2010. Population responses of oribatids and enchytraeids to ectomycorrhizal and saprotrophic fungi in plant − soil microcosms. Soil Biol. Biochem. 42, 978–985.
Reynoldson, T.B., 1939. Enchytraeid worms and the bacteria bed method of sewage treatment. Ann. Appl. Biol. 26, 138–164.
Rodriguez, P., Arrate, J.A., Martinez-Madrid, M., 2002. Life history of the oligochaete *Enchytraeus coronatus* (Annelida, Enchytraeidae) in agar culture. Invertebr. Biol. 121, 350–356.
Sami, A.J., Anwar, M.A., Rehman, F.U., Shakoori, A.R., 2011. Digestive cellulose hydrolyzing enzyme activity (endo- β-1, 4- D-glucanase) in the gut and salivary glands of blister beetle, *Mylabris pustulata*. Pak. J. Zool. 43, 393–401.
Schaerffenberg, B., Tendl, H., 1951. Untersuchungen über das Verhalten der Enchytraeiden gegenüber dem Zuckerrübennematoden *Heterodera schachtii* (Schm.). Z. Angew. Entomol. 32, 476–488.
Schaerffenberg, B., 1950. Untersuchungen über die Bedeutung der Enchytraeiden als Humusbildner und Nematodenfeinde. Z. Pflanzenkrankh. Pfl. schutz 57, 183–191.
Scheu, S., Falca, M., 2000. The soil food web of two beech forests (*Fagus sylvatica*) of contrasting humus type: stable isotope analysis of a macro- and mesofauna-dominated community. Oecologia 123, 285–296.
Schlaghamerský, J., Krawczynski, R., 2015. Does carcass decomposition affect soil-dwelling enchytraeids? Soil Organ. 87, 91–100.
Schlegel, M., Steinbrück, G., Kramer, M., Brockmeyer, V., 1991. Restriction fragment patterns as molecular markers for species identification and phylogenetic analysis in the genus *Enchytraeus* (Oligochaeta). Z. Zool. Syst. Evol. 29, 362–372.
Schmelz, R.M., Collado, R., 2010. A guide to European terrestrial and freshwater species of Enchytraeidae (Oligochaeta). Soil Organ. 82, 1–176.
Schmelz, R.M., Niva, C.C., Römbke, J., Collado, R., 2013. Diversity of terrestrial Enchytraeidae (Oligochaeta) in Latin America: current knowledge and their potential for future research. Appl. Soil Ecol. 69, 13–20.
Schmidt, O., Curry, J.P., Dyckmans, J., Rota, E., Scrimgeour, C.M., 2004. Dual stable isotope analysis ($\delta^{13}C$ and $\delta^{15}N$) of soil invertebrates and their food sources. Pedobiologia 48, 171–180.
Schöne, C., 1971. Über den Einfluß von Nahrung und Substratsalinität auf Verhalten auf Verhalten, Fortpflanzung und Wasserhaushalt von *Enchytraeus albidus*enle (Oligochaeta). Oecologia 6, 254–266.
Seipke, R.F., Kaltenpoth, M., Hutchings, M.I., 2012. *Streptomyces* as symbionts; an emerging and widespread theme? FEMS Microbiol. Rev. 36, 862–876.
Severon, T., Joschko, M., Barkusky, D., Graefe, U., 2012. The impact of conventional and reduced tillage on the Enchytraeidae population in sandy soil and their correlation with plant residue and earthworms. Landbauforschung − vTI Agric. For. Res. 357, 45–52 Special Issue.
Shain, D.H., Mason, T.A., Farrell, A.H., Michalewicz, L.A., 2001. Distribution and behavior of ice worms (*Mesenchytraeus solifugus*) in south-central Alaska. Can. J. Zool. 79, 1813–1821.
Shtina, E.A., Kozlovskaya, L.S., Nekrasova, K.A., 1981. Relations of soil oligochaetes and algae. Ekologiya 1, 55–60.
Springett, J.A., Latter, P.M., 1977. Studies on the microfauna of blanket bog with particular reference to Enchytraeidae. I. Field and laboratory tests of micro-organisms as food. J. Anim. Ecol. 46, 959–974.
Springett, J.A., 1964. A method for culturing Enchytraeidae. Oikos 15, 175–177.
Springett, J.A., 1967. An Ecological Study of Moorland Enchytraeidae. Durham University, Durham (Ph.D. thesis).
Standen, V., Latter, P.M., 1977. Distribution of a population of *Cognettia sphagnetorum* (Enchytraeidae) in relation to microhabitats in a blanket bog. J. Anim. Ecol. 46, 213–229.
Standen, V., 1978. The influence of soil fauna on decomposition by micro-organisms in blanket bog litter. J. Anim. Ecol. 47, 25–38.
Stark, C.J., Bonocora, R.P., Hoopes, J.T., Nelson, D.C., 2010. Bacteriophage lytic enzymes as antimicrobials. In: Sabour, P.M., Griffiths, M.W. (Eds.), Bacteriophages in the Control of Food-and Waterborne Pathogens. ASM Press, Washington, pp. 137–156.
Stephenson, J., 1922. The Oligochaeta of the Oxford University Spitsbergen expedition. Proc. Zool. Soc. Lond. 74, 1109–1138.
Ştefan, V., 1990. Some studies on the relations between Enchytraeidae and soil microflora. Rev. Roum. Biol. Anim. (ser. Biol. Anim.) 35, 101–104.
Šustr, V., Chalupský, J., 1996. Activity of digestive enzymes in two species of potworms (Oligochaeta, Enchytraeidae). Pedobiologia 40, 255–259.
Šustr, V., Chalupský, J., Krištůfek, V., 1997. Effects of artificial acidification and liming on the activity of digestive enzymes in *Cognettia sphagnetorum* (Vejdovský, 1878) (Annelida, Enchytraeidae). Biol. Fertil. Soils 24, 227–230.
Stephenson, J., 1930. The Oligochaeta. University Press, Oxford: Oxford.
Stoklasa, J., 1897. Sind die Enchytraeiden Parasiten der Zuckerrürbe? (Mitteilungen aus der Versuchsstation für Zuckerindustrie in Prag). Zbl. Bakt. Parasitenkd. Abt. 3 (2), 108–110.
Torii, T., 2015. Descriptions of two new and one newly recorded enchytraeid species (Clitellata, Enchytraeidae) from the Ozegahara Mire, a heavy snowfall highmoor in Central Japan. Zootaxa 4000, 473–482.
Toutain, F., Villemin, G., Albrecht, A., Reisinger, O., 1982. Etude ultrastructurale des processus de biodègradation: II. Modéle enchytraeides-litière de feuillus. Pedobiologia 23, 145–156.
Tynen, M.J., 1969. Littoral distribution of *Lumbricillus reynoldsoni* Backlund and other Enchytraeidae (Oligochaeta) in relation to salinity and other factors. Oikos 20, 41–53.
Urbášek, F., Chalupský Jr, J., 1991. Activity of digestive enzymes in four species of Enchytreideae (Oligochaeta). Rev. Ecol. Biol. Sol. 28, 145–154.
Urbášek, F., Chalupský Jr, J., 1992. Effects of artificial acidification and liming on biomass and on the activity of digestive enzymes in Enchytraeidae (Oligochaeta): Results of an ongoing study. Biol. Fertil. Soils 14, 67–70.
Vaçulik, A., Kounda-Kiki, C., Sarthou, C., Ponge, J.F., 2004. Soil invertebrate activity in biological crusts on tropical inselbergs. Eur. J. Soil Sci. 55, 539–549.
van Capelle, C., Schrader, S., Brunotte, J., 2012. Tillage induced changes in functional diversity of soil biota − A review with a focus on German data. Eur. J. Soil Biol. 50, 165–181.
Vanha, J., Stoklasa, J., 1896. Die Rüben-Nematoden (*Heterodera, Dorylaimus* und *Tylenchus*) mit Anhang über die Enchytraeiden. Verlagsbuchhandlung Paul Parey, Berlin.
Vavoulidou, E., Römbke, J., Sidiras, N., Bilasis, D., Tsigo, R., 1999. Effects of three different soil cultivation and fertilization treatments on earthworms and enchytraeids. In: Schmelz, R.M., Sühlo, K. (Eds.), Newsletter on Enchytraeidae No. 6, Proceedings of the 3rd International Symposium on Enchytraeidae. Osnabrück. pp. 91–100.
Vivien, R., Wyler, S., Lafont, M., Pawlowski, J., 2015. Molecular barcoding of aquatic oligochaetes: implications for biomonitoring. PLoS One. http://dx.doi.org/10.1371/journal.pone.0125485.
Vollmer, W., Joris, B., Charlier, P., Foster, S., 2008. Bacterial peptidoglycan (murein) hydrolases. FEMS Microbiol. Rev. 32, 259–286.
Watzinger, A., 2015. Microbial phospholipid biomarkers and stable isotope methods help reveal soil functions. Soil Biol. Biochem. 86, 98–107.
Westheide, W., Bethke-Beilfuss, D., 1991. The sublethal enchytraeid test system: guidelines and some results. In: Esser, G., Overdiek, D. (Eds.), Modern Ecology: Basic and Applied Aspects. Elsevier, Amsterdam, pp. 497–508.
Westheide, W., Bethke-Beilfuss, D., Gebbe, J., 1991. Effects of benomyl on reproduction and population structure of enchytraeid oligochaetes (Annelida) − Sublethal tests on agar and soil. Comp. Biochem. Physiol. C 100, 221–224.
Whitfield, D.W.A., 1977. Energy budgets and ecological efficiencies on Truelove Lowland. In: Bliss, L.C. (Ed.), Truelove Lowland, Devon Island, Canada: A High Arctic Ecosystem. University of Alberta Press, Edmonton, pp. 607–620.

61:7808343995

Whitley, L.S., Seng, T.N., 1976. Studies on the bacterial flora of tubificid worms. Hydrobiologia 48, 79–83.
Wolters, V., 1988. Effects of *Mesenchytraeus glandulosus* (Oligochaeta, Enchytraeidae) on decomposition processes. Pedobiologia 32, 387–398.
Zachariae, G., 1963. Was leisten Collembolen für den Waldhumus? In: Doeksen, J., van der Drift, J. (Eds.), Soil Organisms. North-Holland Publishing Co., Amsterdam, pp. 109–124.
Zhao, C., Li, Z., Li, T., Zhang, Y., Bryant, D.A., Zhao, J., 2015. High-yield production of extracellular type-cellulose by the cyanobacterium *Synechococcus* sp. PCC 7002. Cell Discovery. http://dx.doi.org/10.1038/celldisc.2015.4.
Zimmermann, A., 1899. Over de Enchytraeiden en haar Vorkommen in de Koffiewortels (Über die Enchyträiden und ihr Vorkommen in Kaffeewurzeln). Z. Pflanzenkr. Organ für die Gesamtinteressen des Pflanzenschutzes 9, 168–170.
Zimmermann, P., 1976. Mechanische Einflüsse von *Enchytraeus albidus* auf organische und mineralische Komponenten des Bodens. University of Düsseldorf, Düsseldorf, Germany (Diploma thesis).
Zirbes, L., Mescher, M., Vrancken, V., Wathelet, J.P., Verheggen, F.J., et al., 2011. Earthworms use odor cues to locate and feed on microorganisms in soil. PLoS One. http://dx.doi.org/10.1371/journal.pone.0021927.

62:3088909570

Biochimie 221 (2024) 38–59

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Discovery and characterization of the α-amylases cDNAs from *Enchytraeus albidus* shed light on the evolution of "*Enchytraeus-Eisenia* type" Amy homologs in Annelida

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ARTICLE INFO

Article history:
Received 17 May 2023
Received in revised form
2 December 2023
Accepted 13 January 2024
Available online 17 January 2024

Handling Editor: Dr Y Nakamura

Keywords:
Potworms
Alpha-amylase
Polymorphism
Flexible loop
Annelida phylogeny

ABSTRACT

Although enchytraeids have gained popularity in scientific research, fundamental questions regarding their feeding ecology and biology remain largely unexplored. This study investigates α-amylases, major digestive enzymes responsible for hydrolyzing starch and similar polysaccharides into sugars, in *Enchytraeus albidus*. Genetic data related to α-amylases is currently lacking for the family Enchytraeidae but also for the entire Annelida. To detect and identify coding sequences of the expressed α-amylase genes in COI-monohaplotype culture (PL-A strain) of *E. albidus*, we used classical "gene fishing" and transcriptomic approaches. We also compared coding sequence variants of α-amylase retrieved from transcriptomic data related to freeze-tolerant strains. Our results reveal that *E. albidus* possesses two distinct α-amylase genes (Amy I and Amy II) that are homologs to earthworm *Eisenia fetida* Ef-Amy genes. Different strains of *E. albidus* possess distinctive alleles of α-amylases with unique SNP patterns specific to a particular strain. Unlike Amy II, Amy I seems to be a highly polymorphic and multicopy gene. The domain architecture of the putative Amy proteins was found the same as for classical animal α-amylases with ABC-domains. A characteristic feature of Amy II is the lack of GHGA motif in the flexible loop region, similarly to many insect amylases. We identified "*Enchytraeus-Eisenia* type" α-amylase homologs in other clitellates and polychaetes, indicating the ancestral origin of Amy I/II proteins in Annelida. This study provides the first insight into the endogenous non-proteolytic digestive enzyme genes in potworms, discusses the evolution of Amy α-amylases in Annelida, and explores phylogenetic implications.

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1. Introduction

Enchytraeids, commonly known as potworms, play a crucial role in the decomposition of organic matter and soil structure formation [1]. In recent years, a growing interest in enchytraeids, especially of the genus *Enchytraeus*, as model organisms can be observed, *inter alia*, in ecological and ecotoxicological studies [2]. However, the trophic biology of this annelid group is still poorly understood. A recent review paper on enchytraeids' feeding preferences revealed that many characteristics of their trophic biology remain disputable and need further study [3]. One of the important long-standing issues, which exploration has been ceased for circa two decades, is the digestive capacity of enchytraeids. To date, digestive enzyme activities in enchytraeids have been studied based on traditional biochemical [4–9] or histochemical methods [10]. However, in many mesofaunal invertebrates the contribution of microbial enzymatic apparatus cannot be excluded, and the activity of the microflora can even conceal the endogenous digestive capacity of animals [3,7,129]. While earlier studies had indicated that enchytraeids are capable of digesting proteins, disaccharides, and some polysaccharides, no attempt has been made to distinguish whether the origin of digestive enzymes is the enchytraeids themselves or microorganisms [6–9]. To our knowledge, genetic techniques have not been previously used to detect the endogenous expression of digestive enzyme genes *per se* in enchytraeids. Among potworms, *Enchytraeus albidus* is one of the most known and ecologically relevant soil and marine littoral species [11]. Apart from being a subject of numerous different biological research including

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https://doi.org/10.1016/j.biochi.2024.01.008
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developmental biology (e.g. Ref. [12]) and aquaculture [13,14], it has been employed (alongside with *Enchytraeus crypticus*) as a standard test organism for environmental risk assessment (e.g. OECD Test No. 220: Enchytraeid Reproduction Test) [15]. Even though it is a scientifically and economically important annelid species, data regarding its digestive capacity is still very scarce and limited to only a few old studies [4,6,16]. It is worth noting that a recent molecular taxonomy study has revealed that, in fact, *E. albidus* was a cryptic complex of at least nine morphologically similar and closely related species [11,17]. This potentially complicates the drawing of reliable conclusions related to the digestive capacity of particular species. In the last review [3], it was suggested that the discovery of cryptic diversity in another popular model enchytraeid *Cognettia sphagnetorum* complex (now *Chamaedrilus* spp.) could explain differences obtained among several experiments related to its ecology and food preferences. The situation can be similar in the case of previously unrecognized species within *E. albidus* complex [11]. Even closely related species can possess different enzyme repertoires, including α-amylase arrangements [18]. Although α-amylases are generally known to be nonexclusively widespread in the animal kingdom, and their genomic or coding sequences have been identified in many groups of invertebrates, surprisingly, there is a substantial lack of that kind of data related not only to Enchytraeidae family but to entire phylum Annelida. To date, α-amylases from annelids seem to be more deeply studied only in the earthworm *Eisenia fetida* [19,20], while coding sequences of these important enzymes just for a few polychaetes are readily available in public databases. Because of data scarcity, Annelida amylase sequences are not well represented [21,22] and are only used marginally in protein evolution studies [23–25]. Thus, the evolutionary relationships of amylases in annelids remain unsolved.

Here, we present the first report of complete coding sequences (complete CDSs) for non-proteolytic digestive enzyme genes in representatives of Enchytraeidae family. We performed molecular cloning and characterization of coding sequences of the α-amylase genes from *Enchytraeus albidus*. In addition to the classical approach, we performed *de novo* transcriptome assembly of *E. albidus* PL-A specimens originating from COI-monohaplotype culture and retrieved additional coding sequence variants of α-amylase genes (Amy I and Amy II) from transcriptomic data related to freeze-tolerant German (G) and Greenlandic (N) strains [26], and compared all sequences across the strains. Secondly, we present a basic bioinformatic characterization of the predicted α-amylase proteins of *E. albidus*, including structural modeling. Moreover, we established an evolutionary context for homologous Amy proteins by retrieving close orthologs expressed in other annelid species and integrating them into the maximum likelihood tree, trying to answer the question of their origin and evolution. Additionally, we found that the phylogenetic analysis of annelid α-amylases, neglected genes within a neglected group expressing important digestive enzymes, could provide a compelling opportunity to contribute to the ongoing heated debate concerning the positioning of certain taxa and the overall phylogeny of Annelida.

2. Material and methods

2.1. Animal material

Adult specimens of *E. albidus* from COI-barcoded (Acc. MK044803-MK044805) lab culture were used for DNA & RNA extraction. Animals were kept in a small plastic box filled with defaunized garden soil and fed twice weekly with fish food flakes. Lab culture (mixed population) has been established from stock culture purchased from the commercial seller. The same commercial source has been previously chosen by Urbisz and co-workers [12]. The COI-monohaplotype culture (PL-A strain) was derived from a single cocoon incubated on a non-nutrient agarose plate. After hatching, specimens were transferred from agarose medium to defaunized garden soil and maintained as described above.

2.2. DNA barcoding of the lab and monohaplotype cultures

The DNA extraction was performed with GeneMatrix Tissue DNA Purification Kit (EURx) according to manufacturer's protocol for fresh animal tissue with Proteinase K and RNase A digestion step. PCR amplification of cytochrome *c* oxidase subunit I (COI) fragment was performed using Color OptiTaq PCR Master Mix (EURx), with the following two "universal" primers: LCO1490 and HCO2198 [128] and 1 μl of extracted DNA as a template in a total volume of 50 μl. Reactions were proceeded in Biometra thermocycler with thermal cycling conditions set up as described by Martinsson and Erséus [27]. To confirm amplification, PCR products were run on a 1.2 % agarose gel in TBE buffer with the addition of SimplySafe (EURx). Amplification products were sent to GenoMed (Warsaw, Poland) and sequenced in both directions.

2.3. RNA extraction and cDNA synthesis for a classical approach

Before RNA extraction potworms were transferred to non-nutrient 1.2 % agar in tap-water plates for a time to empty gut contents which can affect RNA quality or interfere with cDNA synthesis/PCR assay. Then, individuals were killed by immersion into Fix RNA reagent (EURx, Poland) in microcentrifuge tubes. Fixed specimens were carefully separated from fixative before proceeding with RNA purification steps by pipettor. Total RNA was extracted from the whole specimens with GeneMATRIX Universal RNA Purification Kit (EURx, Poland) according to manufacturer protocol for fresh animal tissue and manual homogenization. Initially, one to five individuals were used per pooled sample from mixed culture (5 samples, 15 specimens total), while five single PL-A strain specimens were used for PCR validation of RNA-seq data. The quality and concentration of extracted RNA were checked with NanoDrop 2000. A half microgram of RNA primed with oligo(dT)$_{20}$ was reverse transcribed following the instructions provided with the NG dART RT kit (EURx) to synthesize cDNA. The reaction was terminated by incubating at 85 °C for 5 min.

2.4. PCR-amplification of the core region of α-amylase I CDS

Complete cDNAs from *Apis mellifera* (Acc. AB022908), *Drosophila melanogaster* (Acc. AY322195), *Eisenia fetida* (Acc. LC055495), *Gallus gallus* (Acc. NM_001001473), *Panulirus argus* (Acc. LK937698) and *Marsupenaeus japonicus* (Acc. KJ147432) were retrieved from GenBank/NCBI. Sequences were aligned by ClustalW in MEGA7 [28]. A degenerate consensus sequence for a given DNA alignment was generated with the web-based program GeneFisher2 [29]. The conserved fragments were searched manually for designing degenerate primers. OligoAnalyzer Tool (https://eu.idtdna.com/calc/analyzer) and Sequence Manipulation Suite [30] were used for primer analysis. PCR amplification of the core region of α-amylase was performed initially using a pair of degenerate primers: AmyF and AmyR, and subsequently with a pair of primers with a lower degeneracy: tgAmyF and agAmyR (see Table 1). These two sets of primers later turned out to be imperfectly matched to the template; however, specific amplification of the core region was possible. Each PCR mixture consisted of the following components in a total volume of 50 μl: Color OptiTaq PCR Master Mix (2 ×) (final concentration: 1.25 U OptiTaq DNA Polymerase, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP), 0.6 μM of forward and reverse degenerate primers (or 0.2 μM of forward and reverse non-degenerate

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Table 1
Primers used in this study and their application.

Primer name	Direction	Sequence (5′–>3′)	Application
LCO1490	Forward	GGTCAACAAATCATAAAGATATTGG	COI barcoding
HCO2198	Reverse	TAAACTTCAGGGTGACCAAAAAATCA	COI barcoding
AmyF	Forward	ATsGTsCAyyTsTTyGArTGG	amplification of Amy I core region
AmyR	Reverse	CmvGArATvACrTCrCArTA	amplification of Amy I core region
tgAmyF	Forward	ATsGTsCAyyTsTTTGAGTGG	amplification of Amy I core region
agAmyR	Reverse	CmvGArATvACrTCACAGTA	amplification of Amy I core region
Amy1014R	Reverse	ACATGTTTGCCTCAAAGAAGGT	amplification of Amy I core region
Amy993F	Forward	ACCTTCTTTGAGGCAAACATGT	one-sided PCR
oligo(dT)30	Reverse	oligo$(dT)_{30}$	one-sided PCR
PAmy520R	Reverse	GACTTGGTTTGCATCGTTGTAA	5′-cRACE
Amy499F	Forward	TTACAACGATGCAAACCAAGTC	5′-cRACE, core region amplification
Amy364R	Reverse	AGTCATGTGGTTGAATACCC	5′-cRACE
AmyStrF	Forward	ATGCTGTCACTGATTGTGTTTTGTC	amplification of Amy I full CDS
AmyEndR	Reverse	TCAGACATGTAGAGCAATCATGG	amplification of Amy I full CDS
Amy660F	Forward	AGATCTGGAGGCGTTGTATGG	Amy I clone sequencing
Amy838R	Reverse	CATCACCGAGATTCTTTCCGTG	Amy I clone sequencing
T7LongFrw	Forward	TAATACGACTCACTATAGGGCGA	Amy I clone sequencing
SP6LongRev	Reverse	ATTTAGGTGACACTATAGAATACTCA	Amy I clone sequencing
AmyII51F	Forward	CAACTCTCAGTACTTTGGCACGTAC	amplification of Amy II
AmyII958R	Reverse	ATTGAGATCTCCTGTGCGCTGA	amplification of Amy II
AmyIIHybStrF	Forward	ATCTCTCGAACATGAAATCACTA	amplification of Amy II
AmyII626R	Reverse	ACTCCCCAGTCAATCAACTTAT	amplification of Amy II, amplification of Amy II 5′-UTR
AmyII721F	Forward	CCGAGCGGAACAAAGGCTTATG	amplification of Amy II
AmyIIEndShort	Reverse	TTATACATGTAGGGCGACCAT	amplification of Amy II
FAmy1505	Forward	CCATGATTGCTCTACATGTCTGA	amplification of Amy I 3′-UTR
RAmyUTRend	Reverse	ATACGTGACAGACGCACATGTTTAC	amplification of Amy I 3′-UTR
AmyII_5UTR3	Forward	GAGTTACTACTCTAATATATTAGGC	amplification of Amy II 5′-UTR
AmyII1507F	Forward	ATGGTCGCCCTACATGTATAA	amplification of Amy II 3′-UTR
AmyII3UTRlongR	Reverse	GATACATAATATTATTGTCGTCCAC	amplification of Amy II 3′-UTR

primers), and 1 μl of cDNA as template. The thermal profile for amplification was: 1 cycle of 95 °C for 260 s (initial denaturation), 35 cycles each of 95 °C for 40 s, 45 °C for 45 s and 72 °C for 60 s, with a final 72 °C extension for 480 s. Amplification products were sent to GenoMed (Warsaw, Poland) and sequenced in both directions using cgAmyF and agAmyR, respectively. All primers used in this paper are listed in Table 1.

2.5. Amplification of 3' end of α-amylase I CDS

A new series of internal primers was developed based on the sequence obtained from the core region. PCR amplification of 3′ end cDNA of α-amylase was performed with the use of gene-specific forward primer Amy993F (primer site located 518 bp upstream from 3′ end of CDS) and nonspecific reverse primer oligo$(dT)_{30}$ complementary to the 3′ poly(A) tail. The amplification was based on a simplified protocol for the one-sided PCR technique originally described by Ohara et al. [31] and updated by Dorit & Ohara [32]. We found that using oligo$(dT)_{30}$ (instead of oligo$(dT)_{20}$) with a higher annealing temperature (i.e., > 42 °C) increased the specificity of one-sided PCR and significantly reduced the heterogeneity of the final product without the need for additional steps (e.g. a second amplification with nested primer, DNA gel extraction, and reamplification) usually required to rescue the appropriate sequence. The one-sided PCR reaction contained 0.6 μM of forward and reverse primers and the rest of the components as described above. The thermal profile for amplification was: 1 cycle of 95 °C for 180 s (initial denaturation), 35 cycles each of 95 °C for 40 s, 45 °C for 45 s and 72 °C for 60 s, with a final 72 °C extension for 480 s. Five amplification products corresponding with five separate pooled samples (see 2.3 RNA extraction and cDNA synthesis for a classical approach) were sequenced in one direction using Amy993F. All sequences were aligned and 3' end consensus sequence was deduced. A 21 nucleotide primer AmyEndR that covers exactly the last 7 codons of *E. albidus* α-amylase CDS was designed. AmyEndR was validated for amplification in combination with Amy993F and with other internal forward primers.

2.6. Amplification of 5' end of α-amylase I CDS with cRACE

Amplification of 5′ end cDNA of *E. albidus* α-amylase was based on the three-step cRACE method [33] and carried out according to the protocols [33,34] which were slightly modified. A half microgram of RNA was reverse transcribed following the instructions provided NG dART RT kit (EURx) with a gene-specific 5′-phosphorylated oligonucleotide PAmy520R in total volume of 20 μl at 50 °C. The reaction was terminated by incubating at 85 °C for 15 min. From the resulting cDNA 12.5 μl was taken to the next step. Subsequently, three volumes of 1 × TE Buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) containing 4 μg/ml RNaseA were added. Next, two-fifths of the resulting cDNA mixture was circularized by 20 U of T4 RNA ligase with 5 μl of 10 × reaction buffer (Thermo Scientific) in the presence of 15 % (w/v) polyethylene glycol 4000 (Thermo Scientific) in a total volume of 50 μl at 37 °C for 60 min. To remove residuals of the first-strand synthesis primer and unreacted cDNAs, a 1.5 U of Pfu DNA polymerase (EURx) was added to the reaction at 37 °C for 30 min employing its 3′–5′ exonuclease activity. Finally, a 5 μl aliquot of the reaction was directly used as template for the first amplification by PCR with a pair of inverted primers: Amy364R and Amy499F. Because the amplicon was visible as a single faint band, it was cut from the gel and purified using Gel Purification GPB Mini Kit (GenoPlast Biochemicals). A serial dilution of recovered DNA in nuclease-free water was prepared and used as a template in a second round of PCR (reamplification). The thermal profile for PCR was: 1 cycle of 95 °C for 180 s (initial denaturation), 35 cycles each of 95 °C for 30 s, 45 °C for 30 s and 72 °C for 45 s, with a final 72 °C extension for 480 s. PCR products were sequenced in both directions with the above-mentioned inverted primers. Obtained reads were aligned and 5' end consensus sequence was deduced. Primer AmyStrF that covers exactly the first 25 nucleotides of CDS

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of *E. albidus* α-amylase was designed and validated for amplification in combination with AmyEndR.

2.7. Cloning of full-length coding sequence of α-amylase I

The complete coding sequence of *E. albidus* α-amylase I was amplified using the AmyStrF and AmyEndR primers. The PCR product was cloned into pGEM-T Easy Vector using pGEM-T Easy Vector System cloning kit with JM109 competent *E. coli* cells following manufacturer's protocol (Promega). Seven clones were sequenced in both directions with clone screening (T7long, SP6) and gene-specific (Amy660F, Amy838R) primers to cover the whole ORF.

2.8. Amplification of the coding sequence of α-amylase II

Based on sequences obtained from the RNA-seq data (see below), three sets of primers were designed, i.e., AmyIIHybStrF with AmyII626R, AmyII51F with AmyII958R, and AmyII721F with AmyIIEndShort. These primer pairs were used to amplify and sequence the whole CDS of α-amylase II as three overlapping segments. The thermal profile for all PCRs was: 1 cycle of 95 °C for 180 s (initial denaturation), 35 cycles each of 95 °C for 40 s, 45 °C for 45 s, and 72 °C for 60 s, with a final 72 °C extension for 120 s.

2.9. Sequence analysis

The coding sequences of the Amy I and Amy II were used as queries for BLASTn and BLASTp searches [35] to find homologous sequences in the NCBI database. Sequences were aligned using MAFFT7 [36] and the percent identity was calculated in the Sequence Manipulation Suite [30]. The amino acid sequences were deduced with Translate Tool available on the ExPASy platform (http://us.expasy.org). Putative translated proteins were classified using InterProScan [37] and SMART (a Simple Modular Architecture Research Tool) [38] based on their identified functional signature sequences. Signal peptide sequences were predicted by SignalP 6.0 [39]. The isoelectric point (pI) and molecular weight of the putative mature proteins were determined by Compute pI/MW (ExPASy). MEGA7 was used to calculate genetic distances using uncorrected p-distances with uniform rates among sites. Additionally, evolutionary divergence between sequences was assessed through pairwise distance method with the Poisson correction model. The ratio of non-synonymous to synonymous substitutions (dN/dS) for *E. albidus* Amy I and Amy II was computed using the codeML program in PAML 4.9 package [40], on the Galaxy platform [41].

2.10. Protein modeling, structural alignment and figure rendering

The 3D structure of proteins was homology-based modeled with SWISS-MODEL and automatic search for templates [42]. In all cases the 6m4k X-ray crystal structure of wild-type α-amylase I from *Eisenia fetida* was found to be the best and used as the mold. After modeling, the quality and validation of the model were evaluated by several structure assessment methods: GMQE, QMEANDisCo, and QMEAN Z-scores. Primary and secondary structure alignments of Amy I or Amy II with Ef-Amy I, were drawn using Jalview [43] and ESPript [44], respectively. Figures of the tertiary structure of proteins were rendered using SWISS-MODEL and UCSF ChimeraX [45].

2.11. Generation of RNA-seq data and transcriptome assembly for E. albidus

Live specimens of *E. albidus* PL-A strain were sent to A&A Biotechnology (Gdańsk, Poland) for RNA extraction. RNA extraction was performed from 4 adult specimens as a pooled sample using Total RNA Mini Kit (A&A Biotechnology) and DNase treatment. To generate RNA-seq reads, the obtained RNA sample was sent through A&A Biotechnology to Macrogen Europe (Amsterdam, Netherlands) for cDNA library preparation with TruSeq Stranded mRNA LT Sample Prep Kit and paired-end sequencing with Illumina platform (NovaSeq 6000; 2 × 151 bp reads). In parallel, raw RNA-sequencing data (Illumina HiSeq 2500 runs) of two freeze-tolerant strains (Germany-G and Greenland-N) of *E. albidus* [26] were retrieved from the NCBI Sequence Read Archive (SRA: SRP108369). Sequence quality control of reads from all sources was performed using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapter removal and quality trimming were performed with BBDuk plugin in Geneious Prime with the following settings: trim adapters, default settings; trim partial adapters from ends with kmer length 11; trim low quality both ends, minimum quality 20; trim adapters based on paired read overhangs: minimum overlap 24. Each transcriptome was assembled separately using Trinity [46,47] integrated in OmicsBox suite using the default k-mer length settings. Transcriptomes were processed using TransDecoder (http://transdecoder.github.io) in OmicsBox with default settings to detect coding regions. Transdecoder-predicted ORFs were translated into amino acid sequences at least 100 amino acids in length and annotated using GhostKOALA automatic annotation and KEGG mapping service (http://www.kegg.jp/ghostkoala/) [48]. The obtained data was screened for α-amylase (EC 3.2.1.1).

2.12. Data for phylogenetic analysis

Additional 92 transcriptomes originating from other annelids were assembled *de novo* in the same manner as described above, based on data retrieved from the NCBI SRA (Supplementary Table S1). Sequences homologous to *Eisenia fetida* α-amylases (treated as reference sequences) were identified by BLASTp search. Additional sequences were also retrieved from other sources: NCBI Transcriptome Shotgun Assembly Sequence Database, GenBank, Jékely lab transcriptomes databases for *Platynereis dumerilii* and *Alvinella pomepjana* (http://jekelylab.ex.ac.uk/blast/) [49,50], and supplementary data from another paper [51]. Moreover, in a few cases (*Aporrectodea caliginosa, Enchytraeus crypticus, Lepidonotus clava,* and *Metaphire vulgaris*), homologous sequences were identified by tBLASTn hit in genomes available at the NCBI Assembly database and predicted with AUGUSTUS [52] integrated in OmicsBox suite.

2.13. Phylogenetic analysis

Mature protein sequences were aligned using MAFFT 7 with automatic assignment of alignment strategy and with the number of iterations set to 1000 at GUIDANCE2 server [53] with 100 bootstrap repeats. A web server version of IQ-TREE [54] was used to estimate the best-fitting model of amino acid evolution and, subsequently, to construct a maximum likelihood tree. The main tree (final data set) was constructed using the model WAG + F + I + G4 as suggested by IQ-TREE, and with 1000 replications. Ultrafast bootstrap (UFBoot) and SH-like approximate likelihood ratio test (SH-aLRT) support values were calculated using 3000 replicates with default settings. The tree was rooted at *Owenia fusiformis* (Oweniidae) putative amylase sequences (Acc. CAH1795064 and CAH1797073) following previous phylogenetic hypotheses for Annelida (e.g. Refs. [55,56]) and visualized with iTOL [57] with a further edition in Adobe Illustrator.

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3. Results

3.1. Characterization of Amy I and Amy II CDSs and putative proteins

Based on the classical "gene fishing" approach involving reverse transcription PCR and PCR with degenerate primers, we amplified a core region of the coding sequence of α-amylase I from *Enchytraeus albidus*. A similar initial primer set, but with higher degeneracy, was previously used to characterize the α-amylase from the carnivorous crustacean *Panulirus argus* [58]. Relatively inexpensive techniques (one-sided PCR, three-step cRACE) were successfully applied to unravel the sequence of both ends of the target cDNA. The predicted ORF of α-amylase I was found to consist of 1527 bp encoding 508 amino acid residues. The sequencing of the full CDS clones derived from the mixed culture of *E. albidus* revealed the presence of at least five different alleles of Amy I (Amy I Clone1 – Amy I Clone5) in the population and the necessity of the investigation of purer genetic lineage to ensure that no cryptic species specimen was accidently included in the analysis. The RNA-sequencing of pooled sample from the PL-A strain revealed two Amy I alleles. PCR validation from five corresponding specimens revealed another new allele, and it confirmed the sequence of Amy I-Clone1, but not exactly the variants from PL-A transcriptomic data. Seven additional alleles, four from the N strain and three from the G strain, respectively, were retrieved from transcriptomes of freeze-tolerant strains. All identified alleles differ from each other by a set of single nucleotide polymorphisms unique for a given strain, leading in some but not all cases to variations in the predicted amino acid sequence of the putative protein. To sum up, fifteen alleles that encode eleven protein variants of Amy I were discovered (Supplementary Data S1).

Screening of transcriptomes assembled for the PL-A, G, and N strains of *Enchytraeus albidus* revealed the presence of a second α-amylase gene (Amy II). Similarly to Amy I, the predicted ORF of α-amylase II was found to consist of 1527 bp encoding 508 amino acid residues. The expression of Amy II for PL-A strain was confirmed by PCR in corresponding specimens. However, two slightly different variants of CDS were obtained. One of these sequences differs from RNA-Seq-derived sequence only by a single nucleotide, which could result from a base-calling error (though Sanger sequencing was high quality in this region). Incorporating future genomic data would be the most proper solution to address the issue. One PCR-tested specimen was heterozygous in Amy II. Seven additional alleles, three from the N strain and four from the G strain, respectively, were retrieved from transcriptomes of freeze-tolerant strains. A total of ten alleles that encode six different pre-protein variants of Amy II were identified (Supplementary Data S2). After cleavage of the signal sequence only four putative mature Amy II proteins remained unique. The differences between Amy II alleles were related to several single nucleotide polymorphisms, and similarly to Amy I, they were unique for a given strain at the DNA-level.

To gain further insight into the evolutionary pressures on Amy genes, we calculated the dN/dS ratio, a popular measure of evolutionary pressures on protein-coding regions. For Amy I, we found a dN/dS ratio of 0.11158, and for Amy II, the ratio was 0.12829. These results indicate that both Amy genes are under purifying selection, suggesting that changes in their coding sequences could be detrimental.

The global alignment of Amy I with Amy II (Fig. 1) shows that their coding sequence variants share 67–68 % nucleotide identity and 70–71 % amino acid identity. Both *E. albidus* amylases share a high sequence identity with Ef-Amy I (Acc. LC426728 and LC055495) and Ef-Amy II (Acc. LC594654) from the earthworm *Eisenia fetida*. Amy I shares 69 % nucleotide and 73–74 % amino acid identity with Ef-Amy I, as well as 68–69 % nucleotide and 72 % amino acid identity with Ef-Amy II, respectively. Furthermore, Amy II shares 65 % nucleotide and 66 % amino acid identity with Ef-Amy I, as well as 65–66 % nucleotide and 66–67 % amino acid identity with Ef-Amy II, respectively. The uncorrected p-distance for deduced protein sequences of Amy I varied from 0 to 1.38 %, while for Amy II varied from 0 to 0.98 %. Pairwise sequence divergence of unique Amy I and Amy II pre-proteins was shown in Table 2. Signal peptides predicted by SignalP 6.0 for Amy I and Amy II comprised 16 (cleavage site: ASA-QY) and 19 (cleavage site: TNS-QY) amino acid residues, respectively. The molecular mass of the mature Amy I and Amy II proteins was estimated to be 54.3–54.5 kDa and 54.5–54.6 kDa, while the theoretical isoelectric point (pI) for them was estimated to be 5.15–5.45 and 4.71–4.78, respectively.

3.2. Domains architecture and three-dimensional models of Amy I and Amy II

Both *E. albidus* amylases were classified as belonging to Glycoside Hydrolase Family 13 by InterProScan and SMART. According to BLASTp analysis results, they share slightly higher similarity with non-insect animal amylases than with insect amylases (GH13_15). Therefore, they should be classified in the subfamily GH13_24 ("vertebrate amylases"), as proposed by Tsukamoto et al. [20] for *E. fetida* Amy amylases. It should be noted that the original distinction of animal glycoside hydrolases GH13 into two subfamilies by Stam et al. [21] based on phylogenetic relationships is somewhat artificial and obsolete (see Ref. [59]).

The domains arrangement of Amy I and Amy II were found to be typical, like in many other known GH13 α-amylases, with distinguishable A, B, and C domains [60,61] (Fig. 2A). Domain A is represented as a catalytic $(\alpha/\beta)_8$-barrel (TIM barrel). Domain B is located between the third β-strand and the third α-helix of the TIM barrel. It is a loop region with two well-defined antiparallel β-strands connected by a β-turn. The C-terminal domain (domain C) is formed by eight beta-strands arranged as a Greek key β-barrel (Fig. 2B). Interestingly, despite the amino acid sequence divergence between Amy I and Amy II, the predicted secondary structure (Figs. 3 and 4) is identical for both paralogs. The differences in tertiary structure models are minor (Fig. 2C), while the generated models were high quality (Supplementary Table S2). Accordingly, the three invariant catalytic residues of the active site (Asp196, Glu232, and Asp299 in both Amy I and Amy II) are present. Residues involved in binding calcium (Asn101, Arg157, Asp166, His200 in both Amy I and Amy II) and chlorine (Arg194, Asn297, Arg337 in Amy I; Arg194, Asn297, Arg334 in Amy II) ions are conserved with those in Ef-Amy I [19]. Moreover, the Gly-rich loop (also known as "flexible loop") which protrudes near the catalytic cleft and contains the Gly-His-Gly-Ala (GHGA) motif, is present in Amy I and in Ef-Amy I, while it is absent in parallel Amy II and Ef-Amy II [19,20,59]. Based on predicted models, in both *E. albidus* Amy amylases twelve cysteine residues may form six disulfide bridges, similarly to *E. fetida* Amy I (see UniProtKB accession number: A0A173N065). This is in contrast to pig pancreatic α-amylase, which contains five disulfide bridges [62]. Additional modeling and analysis of α-amylases retrieved for other annelid species in our study, specifically ESPript-DSSP prediction of positions of disulfide bridges [44] combined with evaluation of the bonds using Disulfide by Design 2.0 [63], indicates that the extra disulfide bond in domain C is conserved among Clitellata (see Supplementary Fig. S1 and Supplementary Table S3).

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Amy_I_Clone2                                ..............---...................................................................................... 100
Amy_I_Clone5                                ..............---...................................................................................... 100
Amy_I_N_DN3586c1g1i1                        ..............---...................................................................................... 100
Amy_I_PL-A_DN24177c0g2i4                    ..............---...................................................................................... 100
Amy_I_PL-A_EH1                              ..............---.......................................................N.............................. 100
Amy_I_N_DN3586c1g1i4                        ..............---...................................................................................... 100
Amy_I_G_DN2057c0g1i1                        ..............---...................................................................................... 100
Amy_I_G_DN2057c0g1i6_Amy_I_N_DN1864c0g1i1   ..............---...................................................................................... 100
Amy_I_PL-A_EH5                              ..............---...................................................................................... 100
Amy_I_PL-A_DN24177c0g2i3                    ..............---...................................................................................... 100
Amy_I_Clone3                                ..............---.......................................................N.............................. 100
Amy_II_G_DN3622c1g1i7                       .K..VILLVG.TL.GRTNS..F.T....S....V...........QR.................PM.H.IV.......W........S.N....SEQ...D.. 103
Amy_II_G_DN47824c0g1i2_Amy_II_PL-A_EH1      .K..VILLVG.TL.GPTNS..F.T....S....V...........QR.................PM.H.IV.......W........S.N....SEQ...D.. 103
Amy_II_N_DN1688c0g2i1                       .K..VILLVG.TL.GPTNS..F.T....S....V...........QR.................PM.H.IV.......W........S.N....SEQ...D.. 103
Amy_II_N_DN1688c0g1i1                       .K..VILLVG.TL.GPTNS..F.T....S....V...........QS.................PM.H.IV.......W........S.N....SEQ...D.. 103
Amy_II_N_DN1688c0g2i4                       .K..LILL.G.TL.GPTNS..F.T....S....V...........QR.................PM.H.IV.......W........S.N....SEQ...D.. 103
Amy_II_PL-A_DN59470c0g1i1                   .K..VILLVG.TL.GPTNS..F.T....S....V...........QS.................PM.H.IV.......W........S.N....SEQ...D.. 103
Consensus                                   * **::: : *::*   .:**:*:****:****.***********: *****************  *:*::*:*****:********.*.****.: ***.**

Amy_I_Clone2                                ....................................................................................................... 203
Amy_I_Clone5                                ....................................................................................................... 203
Amy_I_N_DN3586c1g1i1                        ....................................................................................................... 203
Amy_I_PL-A_DN24177c0g2i4                    ....................................................................................................... 203
Amy_I_PL-A_EH1                              ....................................................................................................... 203
Amy_I_N_DN3586c1g1i4                        ....................................................................................................... 203
Amy_I_G_DN2057c0g1i1                        ....................................................................................................... 203
Amy_I_G_DN2057c0g1i6_Amy_I_N_DN1864c0g1i1   ....................................................................................................... 203
Amy_I_PL-A_EH5                              ....................................................................................................... 203
Amy_I_PL-A_DN24177c0g2i3                    ....................................................................................................... 203
Amy_I_Clone3                                ...................................................E................................................P.. 203
Amy_II_G_DN3622c1g1i7                       GA..D...Y..I.D.I.......A.........DG.ARD.....F.PF..N.PEQ.P.AS...E..G.PI.............R.GTA..QDQ.AGY..K..D 206
Amy_II_G_DN47824c0g1i2_Amy_II_PL-A_EH1      GA..D...Y..I.D.I.......A.........DG.ARD.....F.PF..N.PEQ.P.AS...E..G.PI.............R.GTA..QDQ.AGY..K..D 206
Amy_II_N_DN1688c0g2i1                       GA..D...Y..I.D.I.......A.........DG.ARD.....F.PF..N.PEQ.P.AS...E..G.PI.............R.GTA..QDQ.AGY..K..D 206
Amy_II_N_DN1688c0g1i1                       GA..D...Y..I.D.I.......A.........DG.ARD.....F.PF..N.PEQ.P.AS...E..G.PI.............R.GTA..QDQ.AGY..K..D 206
Amy_II_N_DN1688c0g2i4                       GA..D...Y..I.D.I.......A.........DG.ARD.....F.PF..N.PEQ.P.AS...E..G.PI.............R.GTA..QDQ.AGY..K..D 206
Amy_II_PL-A_DN59470c0g1i1                   EA..D...Y..I.D.I.......A.........DG.ARD.....F.PF..N.PEQ.P.AS...E..G.PI.............R.GTA..QDQ.AGY..K..D 206
Consensus                                    :**:***:**:*.*:*******:*********:.*:..*****:*  **: . :* *:.***:**.*. *************.*.: **.. *:.:**  *.

Amy_I_Clone2                                ......................................G.............................L.................................. 306
Amy_I_Clone5                                ....................................................................................................... 306
Amy_I_N_DN3586c1g1i1                        ....................................................................................................... 306
Amy_I_PL-A_DN24177c0g2i4                    ......................................................Q................................................ 306
Amy_I_PL-A_EH1                              ....................................................................................................... 306
Amy_I_N_DN3586c1g1i4                        ....................................................................................................... 306
Amy_I_G_DN2057c0g1i1                        ....................................................................L.................................. 306
Amy_I_G_DN2057c0g1i6_Amy_I_N_DN1864c0g1i1   ....................................................................L.................................. 306
Amy_I_PL-A_EH5                              ......................................................Q................................................ 306
Amy_I_PL-A_DN24177c0g2i3                    ......................................................Q................................................ 306
Amy_I_Clone3                                ....................................................................L.................................. 306
Amy_II_G_DN3622c1g1i7                       W......L..A.....A.VSDTFAK.HD..A....S..KA.V......W...AVSG.E..GI......R....I...V...F...M.....Y..G....N.L. 309
Amy_II_G_DN47824c0g1i2_Amy_II_PL-A_EH1      W......L..A.....A.VSDTFAK.HD..A....S..KA.V......W...AVSG.E..GI......R....I...V...F...M.....Y..G....N.L. 309
Amy_II_N_DN1688c0g2i1                       W......L..A.....A.VSDTFAK.HD..A....S..KA.V......W...AVSG.E..GI......R....I...V...F...M.....Y..G....N.L. 309
Amy_II_N_DN1688c0g1i1                       W......L..A.....A.VSDTFAK.HD..A....S..KA.V......W...AVSG.E..GI......R....I...V...F...M.....Y..G....N.L. 309
Amy_II_N_DN1688c0g2i4                       W......L..A.....A.VSDTFAK.HD..A....S..KA.V......W...AVSG.E..GI......R....I...V...F...M.....Y..G....N.L. 309
Amy_II_PL-A_DN59470c0g1i1                   W......L..A.....A.VSDTFAK.HD..A....S..KA.V......W...AVSG.E..GI......R....I...V...F...M.....Y..G....N.L. 309
Consensus                                    ******:**.*****.*:.  :..*::**:****:** .*:****** ***.:..*:** :****** ****:***:*** ***:*****:**.****.* *

Amy_I_Clone2                                ....................................................................................................... 409
Amy_I_Clone5                                ..............................................................................N........................ 409
Amy_I_N_DN3586c1g1i1                        ..............................................................................N........................ 409
Amy_I_PL-A_DN24177c0g2i4                    ..............................................................................N........................ 409
Amy_I_PL-A_EH1                              ..............................................................................N........................ 409
Amy_I_N_DN3586c1g1i4                        ....................................................................................................... 409
Amy_I_G_DN2057c0g1i1                        ....................................................................................................... 409
Amy_I_G_DN2057c0g1i6_Amy_I_N_DN1864c0g1i1   ..............................................................................N........................ 409
Amy_I_PL-A_EH5                              ..............................................................................N........................ 409
Amy_I_PL-A_DN24177c0g2i3                    ..............................................................................N........................ 409
Amy_I_Clone3                                ....................................................................................................... 409
Amy_II_G_DN3622c1g1i7                       .F.......T..---T.DLNS....RQS.........Q..............Y.QQD.QD.R.H.E.I....S..S.S.V...D................... 409
Amy_II_G_DN47824c0g1i2_Amy_II_PL-A_EH1      .F.......T..---T.DLNS....RQS.........Q..............Y.QQD.QD.R.H.E.I....S..S.S.V...D................... 409
Amy_II_N_DN1688c0g2i1                       .F.......T..---T.DLNS....RQS.........Q..............Y.QQD.QD.R.H.E.I....S..S.S.V...D................... 409
Amy_II_N_DN1688c0g1i1                       .F.......T..---T.DLNS....RQS.........Q..............Y.QQD.QD.R.H.E.I....S..S.S.V...D................... 409
Amy_II_N_DN1688c0g2i4                       .F.......T..---T.DLNS....RQS.........Q..............Y.QQD.QD.R.H.E.I....S..S.S.V...D................... 409
Amy_II_PL-A_DN59470c0g1i1                   .F.......T..---T.DLNS....RQS.........Q..............Y.QQD.QD.R.H.E.I....S..S.S.V...D................... 409
Consensus                                   *:*******.**   :*.:.:**** ::*********:************** *  .:* :* * *:*:****.** *::.***:*******************

Amy_I_Clone2                                ..................................................................G................................ 508
Amy_I_Clone5                                .........................K......................................................................... 508
Amy_I_N_DN3586c1g1i1                        ................................................................................................... 508
Amy_I_PL-A_DN24177c0g2i4                    .........................K......................................................................... 508
Amy_I_PL-A_EH1                              .........................K......................................................................... 508
Amy_I_N_DN3586c1g1i4                        ................................................................................................... 508
Amy_I_G_DN2057c0g1i1                        ................................................................................................... 508
Amy_I_G_DN2057c0g1i6_Amy_I_N_DN1864c0g1i1   ................................................................................................... 508
Amy_I_PL-A_EH5                              .............................................................P..................................... 508
Amy_I_PL-A_DN24177c0g2i3                    ......................................Y............................................................ 508
Amy_I_Clone3                                ................................................................................................... 508
Amy_II_G_DN3622c1g1i7                       .V................................NG.L.........E.Q.....T....P...D....A.....S....Q.L.....A....V..... 508
Amy_II_G_DN47824c0g1i2_Amy_II_PL-A_EH1      .V................................NG.L.........E.Q.....T....P...D....A.....S....Q.L.....A....V..... 508
Amy_II_N_DN1688c0g2i1                       .V................................NG.L.........E.Q.....T....P...D....A.....S....Q.L.A...A....V..... 508
Amy_II_N_DN1688c0g1i1                       .V................................NG.L.........E.Q.....T....P...D....A.....S....Q.L.A...A....V..... 508
Amy_II_N_DN1688c0g2i4                       .V................................NG.L.........E.Q.....T....P...D....A.....S....Q.L.A...A....V..... 508
Amy_II_PL-A_DN59470c0g1i1                   .V................................NG.L.........E.Q.....T....P...D....A.....S....Q.L.....A....V..... 508
Consensus                                   *.***********************.*********..*: ******** * *****:*****.***:.**:*****:***** * *:**:*****:****
```

Fig. 1. Amino acid sequence alignment of Amy I and Amy II pre-protein variants from *E. albidus*. Symbols in the consensus sequence indicate: fully conserved residue (*), conservative substitution (:), and semi-conservative substitution (.).

3.3. Transcriptomic data for other annelids and phylogenetic analysis of α-amylase

The final amylase data set comprised 159 mature protein sequences for 61 taxa, including *E. albidus* (Supplementary Data S3). The primary source of additional data for annelids was SRA. Homologous sequences of sufficient length (complete or almost complete 5′-partial sequences) were recovered from 45 out of a total of 94 SRA-derived transcriptomes. The remaining sequences were retrieved from TSA (9 sequences), Stiller et al. [51] supplementary data (9 sequences), GenBank (7 sequences), AUGUSTUS gene prediction for selected Annelida genomes (6 sequences), and Jékely lab transcriptomes (5 sequences). Resulting sequences were screened against GenBank using BLASTp, while *Eisenia fetida* α-amylases (Acc. BAV13234, BBG56860, and BCN16346) were picked as reference sequences for "*Enchytraeus-Eisenia* type" Amy homologs. Almost all BLASTp hits have reported E-value of 0, except those for *Terebellides* sp. and *Alvinella pompejana* ($5e^{-180}$ and $2e^{-179}$,

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Table 2
Estimates of evolutionary divergence between sequences of Amy I and Amy II pre-proteins.

	Seq No	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Amy_II_PL-A_DN59470c0g1i1	**1**		**0,003**	**0,003**	**0,003**	**0,004**	**0,003**	**0,027**	**0,028**	**0,028**	**0,028**	**0,027**	**0,028**	**0,027**	**0,028**	**0,027**	**0,028**	**0,028**
Amy_II_N_DN1688c0g2i1	**2**	**0,006**		**0,002**	**0,002**	**0,003**	**0,003**	**0,028**	**0,028**	**0,028**	**0,028**	**0,028**	**0,028**	**0,028**	**0,028**	**0,028**	**0,028**	**0,028**
Amy_II_N_DN1688c0g1i1	**3**	**0,004**	**0,002**		**0,003**	**0,003**	**0,003**	**0,028**	**0,028**	**0,028**	**0,028**	**0,028**	**0,028**	**0,028**	**0,028**	**0,028**	**0,028**	**0,028**
Amy_II_G_DN47824c0g1i2_Amy_II_PL-A_EH1	**4**	**0,004**	**0,002**	**0,004**		**0,003**	**0,002**	**0,027**	**0,028**	**0,028**	**0,028**	**0,027**	**0,028**	**0,027**	**0,028**	**0,027**	**0,028**	**0,028**
Amy_II_N_DN1688c0g2i4	**5**	**0,010**	**0,004**	**0,006**	**0,006**		**0,004**	**0,027**	**0,027**	**0,027**	**0,027**	**0,027**	**0,027**	**0,027**	**0,027**	**0,027**	**0,028**	**0,027**
Amy_II_G_DN3622c1g1i7	**6**	**0,006**	**0,004**	**0,006**	**0,002**	**0,008**		**0,027**	**0,028**	**0,028**	**0,028**	**0,027**	**0,028**	**0,027**	**0,028**	**0,027**	**0,028**	**0,028**
Amy_I_Clone2	**7**	**0,338**	**0,341**	**0,341**	**0,338**	**0,338**	**0,338**		**0,004**	**0,005**	**0,004**	**0,003**	**0,005**	**0,003**	**0,003**	**0,005**	**0,005**	**0,004**
Amy_I_Clone5	**8**	**0,341**	**0,344**	**0,344**	**0,341**	**0,341**	**0,341**	**0,010**		**0,002**	**0,002**	**0,003**	**0,002**	**0,003**	**0,003**	**0,003**	**0,003**	**0,005**
Amy_I_PL-A_DN24177c0g2i4	**9**	**0,341**	**0,344**	**0,344**	**0,341**	**0,341**	**0,341**	**0,012**	**0,002**		**0,003**	**0,003**	**0,003**	**0,004**	**0,003**	**0,003**	**0,003**	**0,005**
Amy_I_N_DN3586c1g1i1	**10**	**0,338**	**0,341**	**0,341**	**0,338**	**0,338**	**0,338**	**0,008**	**0,002**	**0,004**		**0,002**	**0,003**	**0,003**	**0,002**	**0,003**	**0,003**	**0,004**
Amy_I_N_DN3586c1g1i4	**11**	**0,336**	**0,338**	**0,338**	**0,336**	**0,336**	**0,336**	**0,006**	**0,004**	**0,006**	**0,002**		**0,003**	**0,002**	**0,003**	**0,003**	**0,003**	**0,004**
Amy_I_PL-A_EH1	**12**	**0,344**	**0,347**	**0,347**	**0,344**	**0,344**	**0,344**	**0,012**	**0,002**	**0,004**	**0,004**	**0,006**		**0,004**	**0,003**	**0,004**	**0,004**	**0,004**
Amy_I_G_DN2057c0g1i1	**13**	**0,336**	**0,338**	**0,338**	**0,336**	**0,336**	**0,336**	**0,004**	**0,006**	**0,008**	**0,004**	**0,002**	**0,008**		**0,002**	**0,004**	**0,004**	**0,003**
Amy_I_G_DN2057c0g1i6_Amy_I_N_DN1864c0g1i1	**14**	**0,338**	**0,341**	**0,341**	**0,338**	**0,338**	**0,338**	**0,006**	**0,004**	**0,006**	**0,002**	**0,004**	**0,006**	**0,002**		**0,003**	**0,003**	**0,004**
Amy_I_PL-A_EH5	**15**	**0,336**	**0,338**	**0,338**	**0,336**	**0,336**	**0,336**	**0,012**	**0,006**	**0,004**	**0,004**	**0,006**	**0,008**	**0,008**	**0,006**		**0,003**	**0,005**
Amy_I_PL-A_DN24177c0g2i3	**16**	**0,341**	**0,344**	**0,344**	**0,341**	**0,341**	**0,341**	**0,012**	**0,006**	**0,004**	**0,004**	**0,006**	**0,008**	**0,008**	**0,006**	**0,004**		**0,005**
Amy_I_Clone3	**17**	**0,344**	**0,347**	**0,347**	**0,344**	**0,344**	**0,344**	**0,010**	**0,012**	**0,014**	**0,010**	**0,008**	**0,010**	**0,006**	**0,008**	**0,014**	**0,014**	

The number of amino acid substitutions per site are shown. Standard error estimates are shown above the diagonal. Analysis was conducted in MEGA7 using the Poisson correction model and involved 17 amino acid sequences. All ambiguous positions were removed for each sequence pair. There were a total of 511 positions in the final dataset.

respectively). To avoid long-branch attraction during the generation of the tree, marginally represented Spionida (*Pygospio elegans* GFPL01022367 and *Boccardia proboscidea* SRR2057014) and Echiura (*Urechins unicinctus* SRR13188955) sequences had to be removed sadly from our final data set. Moreover, a few other initially identified homologous sequences had been excluded from the data set. For example, protein sequences of α-amylase for *Chaetogaster diaphanus* (SRR10997419) were recovered as slightly truncated at the N-terminus. In addition, some extra sequences found in *Randiella* sp. and *Terebellides* sp. transcriptomes were probably contaminants from other annelids and were not included in the final data set.

After rooting at Oweniidae, represented by *Owenia fusformis*, the tree split into two superclades (Fig. 5). The first superclade grouped putative Amy proteins from: *Dimorphilus gyrociliatus* (Dinophilidae), basal branching lineages (Amphinomidae, Sipuncula) sensu Weigert and Bleidorn [64], and descendant Errantia. In fact, *D. gyrociliatus* was recently recovered as a member of basal branching annelids (see Ref. [65]), contrary to the previous placement of Dinophilidae within Sedentaria [64]. Within Errantia, two main clades were recovered. The first one contained a single sequence predicted from the genome (Acc. GCA_936440205) of the polynoid scale worm *Lepidonotus clava*. Despite being underrepresented in our analysis, the Polynoidae Amy sequence was essential for maintaining the tree's stability and preventing long branch attraction. In the second main clade of Errantia, Eunicida (Dorvilleidae, Oenonidae, Lumbrineridae) and the remaining Phyllodocida (Nereididae and Syllidae) sequences were recovered as sister sub-clades. Interestingly, a peculiar feature for species in this clade was the presence of out-paralogs pairs with preserved flexible loops. Thus, we considered both types of these proteins as Amy I amylases. The second superclade of the Amy tree consisted of Clitellata nested within Sedentaria. Within Sedentaria, monophyletic Terebeliformia residing on a long branch was placed as the most basal group. The latter was recovered as a sister group to the remaining Sedentaria. The clade comprising Capitellidae, Arenicolidae, Aeolosomatidae, and Hrabeillidae was recovered as sister to Opheliidae plus Clitellata.

As a sidenote, it should be remembered that *Hrabeiella periglandulata* (Annelida, Hrabeillidae), an enigmatic soil species, was considered Annelida *incertae sedis* for a long time. Still, a recent phylogenomic analysis [66] showed that Hrabeiellidae and Aeolosomatidae form a sister group to Clitellata. As Clitellata is nested within Sedentaria, it is logical now to classify *Hrabeiella* and *Aeolosoma* as sedentarian polychaetes and not Oligochaeta *sensu stricto* within Clitellata (see Refs. [66,67]; cf [68]). This conclusion is further supported by the inferred α-amylases phylogeny, which places *Hrabeiella* as a sister to *Aeolosoma* within a well-supported clade that also includes other Sedentarian lineages. Although we found Opheliidae as a sister to Clitellata, similar to Struck et al. [69], and contrary to Erséus et al. [66], nonetheless our phylogenetic tree still supports the close association between Hrabeiellidae and Aeolosomatidae found by the latter authors.

Within Clitellata, Amy protein sequences were separated into two clades: (1) Enchytraeidae and (2) the rest of Clitellata (Capilloventridae, Phreodrilidae, Naididae, Randiellidae, Crassiclitellata, Lumbriculidae, and Branchiobdellidae). Enchytraeidae clade was highly resolved, and sequences originating from *Enchytraeus* and *Mesenchytraeus* were separated into two subclades containing either Amy I or Amy II paralogs and further divided into species-specific clusters. However, it should be noted here that sequences obtained for different *Enchytraeus crypticus* (Ec) samples (SRR10997417 versus assembly GCA_905160935/TSA GALF00000000) were divergent. Depending on compared isoforms, amino acid p-distances varied, ranging between 12.4 and

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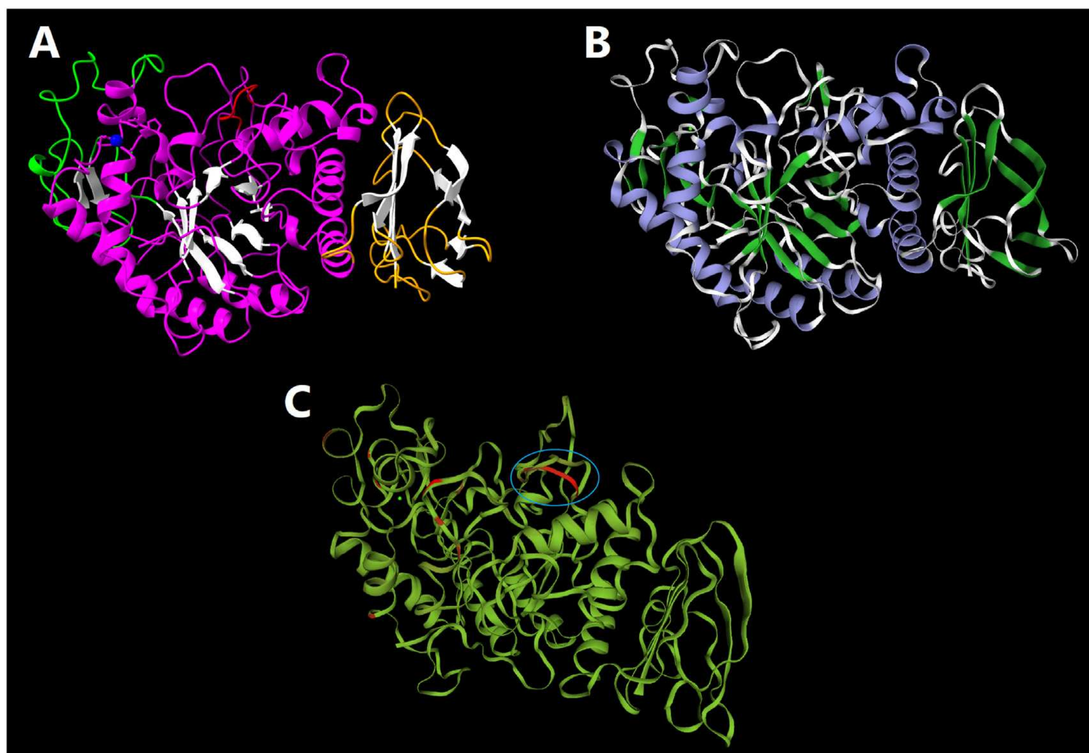

Fig. 2. Three-dimensional models of Amy I and Amy II amylases. (A) Domains arrangement of Amy I in ChimeraX. The magenta color marks domain A, the green color marks domain B, and the orange color marks domain C. The red color marks the flexible loop region in Domain A. White arrows indicate β-sheet structures, α-helices are indicated by helical structures, and a blue dot represents the position of the exaggerated calcium ion. (B) Tertiary structure of Amy I and secondary structure element organization in SWISS-MODEL. Green strands represent β-sheet structures, violet helical structures indicate α-helices, and a green dot indicates the position of a calcium ion. (C) Structure comparison of predicted Amy I and Amy II models in SWISS-MODEL. The consensus model of Amy I and the consensus model of Amy II proteins were superimposed. The local differences between models are visible as reddish structure elements. The region in domain A that contains a flexible loop was encircled.

13.4 % for Ec-Amy I and being approximately 12.3 % for Ec-Amy II. Indeed, sequences originating from those two sources of *Enchytraeus crypticus* were recovered as separate groups, which suggests misidentification, cryptic diversity of the species, or highly divergent lineages. The COI sequence retrieved from mitochondrial genome assembly confirms that the nuclear genomic data (Acc. GCA_905160935) of *E. crypticus* [2] originated from the CE2183 isolate (Acc. GU902055). Unfortunately, such information is typically unavailable for many transcriptomic studies, which rely on the selection of polyadenylated RNA transcripts. Apart from Enchytraeidae, the remaining Clitellata were separated into two main clades, although highly supported only by one method (SH-aLRT 89.3/ultrafast bootstrap 43). The first main clade contained Amy I from Capilloventridae and Amy II from Capilloventridae and Phreodrilidae. These together form the sister group to the Naididae. Interestingly, Amy II was the only type of Amy protein found in Phreodrilidae, which was unusual concerning all other analyzed annelid species where Amy I type proteins were dominant. In the second main clade, composed of all the rest of Clitellata, Lumbriculidae with Branchiobdellidae were recovered as a sister group to Randiellidae plus Crassiclitellata. The placement of *Randiella* amylase as sister to Crassiclitellata was favored only by SH-aLRT (71.7) and depended on data and alignment treatment, which sometimes resulted in placing *Randiella* at the most basal position to all clitellates, though distant to Capilloventridae (not shown) (cf [66]). However, the general sequence similarity between *Randiella* and Crassiclitellata amylases, including the absence of indels, was striking. Thus, we considered Randiellidae placement as ambiguous. Within Crassiclitellata, Amy proteins were separated into two clades. The first clade contained Glossoscolecidae, Hormogastridae, and most Megascolecidae species, while the second one contained *Amynthas gracilis* (Megascolecidae) as a sister to Lumbricidae (*Aporrectodea*, *Lumbricus,* and *Eisenia*). Apart from this rather unexpected discrepancy between Amy gene phylogeny and pheretimoid earthworm species phylogeny, an incomplete lineage sorting was identified in both Megascolecidae (see *Metaphire guillelmi*) and Lumbricidae. The latter was especially evident in the case of *Eisenia* spp. where sequences from *E. fetida* and *E. andrei* were not sorted in species-specific nor paralog-specific groups, despite being sampled prominently. Moreover, a common *Eisenia* Amy I isoform (DRR023799_DN2171c0g1i9 identical with Acc. BAV13234 in GenBank) identical at the protein level (including signal peptide) for both species was found. It is worth noting that the sibling species *E. fetida* and *E. andrei* differ at mitogenomes, and complete reproductive isolation between those two species was reported in older works (for details, see Ref. [70]). However, recent studies clearly demonstrated the existence of fertile hybrids with incomplete and asymmetric reproductive isolation [71–73], and interspecific gene flow between parental species [74,75]. Therefore, the latter could explain the discordance of *Eisenia* Amy proteins obtained in our analysis.

4. Discussion

4.1. Gene copy number and polymorphism of α-amylases

Despite α-amylases being major digestive enzymes responsible for hydrolyzing naturally abundant biopolymers such as starch and similar polysaccharides into sugars, they have been little studied in Annelida. The latter is especially true in the case of Enchytraeidae,

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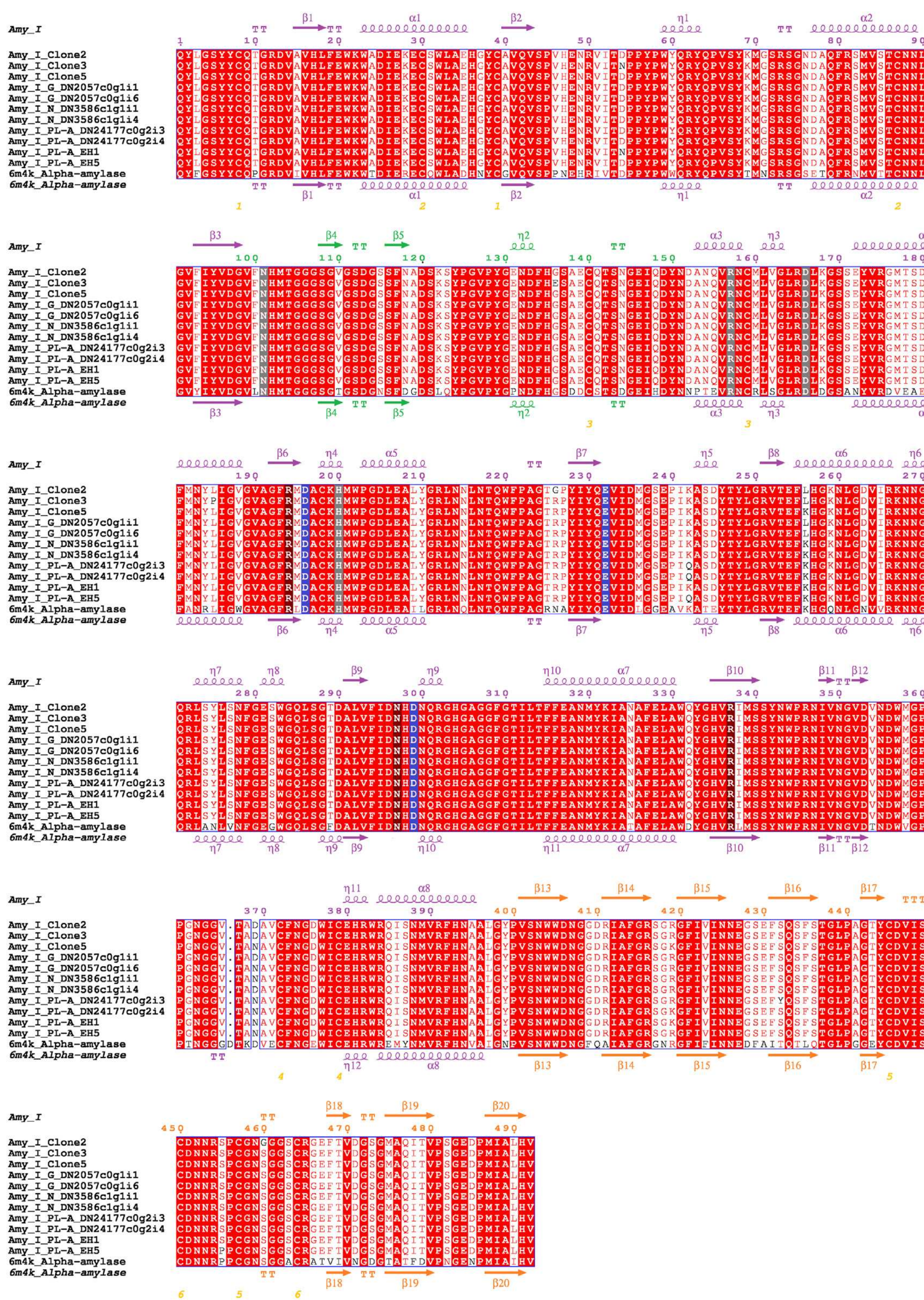

Fig. 3. Primary and secondary structure alignment of *Enchytraeus albidus* Amy I allozymes and Ef-Amy I (Acc. 6m4k) from *Eisenia fetida*. Secondary structure elements of compared α-amylases are indicated above and below the alignment and colored as a function of domains: domain A, purple; domain B, green; domain C, orange. The η symbol refers to a 3_{10}-helix. The α-helices and 3_{10}-helices are displayed as higher and lower squiggles, respectively. β-strands are rendered as arrows, strict β-turns as TT letters and strict α-turns as TTT. The positions of potential disulfide bridges are marked as yellow digits below the alignment. Catalytic triad residues are highlighted in blue, residues involved in binding calcium are highlighted in grey, and those involved in binding chlorine are highlighted in brown. Strictly identical residues are shown as white characters boxed in red, while similar residues within a group are shown as red characters. Middle dots indicate gaps in the sequence alignment.

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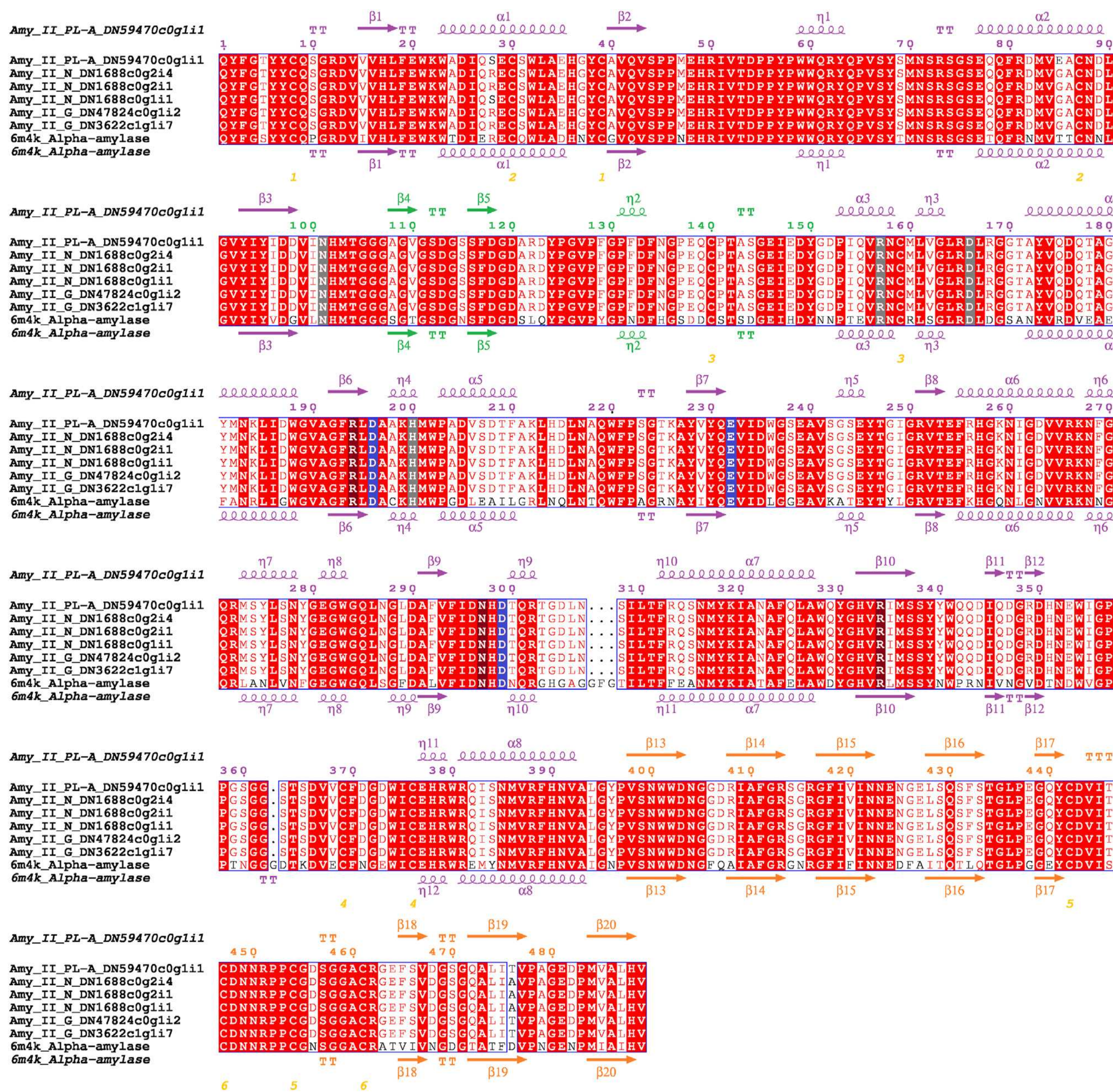

Fig. 4. Primary and secondary structure alignment of *Enchytraeus albidus* Amy II allozymes and Ef-Amy I (Acc. 6m4k) from *Eisenia fetida*. Secondary structure elements of compared α-amylases are indicated above and below the alignment and colored as a function of domains: domain A, purple; domain B, green; domain C, orange. The η symbol refers to a 3_{10}-helix. The α-helices and 3_{10}-helices are displayed as higher and lower squiggles, respectively. β-strands are rendered as arrows, strict β-turns as TT letters and strict α-turns as TTT. The positions of potential disulfide bridges are marked as yellow digits below the alignment. Catalytic triad residues are highlighted in blue, residues involved in binding calcium are highlighted in grey, and those involved in binding chlorine are highlighted in brown. Strictly identical residues are shown as white characters boxed in red, while similar residues within a group are shown as red characters. Middle dots indicate gaps in the sequence alignment.

in which studies of digestive enzymes, including α-amylases, were limited to demonstrating and measuring the enzyme activity. Sporadically, isozyme profiles were used as taxonomic markers for annelids [76,77] and in these studies α-amylases were employed alongside with other enzymes. To date, no molecular characterization or genetic background of digestive enzymes has been investigated in enchytraeids. In the present study, we report that *Enchytraeus albidus* possesses two distinct α-amylase genes (Amy I and Amy II), which are homologous to *Eisenia fetida* (Ef-Amy I and Ef-Amy II) genes [20]. We found that different strains of *E. albidus* possess distinct alleles of Amy I and Amy II with unique SNP patterns specific to a particular strain. No common nucleotide Amy sequence was found for all analyzed strains, which were kept in isolation. Still, the existence of such variants cannot be excluded in other populations with higher intraspecific diversity. Therefore, Amy genes could potentially be helpful in trait-based phylogeography of *E. albidus* lineages. While allelic variation in both Amy genes was found across the species, our analysis of specimens from the COI-monohaplotype culture indicates that Amy I appears to be a highly polymorphic and multicopy gene, in contrast to Amy II. The four Amy I coding sequence variants obtained for PL-A strain specimens originating from a single cocoon indicate gene duplication or even polyploidy. Several populations of different *Enchytraeus* species were reported as polyploids before [78–80], and

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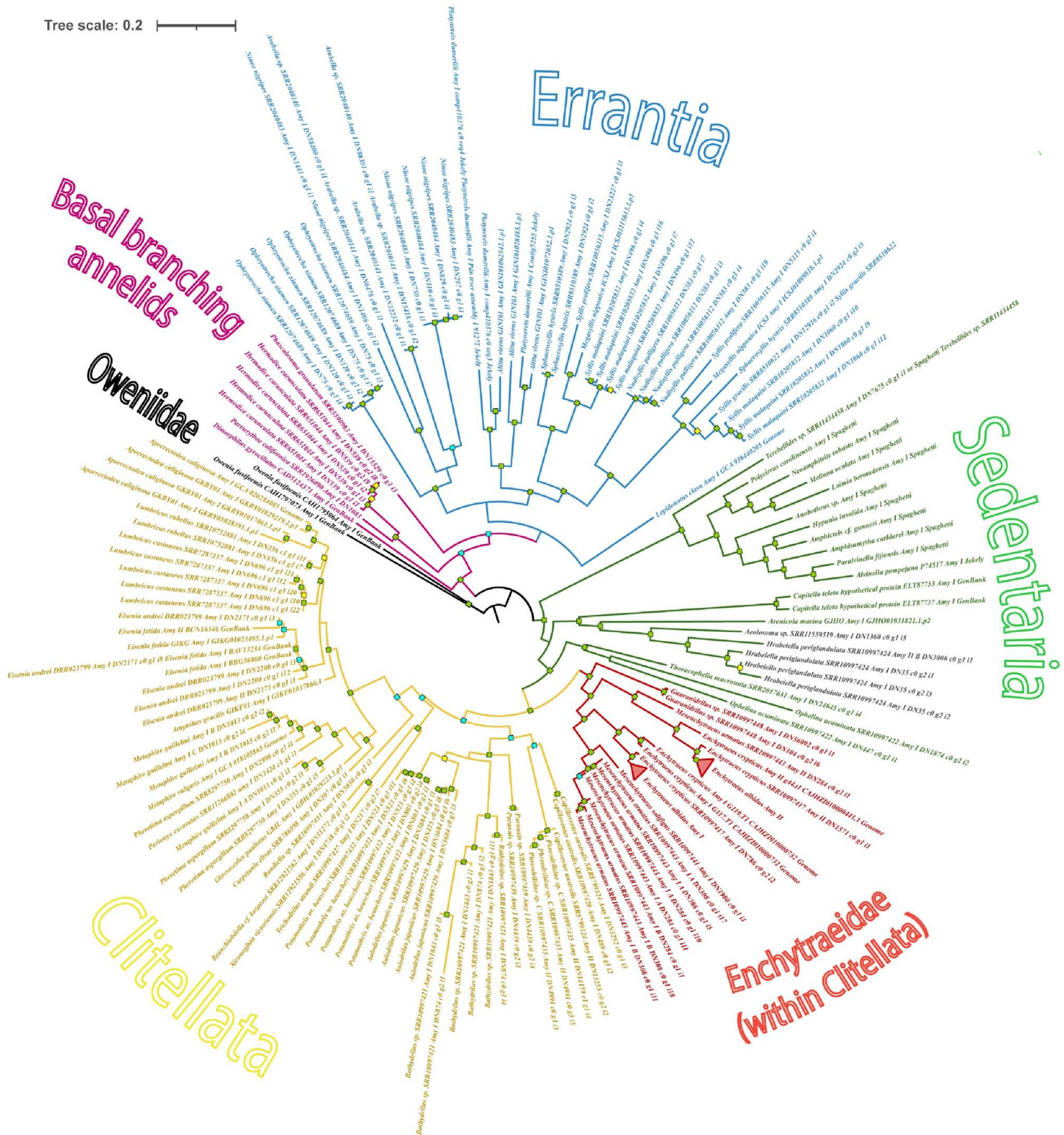

Fig. 5. Best-scoring maximum likelihood main tree (lnL = −37641.896) resulting from the analysis of mature amino acid sequences of the putative *"Enchytraeus-Eisenia* type" Amy proteins in Annelida. Most sequences used in the analysis were retrieved and generated from transcriptomic data. Green squares denote branches with both SH-aLRT and UFBoot values (if ≥ 70) at the respective nodes. Yellow squares indicate support values (if ≥ 70) only for UFBoot, while cyan squares indicate support values (if ≥ 70) only for SH-aLRT. The tree was rooted at *Owenia fusiformis*. The amino acid sequence alignment used to construct the presented tree and IQ-TREE resulting files are included in Supplementary Data S4.

certain polyploid strains, at least in some potworm species, can produce normal spermatozoa and reproduce successfully [81]. However, Christensen [82] reported no variation in chromosome number ($n = 21$) for *E. albidus*, which is therefore considered a diploid species. Thus, each copy of Amy I in *E. albidus* is most likely owned to gene duplication. The four allelic variants obtained in our study could be explained as the presence of two heterozygous loci for Amy I. This is in line with *E. crypticus* in which two copies of Amy I and one copy of Amy II per haploid genome (Acc. GCA_905160935) could be found. Many other animals have multiple copies of amylase genes [59,83]. In drosophilids (Diptera, Drosophilidae), the number of copies can vary from 1 to 6, depending on the species [18,59]. A more starch-rich diet was found to be correlated with the copy number of α-amylase gene in mammalian genomes [84], but there is no clear link between diet and the gene copy number in crustaceans and insects. This copy number can vary greatly between related invertebrate species which share similar diets [59,83]. In addition, different individuals of the same species can

73:1036501724

have a different number of gene copies [85]. However, intraspecific copy number variation of α-amylase gene was probably not explored in invertebrates [59]. An obvious evolutionary advantage of the presence of multiple copies of amylase gene can be increased enzyme production [86]. However, duplications of the gene may also confer flexibility for controlling enzyme synthesis and differential (developmental or tissue-specific) gene expression through fine regulation at the transcriptional level [84,87]. In humans, each copy of AMY1 contains the regulatory sequences necessary for salivary-specific expression [88]. In adult sand fly *Lutzomyia longipalpis* some of the α-amylase genes are down regulated after a blood meal [89,90]. Conversely, in the spiny lobster *Panulirus argus*, which is considered a carnivorous species and possesses only a single α-amylase gene, the gene expression is not affected by changes in starch content in the diet [58]. Some carnivorous species of polychaetes, which lack prey or carrion, may subsist through herbivorous behavior [91]. Such adaptive omnivory could be the reason for the conservation of α-amylase, even in carnivorous invertebrates. On the other hand, some groups of Polychaeta such as Syllidae, well-represented in our phylogenetic analysis, were initially considered mostly carnivorous. However, this broad generalization was later debunked, showing that several of these species are not carnivores [92]. In addition, the fireworm *Hermodice carunculata* (Annelida, Amphinomidae) known for feeding on live cnidarians, and included in our analysis, can use macroalgae as supplementary food source [93,94]. While members of the same group within Polychaeta can belong to very different trophic guilds, typical carnivorous Oligochaeta (sensu Schmelz et al. [67]) are rare outside the leech-like clitellates (Branchiobdellidae, Acanthobdellidae, and Hirudinida) [95]. Interestingly, no amylase gene was found in the genome of the leech *Helobdella robusta* [24]. Furthermore, we identified "*Enchytraeus-Eisenia* type" Amy sequences in branchiobdellids, which are micropredators and can also graze on algae [96,97], but did not find any in the available TSA transcriptomes for acanthobdellids or leeches. Therefore, it seems that α-amylase was not preserved in purely carnivorous clitellates, and the gene loss occurred at least is some hirudinid species.

A few previous studies have reported that α-amylase genes present a high degree of polymorphism in invertebrate species. For example, in a natural population of the tropical shrimp *Penaeus* (syn. *Litopenaeus*) *vannamei* in Panama, 35 different alleles of the "α-amylase II" gene were detected [98]. Similarly, in the oyster *Crassostrea gigas*, 30 different alleles for the α-amylase A gene were recognized [99]. In our analysis, somewhat limited sampling from three different isolated enchytraeid cultures determined 15 alleles of the Amy I gene in *E. albidus*, but it may be expected that there is even higher polymorphism in natural populations. The adaptive significance of α-amylase allelic polymorphism is still little studied in animals, particularly in invertebrates. In *C. gigas*, a correlation between growth and amylase genes polymorphism was found, but no significant differences in allozymes activity were observed. However, high differences in mRNA levels between the two analyzed groups of amylase genotypes suggested that regulatory regions play a major role in this association [100]. On the other hand, a recent study in broilers (*Gallus gallus domesticus*) revealed that several SNPs within the coding sequence of the AMY1A gene, not just in the untranslated regions, can affect food intake and growth [101]. In addition, Hughes and co-workers [102] found that a specific chicken pancreatic α-amylase allozyme had higher activity and lower sensitivity to inhibition by a wheat-derived inhibitor than the other variant. Studies on amylase inhibitors have demonstrated that effective inhibitors form stable complexes with amylases through a couple of dozens of interface-forming residues; thus, multiple incompatible structural changes, rather than a single critical mutation, determine the enzyme's resistance to inhibition [103,104]. The number of amylase inhibitors can be related to the diversity of plants available in the natural habitat [105], and enchytraeids are known to consume various plant materials [3]. A high polymorphism and harboring of multiple copies of the α-amylase gene are adequate adaptations for circumventing the action of amylase inhibitors. Therefore, our results may suggest that both strategies in overcoming these inhibitors, i.e., excessive enzyme production and expression of insensitive allelic variants, play a role in amylolytic activity in *E. albidus*.

4.2. Amy paralogs and the GHGA motif

An intriguing issue is the presence of multiple, often highly divergent α-amylase genes within the same species. In this study, we also reported the expression of the second, divergent (approximately 30 % different in amino acid sequence) α-amylase gene named Amy II in *E. albidus*. The characteristic difference between Amy I and Amy II is that the latter paralog lacks the GHGA motif in the flexible loop region. We used the absence of the GHGA motif and sequence similarity to Ef-Amy II to assign all identified "*Enchytraeus-Eisenia* type" amylases to one of these groups.

The loss of the GHGA motif in animal α-amylases has been reported before and studied in more detail in insects. In true flies (Diptera, Muscomorpha), there is a specific paralog of classical amylases called Amyrel that was first described in *Drosophila* species and shares the loss of the motif. This paralog is highly divergent from other classical α-amylases found in *Drosophila melanogaster*. It shares only about 60 % amino acid identity in this species and its expression is restricted to the larvae midgut. However, it is important to note that the absence of the GHGA motif in the flexible loop region alone does not necessarily determine the Amyrel proteins. Those proteins share a global similarity, specific substitutions, and are grouped in a single cluster, while other non-Amyrel-related amylases in Diptera can also lack this motif. In Hymenoptera, two types of amylases exist, one with conserved flexible loop and another with deletion in the loop, while in Coleoptera, mainly the second type is presented. The recurrent and independent losses of GHGA motif in insect amylases are explained by convergent evolution, but the selective constraints driving these changes remain unknown [59,106].

According to our dataset focused on Annelida, we identified tentative early evolutionary [66] modifications, such as substitutions or incomplete deletions of the GHGA motif in the flexible loop region of Amy proteins, in Spionidae, Echiura, and Capitellidae (Supplementary Data S5). However, it is important to note that amylase sequences were only included for some of the listed taxa in our primary dataset (see section 3.3: Transcriptomic data for other annelids and phylogenetic analysis of α-amylase). Additionally, changes in the GHGA motif in the flexible loop region of Amy proteins were identified in Arenicolidae and Aeolosomatidae. Based on analyzed data, the complete loss of the GHGA motif in the flexible loop and the emergence of Amy II occurred in the polychaete *Hrabeiella periglandulata*. In this case, the entire GHGAGG stretch, not just the GHGA, was lost, and this deletion appears to have occurred independently from that observed in Clitellata. We identified Amy II proteins in clitellate families such as Capilloventridae, Phreodrilidae, and Enchytraeidae, but not in Naididae, Randiellidae, Crassiclitellata (except *Eisenia* spp.), Lumbriculidae, and Branchiobdellidae. Unfortunately, transcriptomic data suitable for digestive enzyme analysis for Proppapidae, Haplotaxidae, and Parvidrilidae is lacking. Our analysis of several transcriptomes of Naididae strongly suggests Amy II gene loss in this family. However, Amy II transcripts are not easy to recover from RNA-seq data. For example, identical Amy I sequences were found in both *Capilloventer australis* samples (SRR10997420, SRR8799324), while

74:8762961145

Amy II was recovered only in one of them. Therefore, it is difficult to reliably assess the presence or absence of the Amy II gene from RNA-seq data without sufficiently deep sequencing (or genomic data support), which indicates that Amy II generally tends to be less expressed than Amy I. While the placement of *Randiella* in our analysis as sister to Crassicletallata may explain the lack of Amy II also in taxa closely associated with the order, it is challenging to reconcile the incongruence with the phylogeny of species. After all, Randiellidae is considered to be one of the evolutionarily oldest groups of Oligochaeta [66,95]. However, it should be mentioned that our screening of the *Randiella* transcriptome assembled based on the only available reads (SRR10997431) revealed the presence of two homologous Amy sequences. These two detected Amy homologs were never placed together or closely associated in phylogenetic analysis. One of these sequences was constantly recovered within Naididae and therefore excluded from further analysis. Nonetheless, Amy II was not found in *Randiella*. However, our concerns were also raised due to the unusually high diversity of other expressed α-amylases found in the transcriptome of *Randiella*. To address this issue, we decided to recover transcripts of eukaryotic 40S ribosomal protein S13 (RPS13e) (Supplementary Data S6), a universally distributed single copy gene [107], and assess the potential contamination of samples used in our phylogenetic analysis. The RPS13e analysis results (Supplementary Fig. S2) demonstrated that *Randiella* transcriptome contains five distinct RPS13e originating from Annelida; therefore, the available RNA-seq reads for this species should be considered contaminated. Our example shows that *Randiella* transcriptome contamination could impact on results of analyses that have included the SRR10997431 sample. RNA-seq is a sensitive technique, and when only transcriptome data is available without genome reference caution should be taken because α-amylases sequences seem prone to contamination (see also [25]). For example, we found a complete sequence of woodlouse *Armadillidium nasatum* α-amylase in publicly available transcriptome of *Eisenia fetida* (Acc. GIKG00000000). As in the cases mentioned above, to solve some contamination issues in transcriptome data, constructing reference databases using both the transcript of interest and a universally expressed single copy gene from RNA-seq data from closely related species can be helpful.

The distribution of Amy II proteins among different families, as indicated by the inferred phylogenetic tree (Fig. 5), suggests independent duplication events within clitellate lineages, as these proteins do not cluster into a single orthologous group (Fig. 6). This scenario also assumes a series of independent and identical GHGA motif deletions in flexible loop of Amy II proteins. Moreover, it implies a co-orthologous relationship between "*Enchytraeus-Eisenia* type" proteins (i.e., Amy I/Amy II) among Clitellata species, as in this scenario there is no duplication of Amy gene in the most recent common ancestor.

However, when the results of Amy I/II distribution are mapped onto the Clitellata phylogeny tree, an alternative evolutionary history hypothesis could be postulated, suggesting that ancestral duplication of the Amy gene may have occurred (Fig. 7). In the latter hypothesis the distribution of Amy I and Amy II could be explained by incomplete lineage sorting and lineage specific gene loss in certain families.

Based on the inferred AmyI/II gene tree, we found that in Enchytraeidae, more precisely in *Enchytraeus* and *Mesenchytraeus*, paralogous pairs of Amy genes diverged before a speciation event in their ancestor and are out-paralogs. Conversely, in *Eisenia* earthworms Amy I and Amy II were found to be in-paralogs with low genetic divergence. Given their high identity within *Eisenia*, it is likely that these in-paralogs originated from a gene duplication event, followed by the deletion in the flexible loop region and several substitutions. Despite thorough sampling at least some other lumbricids such as *Lumbricus* spp., Amy II type amylase was not found in the remaining earthworm species. Therefore, the presence of Amy II in certain members of Crassiclitellata, such as *Eisenia*, could be explained by parallel evolution at the genetic level. This suggests the emergence of a novel *Eisenia*-Amy II type amylase (i.e., Ef-Amy I' ΔGHGA is Ef-Amy II sensu Tsukamoto et al. [20]) following the general loss of the Amy II gene in Crassiclitellata. This hypothesis is supported by an additional phylogenetic analysis of amylases featuring a modified flexible loop region, particularly when considered in conjunction with a comparative analysis of the

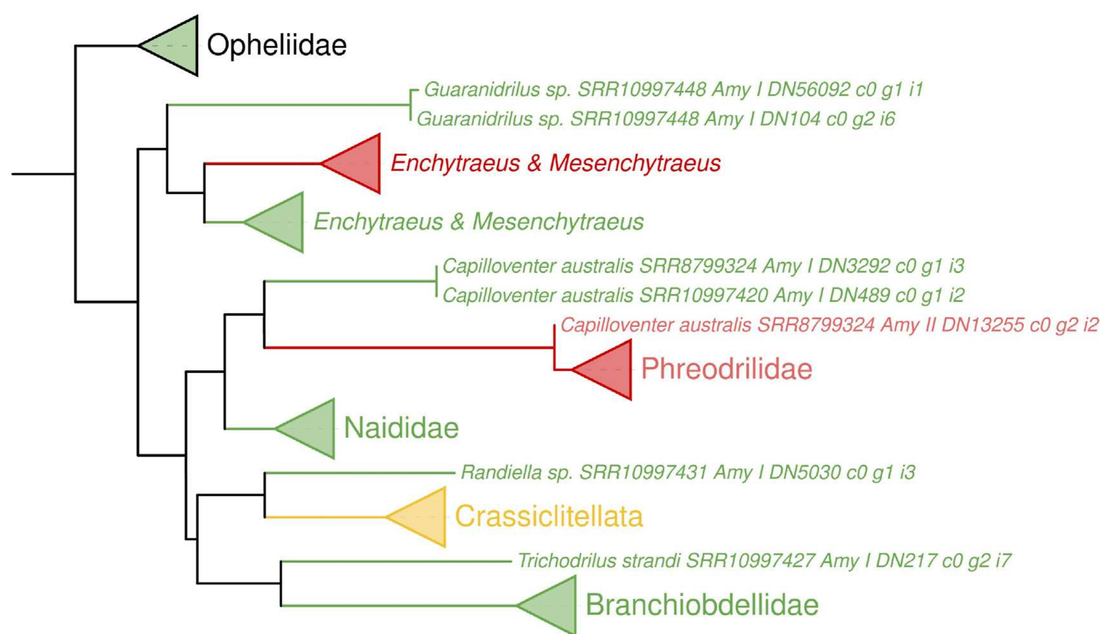

Fig. 6. Phylogenetic relationships and distribution of Amy I and Amy II paralogs among different groups of Clitellata as found in this study. The Amy I lineages are highlighted in green, while the Amy II lineages are highlighted in red. Crassiclitellata clade is collapsed and highlighted in yellow, as most species in this group have Amy I, while Amy II evolved independently from Amy I in *Eisenia* spp.

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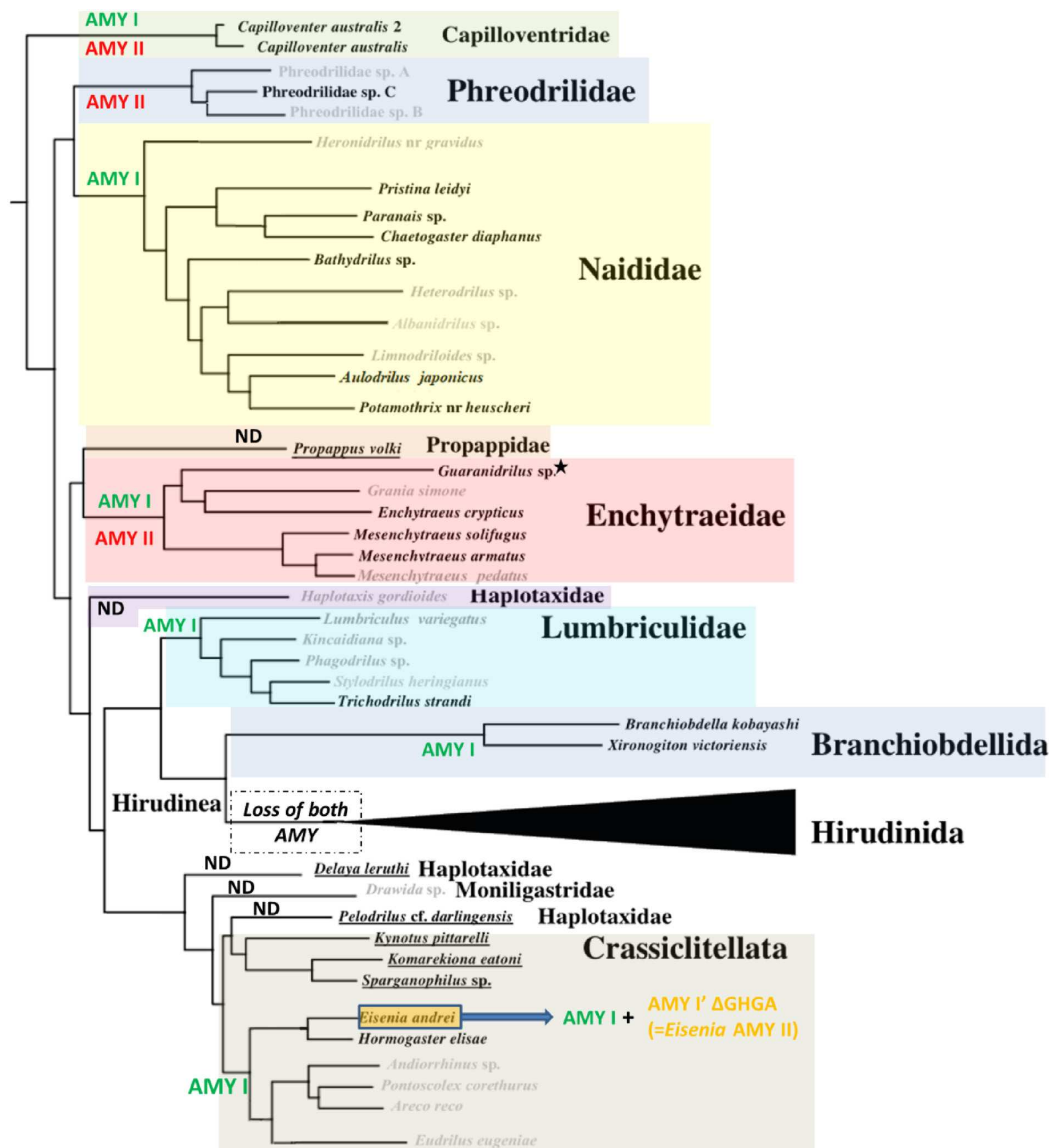

Fig. 7. Evolutionary history hypothesis of "*Enchytraeus-Eisenia* type" amylases in the context of phylogenetic relationships within Clitellata. The distribution of Amy I and Amy II found in this study was mapped onto the recent species phylogeny topology adapted from Ref. [66]. Randiellidae-Parvidrilidae clade was excluded due to the ambiguity surrounding the origin of the recovered *Randiella* amylase sequence. Species analyzed in both studies were indicated in normal font. Species analyzed in both studies, for which the Amy sequence was not recovered or only a short partial sequence was obtained, were marked in greyed font. Species not analyzed for amylase but included for the topology were underlined. ND denotes that no data was analyzed, and the star symbol denotes that only Amy I was found, but the transcriptome was highly incomplete (26.21 % of the BUSCO [108] groups had complete gene representation).

topology of the Amy gene tree and the phylogenetic tree of Clitellata species (see Fig. 8).

The placement of *Eisenia* Amy II proteins outside the clade formed by the remaining clitellates, including the basal Capilloventridae, supports the idea that *Eisenia fetida/andrei* Amy II proteins and Amy II proteins in the remaining clitellates share a pseudoorthologous relationship. This positioning of *Eisenia* Amy II is notably discordant with the overall species phylogeny. Conversely, the minimal discordance within the clade formed by Amy II from the remaining clitellates can be attributed to the sister position of *Capilloventer* and Phreodrilidae sp. This positioning likely results from the very high sequence similarity and identity of Amy II in both taxa, and possibly incomplete lineage sorting. Taking all these into account, our additional phylogenetic analysis of amylases with modified flexible loop region from Sedentaria and basal Clitellata lineages supports the hypothesis that the most recent common ancestor of Clitellata possessed Amy II, apart from Amy I.

Based on the habitat information (Fig. 9) for the analyzed

76:7939332642

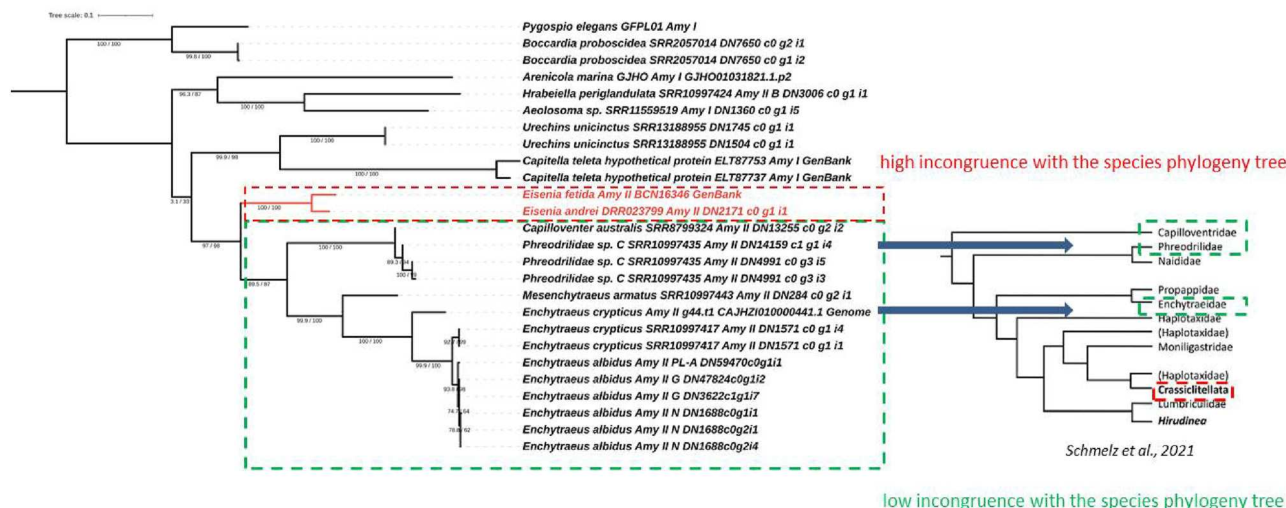

Fig. 8. Best-scoring maximum likelihood resulting from an additional phylogenetic analysis of Amy amylases with a modified flexible loop region from Sedentaria and Clitellata, including *Eisenia* spp. The GHGAGG motif is considered ancestral. The position of Clitellata sequences was compared with the phylogenetic relationships of families [66,67]. Sequences for data matrix were aligned using MUSCLE. The tree was rooted at Spionidae, and SH-aLRT and UFBoot values are shown below the branches. The result of the analysis indicates that Amy II proteins from *Eisenia* spp. form a separated group to Amy II proteins of other clitellates with high support.

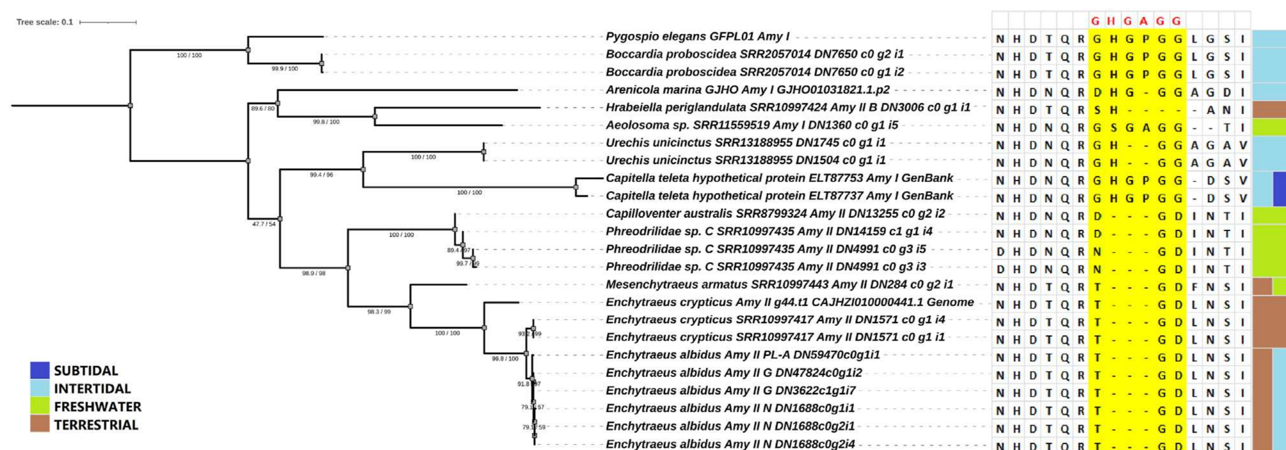

Fig. 9. Best-scoring maximum likelihood tree (lnL = −7713.887) resulting from an additional phylogenetic analysis of Amy amylases with a modified flexible loop region from Sedentaria and Clitellata, and a comparison of habitat characters. The GHGAGG motif is considered ancestral. Sequences were aligned using MUSCLE [111], and the resulting matrix was analyzed using IQ-TREE with default settings. The tree was rooted at Spionidae, and SH-aLRT and UFBoot values are shown below the branches. Habitat characters were assigned based on the literature [112–114] and the coauthors' personal knowledge.

species, we propose a hypothetical scenario in which the transition from marine to freshwater and terrestrial environments drove the deletion in the flexible loop of Amy proteins in Clitellates. However, the explanation of the selection pressures influencing this phenomenon remains unknown, especially since the exact biological role of Amy II and other amylases lacking the GHGA motif in animals is still elusive. It is known from studies on *E. fetida* [19,20] that both Amy I and II proteins are halophilic and cold-adapted enzymes, which might also be expected in *E. albidus* and *Mesenchytraeus solifugus* (partial Amy II sequence was found for this species), given the living environment of the latter species [109,110] (though confirmation of these properties requires experimental evidence). Therefore, changes in the salinity of the environment or temperature were probably not crucial evolutionary constraints driving adaptations of Amy amylases. According to Erséus and co-workers [66] the radiation of freshwater annelids that began in the Devonian was possibly facilitated by the contemporaneous evolution of land plants. The latter led to an increased production of organic matter deposited in freshwater sediments. Amy II could potentially counteract the increased number of plant inhibitors in the environment because the flexible loop plays a role in the recognition of substrates and inhibitors [20]. Further studies are needed to test this hypothesis.

A recent study of the biochemical properties of Amyrel from *D. melanogaster* [115] revealed that this GHGA motif-lacking enzyme possesses both hydrolytic α-amylase activity (EC 3.2.1.1) and a 4-α-glucanotransferase (EC 2.4.1.25) transglycosylation activity. Unlike canonical animal α-amylases, the enzymatic hydrolysis of starch catalyzed by Amyrel produces a complex mixture of maltooligosaccharides. The engineered deletion mutants ΔGHGA of porcine pancreatic and classical *D. melanogaster* α-amylases result in reaction patterns similar to those of Amyrel [115]. Recently, transglycosylation activity was reported in LDAmy, another α-amylase with a deletion in the flexible loop, derived from the Colorado potato beetle (*Leptinotarsa decemlineata*) [116]. It is worth mentioning that transglycosylation activity was also suggested in

77:6876710015

Ef-Amy II based on the hydrolysis product patterns [20]. In contrast to Amyrel, which has weak amylolytic activity (30–50 times lower than the classical *Drosophila* α-amylases) [117], both *E. fetida* Amy amylases exhibit similarly high activity, though their substrate specificities are different from each other [20]. Thus, the adaptive significance of Amy II in annelids could be related to the effective utilization of starch and similar polysaccharides from a broader range of food sources in their natural environment.

It would be interesting to study further the gene expression and regulation of Amy I and Amy II in *E. albidus*, including food-dependent gene expression, using RT-qPCR and expression vectors. This would allow for a comparison of the expression of both paralogs and their enzymatic properties to other described amylases in Annelida, offering deeper insight into the adaptive

Fig. 10. Best-scoring maximum likelihood tree resulting from the analysis of mature amino acid sequences of the putative "*Enchytraeus-Eisenia* type" Amy proteins, along with other divergent types of α-amylases revealed from screening the transcriptomes of Annelida (Supplementary Data S7). Additionally, BLASTp-identified homologs expressed in the brachiopod *Lingula anatina* (Acc. XP_013384479) and the Atlantic horseshoe crab *Limulus polyphemus* (Acc. XP_022250530) were included. The amino acid alignment was curated with BMGE [119] using default parameters via the online server NGPhylogeny.fr [120], and the tree was constructed using IQ-TREE. The tree was rooted at Echinodermata sequences. Green squares denote branches with both SH-aLRT and UFBoot values (if $\geq$ 70) at the respective nodes.

78:3921233129

Fig. 11. Alignment of Amy II coding sequences revealed in *Capilloventer australis* and Phreodrilidae sp. C.

significance of these enzymes in annelids and the evolutionary processes that underlie their role in the digestion processes of polysaccharides.

4.3. Divergent α-amylase genes and "Enchytraeus-Eisenia type" α-amylase

Our screening of the transcriptomes of many different species revealed the expression of other types of α-amylase genes in Annelids. These α-amylases share significant similarities with those found in lingulid brachiopod *Lingula anatina* (Acc. XP_013384479), Atlantic horseshoe crab *Limulus polyphemus* (Acc. XP_022250530), and *Platynereis dumerilii* (Acc. AIZ77504). The first two amylases possess an additional protein domain linked to the classical domain C in the C-terminal position and were analyzed in bilateral animals by Da Lage [24]. Orthologous amylase genes for annelids with the extended C-terminal domain are represented in GenBank for *Capitella teleta* (Acc. ELU03285 and ELT92052) and possibly for *Owenia fusiformis* (Acc. CAH1788535, CAH1798950, and CAH1798949). Based on transcriptomic data used in our analysis, these α-amylases are not restricted to polychaetes, but can also be found scattered in clitellates, such as *Capilloventer australis*, *Mesenchytraeus armatus*, *Mesenchytraeus solifugus*, *Carpetania elisae*, and more. However, transcriptomic data for *E. albidus* and genomic data for *E. crypticus*

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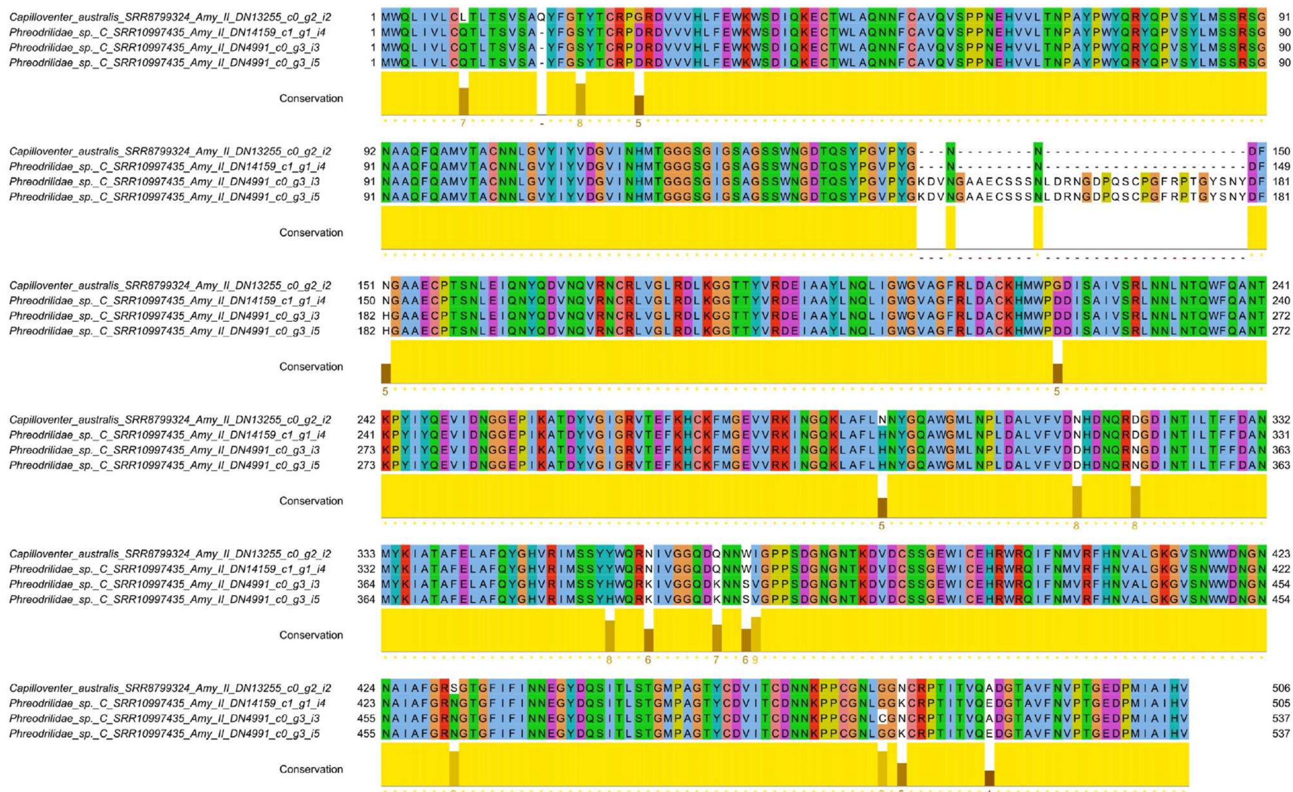

Fig. 12. Alignment of putative Amy II pre-protein sequences for *Capilloventer australis* and Phreodrilidae sp. C.

(Acc. GCA_905160935) lack these amylases. We found that α-amylases with an extended C-terminal domain are present in *Mesenchytraeus* and absent in *Enchytraeus*. Therefore, Amy I and Amy II seem to be the only amylases present in the latter genus.

The extra-domain lacking α-amylase from *Platynereis dumerilii* (Acc. AIZ77504) was used as a digestive system marker gene by Williams et al. [118]. However, it differs from *P. dumerilii* Amy I proteins, which we used in our data set. The former amylase shares more similarity with porcine pancreatic α-amylase (Acc. 1JFH_A) than with "*Enchytraeus-Eisenia* type" amylases. A distinguishing feature of "*Enchytraeus-Eisenia* type" amylases compared to other amylases is the absence of a serine-lysine-leucine (SKL) or similar motifs with a conserved lysine (e.g., AKL, AKV, or SKV) at the carboxyl-terminal end (see also [20]). These motifs are widely distributed in vertebrate pancreatic α-amylases and insect amylases.

Based on a phylogenetic analysis of AmyI/AmyII and the aforementioned non-AmyI/AmyII sequences from Annelida and other taxa (Fig. 10), the presence of Amy proteins in annelids is clearly ancestral. Furthermore, the "*Enchytraeus-Eisenia* type" α-amylase is derived in comparison to other invertebrate α-amylase types.

4.4. "*Enchytraeus-Eisenia type*" α-amylase homologs and annelid phylogeny

Although the evolution of species is typically inferred through phylogenetic analysis based on the relationships between their homologous genetic sequences, it is not uncommon to find that the phylogenetic trees of individual genes do not match the species phylogeny. Apart from methodological issues, this discrepancy can result from various processes such as gene loss, gene duplication, horizontal gene transfer, and incomplete lineage sorting. In addition, unequal evolutionary rates of certain genes in different taxa are also observed [121,122]. Concerted evolution, a process in which evolving paralogs from the same species show higher sequence similarity to each other than either does to orthologs in other species, can also lead to conflicts between specific gene phylogeny and species phylogeny [123,124].

Our study found that "*Enchytraeus-Eisenia* type" α-amylase homologs are present and have evolved across all major groups of Annelida: basal branching annelids, Errantia, Sedentaria, and Oligochaeta, indicating their ancestral origin within the phylum. While the inferred phylogeny of Amy proteins was consistent with particular recent findings regarding the relationship of Aeolosomatidae and Hrabeiellidae, as well as the position of *Dimorphilus* within basal branching annelids (but cf [68]), the obtained topology of the Amy tree showed a few major discrepancies with the currently accepted phylogeny of annelid species. In the phylogeny of species, Annelida is divided into a clade called Pleistoannelida, with Errantia and Sedentaria (including Clitellata) as the highest-ranked sister groups, and a cluster of basal branching lineages [64,65,125]. After rooting the tree at Oweniidae, our analysis of Amy proteins showed that the tree split into two superclades. The first superclade comprises basal branching lineages and the descendant Errantia, and the second superclade comprises Sedentaria, including the Clitellata. This partitioning of the tree does not match well with the species phylogeny. However, we believe that the observed topology results from the lack of Amy sequences in our data set for the old evolutionary groups of Sedentaria, such as Orbiniida or Diurodrilidae. For instance, we only recovered a short partial Amy sequence for *Diurodrilus subterraneus*, and no α-amylase at all was found in the transcriptome of orbiniid *Naineris dendritica*. Although we have incorporated a single Amy sequence from another orbiniid species *Scoloplos armiger* into our data set for

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discussion purposes, it had little effect on the overall topology of the Amy tree (Supplementary Fig. S3, Supplementary Data S8). Our screening of the transcriptomes assembled independently by us and retrieved from supplementary data included in Tilic et al. [126] strongly indicates that Amy proteins are absent in Sabellida due to gene loss. Interestingly, we identified sequences of GH13_1-like acid α-amylases in this taxon, which were studied in bilaterian animals by Desiderato et al. [25]. Therefore, the limited data for some taxa, as well as the loss of genes in others, could have contributed to the observed topology.

An unexpected result of our phylogenetic analysis of Amy proteins is the placement of Enchytraeidae as a sister to all other Clitellata, which is incongruent with the Clitellata species phylogeny [66]. The presence of paralogous gene copies in the data set can cause several problems that impede accurate phylogenetic reconstruction, and the resulting phylogeny may be distorted. A rigorous approach is to remove any paralogous loci from phylogenetic analyses [127]. However, in our dataset, it was not feasible to remove the paralogs, as Amy paralogs in Errantia cannot be unambiguously distinguished from orthologs. Interestingly, the inclusion of paralogous copies (i.e. Amy II) for clitellates resulted in a topology more consistent with the species tree, potentially improving phylogenetic resolution. We observed uneven distribution of Amy paralogs across the analyzed groups of clitellates, which may be due to incomplete lineage sorting or data limitations. To address this issue, more data for underrepresented taxa is required. Furthermore, we noted a remarkable case of high sequence similarity, both in protein and coding sequences, between Amy II in *Capilloventer australis* and Phreodrilidae sp. C (Figs. 11 and 12, Supplementary Data S9). This finding suggests that these taxa may have experienced strong selection pressures acting on the Amy II gene. Thus, it would be interesting to explore the Amy proteins in other members of Capilloventridae and Phreodrilidae to understand better the evolution of "*Enchytraeus-Eisenia* type" α-amylase homologs within Clitellata.

5. Conclusions

- *Enchytraeus albidus* has two distinct α-amylase genes (Amy I and Amy II) that are homologs to earthworm *Eisenia fetida* Ef-Amy genes. Amy genes seem to be the only α-amylases expressed in *Enchytraeus*, in contrast to *Mesenchytraeus.*
- Different laboratory strains of *E. albidus* (PL-A, G, and N) possess different alleles of α-amylases with unique SNP patterns associated with a particular strain.
- Amy I seems to be a highly polymorphic and multicopy gene, unlike Amy II.
- The domain architecture of the putative Amy proteins is typical of other α-amylases with ABC-domains reported in other animals.
- Amy II lacks the GHGA motif in the flexible loop region, similarly to some insect amylases.
- The most recent common ancestor of Clitellata had both Amy I and Amy II paralogs. According to our hypothesis, the transition from marine to freshwater and terrestrial environments drove the deletion in the flexible loop of Amy proteins.
- The loss of GHGA motif in Amy α-amylase is an ancestral feature for clitellates, although modification or even independent deletion in the flexible loop region also occurred in some relatively distant sedentarian lineages.
- "*Enchytraeus-Eisenia* type" α-amylase homologs are present in major groups of Annelida: basal branching annelids, Errantia, Sedentaria, and Oligochaeta, indicating ancestral origin of Amy proteins within the phylum.
- While the inferred phylogeny based on the Amy genes displayed some notable differences from the currently accepted species phylogeny of Annelida, it successfully resolved some long-standing issues regarding the placement and relationships of certain taxa (*Dimorphilus*, *Hrabeiella*, and *Aeolosoma*), in line with the results of recent large multi-locus phylotranscriptomic and phylogenomic analyses.

Funding information

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors, and was realized as part of the statutory activities of the University of Silesia in Katowice.

CRediT authorship contribution statement

Łukasz Gajda: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Agata Daszkowska-Golec:** Software, Validation, Writing – review & editing, Supervision. **Piotr Świątek:** Resources, Writing – review & editing, Supervision.

Data availability

The data underlying this article are available in the supplementary materials. New nucleotide α-amylase sequences generated for *E. albidus* PL-A strain were deposited in GenBank database under accession numbers OQ830661-OQ830669 and OQ843025-OQ843027. Raw RNA-seq reads generated for this study were deposited in the Sequence Read Archive (SRA) at NCBI and can be accessed under the BioProject number PRJNA956247.

Declarations of interest

The authors declare no competing interests.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We would like to express our gratitude to Tjalf de Boer and Martin Holmstrup for providing the core set of transcripts for freeze-tolerant strains at an early stage of the conception of this manuscript. We also extend our thanks to Monica Amorim, Dick Roelofs, and Rüdiger Schmelz for providing detailed answers regarding the identity of the freeze-tolerant *E. albidus* strains in the light of the recent taxonomic revision. All co-authors would like to thank Maciej Pabijan from the Department of Comparative Anatomy, Institute of Zoology and Biomedical Research at Jagiellonian University, and an anonymous reviewer for their valuable and constructive comments that contributed to improving the quality of the paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biochi.2024.01.008.

81:2829555400

References

[1] C. Pelosi, J. Römbke, Enchytraeids as bioindicators of land use and management, Appl. Soil Ecol. 123 (2018) 775–779, https://doi.org/10.1016/j.apsoil.2017.05.014.

[2] M.J. Amorim, Y. Gansemans, S.I. Gomes, F. Van Nieuwerburgh, J.J. Scott-Fordsmand, Annelid genomes: *Enchytraeus crypticus*, a soil model for the innate (and primed) immune system, Lab. Anim. 50 (2021) 285–294, https://doi.org/10.1038/s41684-021-00831-x.

[3] Ł. Gajda, S. Gorgoń, A.Z. Urbisz, Food preferences of enchytraeids, Pedobiologia 63 (2017) 19–36, https://doi.org/10.1016/j.pedobi.2017.06.002.

[4] C.O. Nielsen, Carbohydrases in soil and litter invertebrates, Oikos 13 (1962) 200, https://doi.org/10.2307/3565085.

[5] M.C. Dash, B. Nanda, P.C. Mishra, Digestive enzymes in three species of Enchytraeidae (Oligochaeta), Oikos 36 (1981) 316, https://doi.org/10.2307/3544628.

[6] F. Urbášek, J. Chalupský Jr., Activity of digestive enzymes in 4 species of Enchytraeidae (Oligochaeta), Rev. Ecol. Biol. Sol 28 (1991) 145–154.

[7] F. Urbášek, J. Chalupský, Effects of artificial acidification and liming on biomass and on the activity of digestive enzymes in Enchytraeidae (Oligochaeta): results of an ongoing study, Biol. Fertil. Soils 14 (1992) 67–70, https://doi.org/10.1007/BF00336305.

[8] V. Šustr, J. Chalupský, Activity of digestive enzymes in two species of potworms (Oligochaeta, Enchytraeidae), Pedobiologia 40 (1996) 255–259.

[9] V. Šustr, J. Chalupský, V. Krištůfek, Effects of artificial acidification and liming on the activity of digestive enzymes in *Cognettia sphagnetorum* (Vejdovsky, 1878)(Annelida, Enchytraeidae), Biol. Fertil. Soils 24 (1997) 227–230.

[10] S.R. Gelder, Diet and histophysiology of the alimentary canal of *Lumbricillus lineatus* (Oligochaeta, Enchytraeidae), Hydrobiologia 115 (1984) 71–81.

[11] C. Erséus, M.J. Klinth, E. Rota, P. De Wit, D.R. Gustafsson, S. Martinsson, The popular model annelid *Enchytraeus albidus* is only one species in a complex of seashore white worms (Clitellata, Enchytraeidae), Org. Divers. Evol. 19 (2019) 105–133, https://doi.org/10.1007/s13127-019-00402-6.

[12] A.Z. Urbisz, Ł. Chajec, A. Brąszewska-Zalewska, J. Kubrakiewicz, P. Świątek, Ovaries of the white worm (*Enchytraeus albidus*, Annelida, Clitellata) are composed of 16-celled meroistic germ-line cysts, Dev. Biol. 426 (2017) 28–42, https://doi.org/10.1016/j.ydbio.2017.04.009.

[13] E.A. Fairchild, A.M. Bergman, J.T. Trushenski, Production and nutritional composition of white worms *Enchytraeus albidus* fed different low-cost feeds, Aquaculture 481 (2017) 16–24, https://doi.org/10.1016/j.aquaculture.2017.08.019.

[14] M.E. Holmstrup, S.F. Gadeberg, K. Engell-Sørensen, S. Slotsbo, M. Holmstrup, A new strategy in rearing of European flounder: using live *Enchytraeus albidus* to enhance juvenile growth, J. Insects as Food Feed. 8 (2022) 1333–1341, https://doi.org/10.3920/JIFF2021.0106.

[15] OECD, Test No. 220: Enchytraeid Reproduction Test, OECD, 2016, https://doi.org/10.1787/9789264264472-en.

[16] W. Michaelsen, Oligochaeta, in: G. Grimpe, E. Wagler (Eds.), Die Tierwelt Der Nord- Und Ostsee Tierwelt Der Nordund Ostsee. VI (C1), Akademische Verlagsgesellschaft, Leipzig, 1927, pp. 1–44.

[17] H. Nagy, K. Dózsa-Farkas, T. Felföldi, New insights into the *Enchytraeus albidus* complex (Annelida, Enchytraeidae), with the description of three new species from seashores in Italy and Croatia, Eur. J. Taxon. 870 (2023), https://doi.org/10.5852/ejt.2023.870.2123.

[18] J.-L. Da Lage, A. Van Wormhoudt, M.-L. Cariou, Diversity and evolution of the alpha-amylase genes in animals, Biologia-Bratislava 57 (2002) 181–190.

[19] Y. Hirano, K. Tsukamoto, S. Ariki, Y. Naka, M. Ueda, T. Tamada, X-ray crystallographic structural studies of α-amylase I from *Eisenia fetida*, Acta Crystallogr D Struct Biol 76 (2020) 834–844, https://doi.org/10.1107/S2059798320010165.

[20] K. Tsukamoto, S. Ariki, M. Nakazawa, T. Sakamoto, M. Ueda, Novel cold-adapted raw-starch digesting α-amylases from *Eisenia fetida*: gene cloning, expression, and characterization, Biotechnol. Rep. 31 (2021) e00662, https://doi.org/10.1016/j.btre.2021.e00662.

[21] M.R. Stam, E.G.J. Danchin, C. Rancurel, P.M. Coutinho, B. Henrissat, Dividing the large glycoside hydrolase family 13 into subfamilies: towards improved functional annotations of -amylase-related proteins, Protein Eng. Des. Sel. 19 (2006) 555–562, https://doi.org/10.1093/protein/gzl044.

[22] S. Yan, G. Wu, Analysis on evolutionary relationship of amylases from archaea, bacteria and eukaryota, World J. Microbiol. Biotechnol. 32 (2016) 24, https://doi.org/10.1007/s11274-015-1979-y.

[23] J.-L. Da Lage, F. Maczkowiak, M.-L. Cariou, Phylogenetic distribution of intron positions in alpha-amylase genes of Bilateria suggests numerous gains and losses, PLoS One 6 (2011) e19673, https://doi.org/10.1371/journal.pone.0019673.

[24] J.-L. Da Lage, An optional C-terminal domain is ancestral in α-amylases of bilaterian animals, Amylase 1 (2017), https://doi.org/10.1515/amylase-2017-0003.

[25] A. Desiderato, M. Barbeitos, C. Gilbert, J.-L. Da Lage, Horizontal transfer and gene loss shaped the evolution of alpha-amylases in bilaterians, G3-GENES GENOM, For. Genet. 10 (2020) 709–719, https://doi.org/10.1534/g3.119.400826.

[26] T.E. de Boer, D. Roelofs, R. Vooijs, M. Holmstrup, M.J. Amorim, Population-specific transcriptional differences associated with freeze tolerance in a terrestrial worm, Ecol. Evol. 8 (2018) 3774–3786, https://doi.org/10.1002/ece3.3602.

[27] S. Martinsson, C. Erséus, Cryptic diversity in the well-studied terrestrial worm *Cognettia sphagnetorum* (Clitellata: Enchytraeidae), Pedobiologia 57 (2014) 27–35, https://doi.org/10.1016/j.pedobi.2013.09.006.

[28] S. Kumar, G. Stecher, K. Tamura, MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets, Mol. Biol. Evol. 33 (2016) 1870–1874, https://doi.org/10.1093/molbev/msw054.

[29] R. Giegerich, F. Meyer, C. Schleiermacher, GeneFisher-software support for the detection of postulated genes, Proc. Int. Conf. Intell. Syst. Mol. Biol. 4 (1996) 68–77.

[30] P. Stothard, The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences, Biotechniques 28 (2000) 1102–1104, https://doi.org/10.2144/00286ir01.

[31] O. Ohara, R.L. Dorit, W. Gilbert, One-sided polymerase chain reaction: the amplification of cDNA, Proc. Natl. Acad. Sci. U.S.A. 86 (1989) 5673–5677, https://doi.org/10.1073/pnas.86.15.5673.

[32] R.L. Dorit, O. Ohara, cDNA amplification using one-sided (anchored) PCR, Curr. Protoc. Mol. Biol. 17 (1992), https://doi.org/10.1002/0471142727.mb1506s17, 15.6.1-15.6.10.

[33] K. Dallmeier, J. Neyts, Simple and inexpensive three-step rapid amplification of cDNA 5′ ends using 5′ phosphorylated primers, Anal. Biochem. 434 (2013) 1–3, https://doi.org/10.1016/j.ab.2012.10.031.

[34] J. Vandecraen, P. Monsieurs, M. Mergeay, N. Leys, A. Aertsen, R. Van Houdt, Zinc-induced transposition of insertion sequence elements contributes to increased adaptability of *Cupriavidus metallidurans*, Front. Microbiol. 7 (2016), https://doi.org/10.3389/fmicb.2016.00359.

[35] C. Camacho, G. Coulouris, V. Avagyan, N. Ma, J. Papadopoulos, K. Bealer, T.L. Madden, BLAST+: architecture and applications, BMC Bioinf. 10 (2009) 421, https://doi.org/10.1186/1471-2105-10-421.

[36] K. Katoh, J. Rozewicki, K.D. Yamada, MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization, Briefings Bioinf. 20 (2019) 1160–1166, https://doi.org/10.1093/bib/bbx108.

[37] T. Paysan-Lafosse, M. Blum, S. Chuguransky, T. Grego, B.L. Pinto, G.A. Salazar, M.L. Bileschi, P. Bork, A. Bridge, L. Colwell, J. Gough, D.H. Haft, I. Letunić, A. Marchler-Bauer, H. Mi, D.A. Natale, C.A. Orengo, A.P. Pandurangan, C. Rivoire, C.J.A. Sigrist, I. Sillitoe, N. Thanki, P.D. Thomas, S.C.E. Tosatto, C.H. Wu, A. Bateman, InterPro in 2022, Nucleic Acids Res. 51 (2023) D418–D427, https://doi.org/10.1093/nar/gkac993.

[38] I. Letunic, S. Khedkar, P. Bork, SMART: recent updates, new developments and status in 2020, Nucleic Acids Res. 49 (2021) D458–D460, https://doi.org/10.1093/nar/gkaa937.

[39] F. Teufel, J.J. Almagro Armenteros, A.R. Johansen, M.H. Gíslason, S.I. Pihl, K.D. Tsirigos, O. Winther, S. Brunak, G. von Heijne, H. Nielsen, SignalP 6.0 predicts all five types of signal peptides using protein language models, Nat. Biotechnol. 40 (2022) 1023–1025, https://doi.org/10.1038/s41587-021-01156-3.

[40] Z. Yang, Paml 4: phylogenetic analysis by maximum likelihood, Mol. Biol. Evol. 24 (2007) 1586–1591, https://doi.org/10.1093/molbev/msm088.

[41] The Galaxy Community, the Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2022 update, Nucleic Acids Res. 50 (2022) W345–W351, https://doi.org/10.1093/nar/gkac247.

[42] A. Waterhouse, M. Bertoni, S. Bienert, G. Studer, G. Tauriello, R. Gumienny, F.T. Heer, T.A.P. de Beer, C. Rempfer, L. Bordoli, R. Lepore, T. Schwede, SWISS-MODEL: homology modelling of protein structures and complexes, Nucleic Acids Res. 46 (2018) W296–W303, https://doi.org/10.1093/nar/gky427.

[43] A.M. Waterhouse, J.B. Procter, D.M.A. Martin, M. Clamp, G.J. Barton, Jalview Version 2–a multiple sequence alignment editor and analysis workbench, Bioinformatics 25 (2009) 1189–1191, https://doi.org/10.1093/bioinformatics/btp033.

[44] P. Gouet, E. Courcelle, D.I. Stuart, F. Métoz, ESPript: analysis of multiple sequence alignments in PostScript, Bioinformatics 15 (1999) 305–308, https://doi.org/10.1093/bioinformatics/15.4.305.

[45] E.F. Pettersen, T.D. Goddard, C.C. Huang, E.C. Meng, G.S. Couch, T.I. Croll, J.H. Morris, T.E. Ferrin, U.C.S.F. ChimeraX, Structure visualization for researchers, educators, and developers, Protein Sci. 30 (2021) 70–82, https://doi.org/10.1002/pro.3943.

[46] M.G. Grabherr, B.J. Haas, M. Yassour, J.Z. Levin, D.A. Thompson, I. Amit, X. Adiconis, L. Fan, R. Raychowdhury, Q. Zeng, Z. Chen, E. Mauceli, N. Hacohen, A. Gnirke, N. Rhind, F. di Palma, B.W. Birren, C. Nusbaum, K. Lindblad-Toh, N. Friedman, A. Regev, Full-length transcriptome assembly from RNA-Seq data without a reference genome, Nat. Biotechnol. 29 (2011) 644–652, https://doi.org/10.1038/nbt.1883.

[47] B.J. Haas, A. Papanicolaou, M. Yassour, M. Grabherr, P.D. Blood, J. Bowden, M.B. Couger, D. Eccles, B. Li, M. Lieber, M.D. MacManes, M. Ott, J. Orvis, N. Pochet, F. Strozzi, N. Weeks, R. Westerman, T. William, C.N. Dewey, R. Henschel, R.D. LeDuc, N. Friedman, A. Regev, De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis, Nat. Protoc. 8 (2013) 1494–1512, https://doi.org/10.1038/nprot.2013.084.

[48] M. Kanehisa, Y. Sato, K. Morishima, BlastKOALA and GhostKOALA: KEGG Tools for functional characterization of genome and metagenome sequences, J. Mol. Biol. 428 (2016) 726–731, https://doi.org/10.1016/j.jmb.2015.11.006.

[49] M. Conzelmann, E.A. Williams, K. Krug, M. Franz-Wachtel, B. Macek, G. Jékely, The neuropeptide complement of the marine annelid *Platynereis*

82:7521190669

dumerilii, BMC Genom. 14 (2013) 906, https://doi.org/10.1186/1471-2164-14-906.
[50] T. Holder, C. Basquin, J. Ebert, N. Randel, D. Jollivet, E. Conti, G. Jékely, F. Bono, Deep transcriptome-sequencing and proteome analysis of the hydrothermal vent annelid *Alvinella pompejana* identifies the CvP-bias as a robust measure of eukaryotic thermostability, Biol. Direct 8 (2013) 2, https://doi.org/10.1186/1745-6150-8-2.
[51] J. Stiller, E. Tilic, V. Rousset, F. Pleijel, G.W. Rouse, Spaghetti to a tree: a robust phylogeny for Terebelliformia (Annelida) based on transcriptomes, molecular and morphological data, Biology 9 (2020) 73, https://doi.org/10.3390/biology9040073.
[52] K.J. Hoff, M. Stanke, Predicting genes in single genomes with AUGUSTUS, Curr. Protoc. Bioinformatics. 65 (2019) e57, https://doi.org/10.1002/cpbi.57.
[53] I. Sela, H. Ashkenazy, K. Katoh, T. Pupko, GUIDANCE2: accurate detection of unreliable alignment regions accounting for the uncertainty of multiple parameters, Nucleic Acids Res. 43 (2015) W7–W14, https://doi.org/10.1093/nar/gkv318.
[54] J. Trifinopoulos, L.-T. Nguyen, A. von Haeseler, B.Q. Minh, W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis, Nucleic Acids Res. 44 (2016) W232–W235, https://doi.org/10.1093/nar/gkw256.
[55] A. Weigert, C. Helm, M. Meyer, B. Nickel, D. Arendt, B. Hausdorf, S.R. Santos, K.M. Halanych, G. Purschke, C. Bleidorn, T.H. Struck, Illuminating the base of the annelid tree using transcriptomics, Mol. Biol. Evol. 31 (2014) 1391–1401, https://doi.org/10.1093/molbev/msu080.
[56] T.H. Struck, A. Golombek, A. Weigert, F.A. Franke, W. Westheide, G. Purschke, C. Bleidorn, K.M. Halanych, The evolution of annelids reveals two adaptive routes to the interstitial realm, Curr. Biol. 25 (2015) 1993–1999, https://doi.org/10.1016/j.cub.2015.06.007.
[57] I. Letunic, P. Bork, Interactive Tree of Life (iTOL): an online tool for phylogenetic tree display and annotation, Bioinformatics 23 (2007) 127–128, https://doi.org/10.1093/bioinformatics/btl529.
[58] L. Rodríguez-Viera, E. Perera, J.A. Martos-Sitcha, R. Perdomo-Morales, A. Casuso, V. Montero-Alejo, T. García-Galano, G. Martínez-Rodríguez, J.M. Mancera, Molecular, biochemical, and dietary regulation features of α-amylase in a carnivorous crustacean, the spiny lobster *Panulirus argus*, PLoS One 11 (2016) e0158919, https://doi.org/10.1371/journal.pone.0158919.
[59] J.-L. Da Lage, The amylases of insects, Int. J. Insect Sci. 10 (2018) 117954331880478, https://doi.org/10.1177/1179543318804783.
[60] Š. Janeček, B. Svensson, B. Henrissat, Domain evolution in the alpha-amylase family, J. Mol. Evol. 45 (1997) 322–331, https://doi.org/10.1007/pl00006236.
[61] J.-L. Da Lage, G. Feller, Š. Janeček, Horizontal gene transfer from Eukarya to Bacteria and domain shuffling: the α-amylase model, CMLS Cell. Mol. Life Sci. 61 (2004) 97–109, https://doi.org/10.1007/s00018-003-3334-y.
[62] L. Pasero, Y. Mazzéi-Pierron, B. Abadie, Y. Chicheportiche, G. Marchis-Mouren, Complete amino acid sequence and location of the five disulfide bridges in porcine pancreatic α-amylase, Biochim. Biophys. Acta Protein Struct. Mol. Enzymol. 869 (1986) 147–157, https://doi.org/10.1016/0167-4838(86)90289-X.
[63] D.B. Craig, A.A. Dombkowski, Disulfide by Design 2.0: a web-based tool for disulfide engineering in proteins, BMC Bioinf. 14 (2013) 346, https://doi.org/10.1186/1471-2105-14-346.
[64] A. Weigert, C. Bleidorn, Current status of annelid phylogeny, Org. Divers. Evol. 16 (2016) 345–362, https://doi.org/10.1007/s13127-016-0265-7.
[65] J.M. Martín-Durán, B.C. Vellutini, F. Marlétaz, V. Cetrangolo, N. Cvetesic, D. Thiel, S. Henriet, X. Grau-Bové, A.M. Carrillo-Baltodano, W. Gu, A. Kerbl, Y. Marquez, N. Bekkouche, D. Chourrout, J.L. Gómez-Skarmeta, M. Irimia, B. Lenhard, K. Worsaae, A. Hejnol, Conservative route to genome compaction in a miniature annelid, Nat. Ecol. Evol. 5 (2021) 231–242, https://doi.org/10.1038/s41559-020-01327-6.
[66] C. Erséus, B.W. Williams, K.M. Horn, K.M. Halanych, S.R. Santos, S.W. James, M. Creuzé des Châtelliers, F.E. Anderson, Phylogenomic analyses reveal a Palaeozoic radiation and support a freshwater origin for clitellate annelids, Zool. Scripta 49 (2020) 614–640, https://doi.org/10.1111/zsc.12426.
[67] R.M. Schmelz, C. Erséus, P. Martin, T.V. Haaren, T. Timm, A proposed order-level classification in Oligochaeta (Annelida, Clitellata), Zootaxa 5040 (2021) 589–591, https://doi.org/10.11646/zootaxa.5040.4.9.
[68] G.W. Rouse, F. Pleijel, T. Ekin, Introduction and systematics, in: G. Rouse, F. Pleijel, E. Tilic (Eds.), Annelida, Oxford University Press, 2022, pp. 1–8, https://doi.org/10.1093/oso/9780199692309.003.0001.
[69] T.H. Struck, M.P. Nesnidal, G. Purschke, K.M. Halanych, Detecting possibly saturated positions in 18S and 28S sequences and their influence on phylogenetic reconstruction of Annelida (Lophotrochozoa), Mol. Phylogenet. Evol. 48 (2008) 628–645, https://doi.org/10.1016/j.ympev.2008.05.015.
[70] C. Csuzdi, J. Koo, Y. Hong, The complete mitochondrial DNA sequences of two sibling species of lumbricid earthworms, *Eisenia fetida* (Savigny, 1826) and *Eisenia andrei* (Bouché, 1972) (Annelida, Crassiclitellata): comparison of mitogenomes and phylogenetic positioning, ZooKeys 1097 (2022) 167–181, https://doi.org/10.3897/zookeys.1097.80216.
[71] B. Plytycz, J. Bigaj, A. Osikowski, S. Hofman, A. Falniowski, T. Panz, P. Grzmil, F. Vandenbulcke, The existence of fertile hybrids of closely related model earthworm species, *Eisenia andrei* and *E. fetida*, PLoS One 13 (2018) e0191711, https://doi.org/10.1371/journal.pone.0191711.
[72] B. Plytycz, J. Bigaj, A. Rysiewska, A. Osikowski, S. Hofman, A. Podolak, P. Grzmil, Impairment of reproductive capabilities in three subsequent generations of asymmetric hybrids between *Eisenia andrei* and *E. fetida* from French, Hungarian and Polish laboratory colonies, PLoS One 15 (2020) e0235789, https://doi.org/10.1371/journal.pone.0235789.
[73] A. Podolak, J. Kostecka, S. Hofman, A. Osikowski, J. Bigaj, B. Plytycz, Annual Reproductive performance of *Eisenia andrei* and *E. fetida* 2 in intra- and interspecific pairs and lack of reproduction of isolated virgin earthworms, Folia Biol (Krakow) 68 (2020) 1–6, https://doi.org/10.3409/fb_68-1.01.
[74] B. Plytycz, J. Bigaj, T. Panz, P. Grzmil, Asymmetrical hybridization and gene flow between *Eisenia andrei* and *E. fetida* lumbricid earthworms, PLoS One 13 (2018) e0204469, https://doi.org/10.1371/journal.pone.0204469.
[75] M. Jaskulak, A. Rorat, F. Vandenbulcke, M. Pauwels, P. Grzmil, B. Plytycz, Polymorphic microsatellite markers demonstrate hybridization and interspecific gene flow between lumbricid earthworm species, *Eisenia andrei* and *E. fetida*, PLoS One 17 (2022) e0262493, https://doi.org/10.1371/journal.pone.0262493.
[76] V. Brockmeyer, Isozymes and general protein patterns for use in discrimination and identification of *Enchytraeus* species (Annelida, Oligochaeta)1, J. Zool. Syst. Evol. Res. 29 (1991) 343–361, https://doi.org/10.1111/j.1439-0469.1991.tb00457.x.
[77] H. Schmidt, W. Westheide, Isozymes and general protein patterns as taxonomic markers in the taxon Nephtyidae (Annelida Polychaeta), Mar. Biol. 119 (1994) 31–38, https://doi.org/10.1007/BF00350103.
[78] R.M. Schmelz, R. Collado, M. Myohara, A Taxonomic study of *Enchytraeus japonensis* (Enchytraeidae, Oligochaeta): morphological and biochemical comparisons with *E. bigeminus*, Zool. Sci. 17 (2000) 505–516, https://doi.org/10.2108/0289-0003 (2000)17[505:ATSOEJ]2.0.CO;2.
[79] B. Christensen, H. Glenner, Molecular phylogeny of Enchytraeidae (Oligochaeta) indicates separate invasions of the terrestrial environment, J. Zool. Syst. Evol. Res. 48 (2010) 208–212, https://doi.org/10.1111/j.1439-0469.2009.00558.x.
[80] R. Collado, E. Cordes, R. Schmelz, Microtaxonomy of fragmenting *Enchytraeus* species using molecular markers, with a comment on species complexes in enchytraeids, Turk. J. Zool. 36 (2012), https://doi.org/10.3906/zoo-1002-70.
[81] K.A. Coates, Widespread polyploid forms off *Lumbricillus lineatus* (Müller) (Enchytraeidae: Oligochaeta): comments on polyploidism in the enchytraeids, Can. J. Zool. 73 (1995) 1727–1734. https://doi.org/10.1139/z95-204.
[82] B. Christensen, Studies on cyto-taxonomy and reproduction in the Enchytraeidae, Hereditas 47 (1961) 387–450, https://doi.org/10.1111/j.1601-5223.1961.tb01782.x.
[83] L. Rodríguez-Viera, D. Alpízar-Pedraza, J.M. Mancera, E. Perera, Toward a more comprehensive view of α-amylase across decapods crustaceans, Biology 10 (2021) 947, https://doi.org/10.3390/biology10100947.
[84] P. Pajic, P. Pavlidis, K. Dean, L. Neznanova, R.-A. Romano, D. Garneau, E. Daugherity, A. Globig, S. Ruhl, O. Gokcumen, Independent amylase gene copy number bursts correlate with dietary preferences in mammals, Elife 8 (2019) e44628, https://doi.org/10.7554/eLife.44628.
[85] A. Clop, O. Vidal, M. Amills, Copy number variation in the genomes of domestic animals, Anim. Genet. 43 (2012) 503–517, https://doi.org/10.1111/j.1365-2052.2012.02317.x.
[86] G.H. Perry, N.J. Dominy, K.G. Claw, A.S. Lee, H. Fiegler, R. Redon, J. Werner, F.A. Villanea, J.L. Mountain, R. Misra, N.P. Carter, C. Lee, A.C. Stone, Diet and the evolution of human amylase gene copy number variation, Nat. Genet. 39 (2007) 1256–1260, https://doi.org/10.1038/ng2123.
[87] Y. Matsuo, T. Yamazaki, Genetic analysis of natural populations of *Drosophila melanogaster* in Japan. VI. Differential regulation of duplicated amylase loci and degree of dominance of amylase activity in different environments, Jpn. J. Genet. 61 (1986) 543–558.
[88] C.N. Ting, M.P. Rosenberg, C.M. Snow, L.C. Samuelson, M.H. Meisler, Endogenous retroviral sequences are required for tissue-specific expression of a human salivary amylase gene, Genes Dev. 6 (1992) 1457–1465, https://doi.org/10.1101/gad.6.8.1457.
[89] J.M.C. Ribeiro, E.D. Rowton, R. Charlab, Salivary amylase activity of the phlebotomine sand fly, *Lutzomyia longipalpis*, Insect Biochem. Mol. Biol. 30 (2000) 271–277, https://doi.org/10.1016/S0965-1748(99)00119-8.
[90] S.G. da Costa-Latgé, P. Bates, R. Dillon, F.A. Genta, Characterization of glycoside hydrolase families 13 and 31 reveals expansion and diversification of α-amylase genes in the phlebotomine *Lutzomyia longipalpis* and modulation of sandfly glycosidase activities by *Leishmania* infection, Front. Physiol. 12 (2021) 635633, https://doi.org/10.3389/fphys.2021.635633.
[91] H.H. Checon, E.V. Pardo, A.C.Z. Amaral, Breadth and composition of polychaete diets and the importance of diatoms to species and trophic guilds, Helgol. Mar. Res. 70 (2017) 19, https://doi.org/10.1186/s10152-016-0469-4.
[92] A. Giangrande, M. Licciano, P. Pagliara, The diversity of diets in Syllidae (Annelida : Polychaeta), Cah. Biol. Mar. 41 (2000) 55–65.
[93] A.T. Wolf, M.M. Nugues, C. Wild, Distribution, food preference, and trophic position of the corallivorous fireworm *Hermodice carunculata* in a Caribbean coral reef, Coral Reefs 33 (2014) 1153–1163, https://doi.org/10.1007/s00338-014-1184-8.
[94] A. Schulze, C.J. Grimes, T.E. Rudek, Tough, armed and omnivorous: *Hermodice carunculata* (Annelida: Amphinomidae) is prepared for ecological challenges, J. Mar. Biol. Assoc. U. K. 97 (2017) 1075–1080, https://doi.org/10.1017/S0025315417000091.
[95] T. Timm, Life forms in Oligochaeta: a literature review, Zool. Middle East 58 (2012) 71–82, https://doi.org/10.1080/09397140.2012.10648986.
[96] J. Wierzbicka, P. Śmietana, The food of *Branchiobdella* Odier, 1823 (Annelida) dwelling on crayfish and the occurrence of the fish parasite Argulus Müller,

1785 (Crustacea) on the carapace of *Pontastacus leptodactylus* (Esch.), Acta Ichthyol. Piscatoria 29 (1999) 93–99, https://doi.org/10.3750/AIP1999.29.1.08.
[97] K.S.P. Gale, H.C. Proctor, Diets of two congeneric species of crayfish worm (Annelida: Clitellata: Branchiobdellidae) from western Canada, Can. J. Zool. 89 (2011) 289–296, https://doi.org/10.1139/z11-003.
[98] A. Van Wormhoudt, D. Sellos, Highly variable polymorphism of the alpha-amylase gene family in *Litopenaeus vannamei* (Crustacea Decapoda), J. Mol. Evol. 57 (2003) 659–671, https://doi.org/10.1007/s00239-003-2516-4.
[99] D. Sellos, J. Moal, L. Degremont, A. Huvet, J.-Y. Daniel, S. Nicoulaud, P. Boudry, J.-F. Samain, A. Van Wormhoudt, Structure of amylase genes in populations of Pacific cupped oyster (*Crassostrea gigas*): tissue expression and allelic polymorphism, Mar. Biotechnol. 5 (2003) 360–372, https://doi.org/10.1007/s10126-002-0089-7.
[100] A. Huvet, F. Jeffroy, C. Fabioux, J.Y. Daniel, V. Quillien, A. Van Wormhoudt, J. Moal, J.F. Samain, P. Boudry, S. Pouvreau, Association among growth, food consumption-related traits and amylase gene polymorphism in the Pacific oyster *Crassostrea gigas*, Anim. Genet. 39 (2008) 662–665, https://doi.org/10.1111/j.1365-2052.2008.01776.x.
[101] Z. Zhang, H. Zhong, S. Lin, L. Liang, S. Ye, Z. Xu, C. Ji, Z. Zhang, D. Zhang, X. Zhang, Polymorphisms of AMY1A gene and their association with growth, carcass traits and feed intake efficiency in chickens, Genomics 113 (2021) 583–594, https://doi.org/10.1016/j.ygeno.2020.10.041.
[102] B.L. Hughes, R.G. Suniga, D.G. Yardley, Influence of amylase genotypes on growth rate and feed conversion of chickens, Poultry Sci. 73 (1994) 953–957, https://doi.org/10.3382/ps.0730953.
[103] M.C.M. Da Silva, M.F.G. de Sá, M.J. Chrispeels, R.C. Togawa, G. Neshich, Analysis of structural and physico-chemical parameters involved in the specificity of binding between α-amylases and their inhibitors, Protein Eng. Des. Sel. 13 (2000) 167–177, https://doi.org/10.1093/protein/13.3.167.
[104] I. Kluh, M. Horn, J. Hýblová, J. Hubert, L. Doležková-Marešová, Z. Voburka, I. Kudliková, F. Kocourek, M. Mareš, Inhibitory specificity and insecticidal selectivity of α-amylase inhibitor from *Phaseolus vulgaris*, Phytochemistry 66 (2005) 31–39, https://doi.org/10.1016/j.phytochem.2004.11.001.
[105] O.L. Franco, D.J. Rigden, F.R. Melo, M.F. Grossi-de-Sá, Plant α-amylase inhibitors and their interaction with insect α-amylases, Eur. J. Biochem. 269 (2002) 397–412, https://doi.org/10.1046/j.0014-2956.2001.02656.x.
[106] F. Maczkowiak, J.-L.D. Lage, Origin and evolution of the Amyrel gene in the α-amylase multigene family of Diptera, Genetica 128 (2006) 145–158, https://doi.org/10.1007/s10709-005-5578-y.
[107] C.J. Creevey, T. Doerks, D.A. Fitzpatrick, J. Raes, P. Bork, Universally distributed single-copy genes indicate a constant rate of horizontal transfer, PLoS One 6 (2011) e22099, https://doi.org/10.1371/journal.pone.0022099.
[108] M. Seppey, M. Manni, E.M. Zdobnov, BUSCO: assessing genome assembly and annotation completeness, in: M. Kollmar (Ed.), Gene Prediction, Springer, New York, New York, NY, 2019, pp. 227–245, https://doi.org/10.1007/978-1-4939-9173-0_14.
[109] A.L.P. Silva, M.J.B. Amorim, M. Holmstrup, Adaptations of enchytraeids to single and combined effects of physical and chemical stressors, Environ. Rev. 24 (2016) 1–12, https://doi.org/10.1139/er-2015-0048.
[110] T. Osorio, E.R. Scoma, D.H. Shain, D.S. Melissaratos, L.M. Riggs, V. Hambardikar, M.E. Solesio, The glacier ice worm, *Mesenchytraeus solifugus*, elevates mitochondrial inorganic polyphosphate (PolyP) levels in response to stress, Biology 11 (2022) 1771, https://doi.org/10.3390/biology11121771.
[111] R.C. Edgar, MUSCLE: multiple sequence alignment with high accuracy and high throughput, Nucleic Acids Res. 32 (2004) 1792–1797, https://doi.org/10.1093/nar/gkh340.
[112] J.A. Blake, J.P. Grassle, K.J. Eckelbarger, *Capitella teleta*, a new species designation for the opportunistic and experimental *Capitella* sp. I, with a review of the literature for confirmed records, Zoosymposia 2 (2009) 25–53, https://doi.org/10.11646/zoosymposia.2.1.6.
[113] V.I. Radashevsky, V.V. Pankova, V.V. Malyar, T.V. Neretina, R.S. Wilson, T.M. Worsfold, M.E. Diez, L.H. Harris, S. Hourdez, C. Labrune, C. Houbin, B. Kind, R. Kuhlenkamp, A. Nygren, P. Bonifácio, G. Bachelet, Molecular analysis and new records of the invasive polychaete *Boccardia proboscidea* (Annelida: Spionidae), Medit, Mar. Sci. 20 (2019), https://doi.org/10.12681/mms.20363.
[114] D. Liu, Z. Qin, M. Wei, D. Kong, Q. Zheng, S. Bai, S. Lin, Z. Zhang, Y. Ma, Genome-Wide analyses of heat shock protein superfamily provide new insights on adaptation to sulfide-rich environments in *Urechis unicinctus* (Annelida, Echiura), Int. J. Mol. Sci. 23 (2022) 2715, https://doi.org/10.3390/ijms23052715.
[115] G. Feller, M. Bonneau, J.-L. Da Lage, Amyrel, a novel glucose-forming α-amylase from *Drosophila* with 4-α-glucanotransferase activity by disproportionation and hydrolysis of maltooligosaccharides, Glycobiology 31 (2021) 1134–1144, https://doi.org/10.1093/glycob/cwab036.
[116] C. Hámori, L. Kandra, G. Gyémánt, LDAmy, an α-amylase from Colorado potato beetle (*Leptinotarsa decemlineata*) with transglycosylation activity, Biocatal. Biotransform. 41 (2023) 153–160, https://doi.org/10.1080/10242422.2022.2050707.
[117] G. Claisse, G. Feller, M. Bonneau, J.-L. Da Lage, A single amino-acid substitution toggles chloride dependence of the alpha-amylase paralog amyrel in *Drosophila melanogaster* and *Drosophila virilis* species, Insect Biochem. Mol. Biol. 75 (2016) 70–77, https://doi.org/10.1016/j.ibmb.2016.06.003.
[118] E.A. Williams, M. Conzelmann, G. Jékely, Myoinhibitory peptide regulates feeding in the marine annelid *Platynereis*, Front. Zool. 12 (2015) 1, https://doi.org/10.1186/s12983-014-0093-6.
[119] A. Criscuolo, S. Gribaldo, BMGE (Block Mapping and Gathering with Entropy): a new software for selection of phylogenetic informative regions from multiple sequence alignments, BMC Evol. Biol. 10 (2010) 210, https://doi.org/10.1186/1471-2148-10-210.
[120] F. Lemoine, D. Correia, V. Lefort, O. Doppelt-Azeroual, F. Mareuil, S. Cohen-Boulakia, O. Gascuel, NGPhylogeny.fr: new generation phylogenetic services for non-specialists, Nucleic Acids Res. 47 (2019) W260–W265, https://doi.org/10.1093/nar/gkz303.
[121] K. Andreasen, B.G. Baldwin, Unequal evolutionary rates between annual and perennial lineages of checker mallows (*Sidalcea, Malvaceae*): evidence from 18S–26S rDNA internal and external transcribed spacers, Mol. Biol. Evol. 18 (2001) 936–944, https://doi.org/10.1093/oxfordjournals.molbev.a003894.
[122] T. Bromée, B. Venkatesh, S. Brenner, J.H. Postlethwait, Y.-L. Yan, D. Larhammar, Uneven evolutionary rates of bradykinin B1 and B2 receptors in vertebrate lineages, Gene 373 (2006) 100–108, https://doi.org/10.1016/j.gene.2006.01.017.
[123] N. Galtier, V. Daubin, Dealing with incongruence in phylogenomic analyses, Phil. Trans. R. Soc. B. 363 (2008) 4023–4029, https://doi.org/10.1098/rstb.2008.0144.
[124] S. Wang, Y. Chen, Phylogenomic analysis demonstrates a pattern of rare and long-lasting concerted evolution in prokaryotes, Commun. Biol. 1 (2018) 12, https://doi.org/10.1038/s42003-018-0014-x.
[125] G. Purschke, S. Vodopyanov, A. Baller, T. von Palubitzki, T. Bartolomaeus, P. Beckers, Ultrastructure of cerebral eyes in Oweniidae and Chaetopteridae (Annelida) – implications for the evolution of eyes in Annelida, Zool. Lett. 8 (2022) 3, https://doi.org/10.1186/s40851-022-00188-0.
[126] E. Tilic, E. Sayyari, J. Stiller, S. Mirarab, G.W. Rouse, More is needed—thousands of loci are required to elucidate the relationships of the 'flowers of the sea' (Sabellida, Annelida), Mol. Phylogenet. Evol. 151 (2020) 106892, https://doi.org/10.1016/j.ympev.2020.106892.
[127] R. Ufimov, J.M. Gorospe, T. Fér, M. Kandziora, L. Salomon, M. van Loo, R. Schmickl, Utilizing paralogues for phylogenetic reconstruction has the potential to increase species tree support and reduce gene tree discordance in target enrichment data, Mol. Ecol. Resour. 22 (2022) 3018–3034, https://doi.org/10.1111/1755-0998.13684.
[128] O. Folmer, M. Black, W. Hoeh, R. Lutz, R. Vrijenhoek, DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates, Mol. Marine Biol. Biotechnol. 3 (1994) 294–299.
[129] M. Nozaki, C. Miura, Y. Tozawa, T. Miura, The contribution of endogenous cellulase to the cellulose digestion in the gut of earthworm (*Pheretima hilgendorfi* Megascolecidae), Soil Biol. Biochem. 41 (2009) 762–769. https://doi.org/10.1016/j.soilbio.2009.01.016.

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International Journal of
Molecular Sciences

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Trophic Position of the White Worm (*Enchytraeus albidus*) in the Context of Digestive Enzyme Genes Revealed by Transcriptomics Analysis

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Abstract: To assess the impact of Enchytraeidae (potworms) on the functioning of the decomposer system, knowledge of the feeding preferences of enchytraeid species is required. Different food preferences can be explained by variations in enzymatic activities among different enchytraeid species, as there are no significant differences in the morphology or anatomy of their alimentary tracts. However, it is crucial to distinguish between the contribution of microbial enzymes and the animal's digestive capacity. Here, we computationally analyzed the endogenous digestive enzyme genes in *Enchytraeus albidus*. The analysis was based on RNA-Seq of COI-monohaplotype culture (PL-A strain) specimens, utilizing transcriptome profiling to determine the trophic position of the species. We also corroborated the results obtained using transcriptomics data from genetically heterogeneous freeze-tolerant strains. Our results revealed that *E. albidus* expresses a wide range of glycosidases, including GH9 cellulases and a specific digestive SH3b-domain-containing i-type lysozyme, previously described in the earthworm *Eisenia andrei*. Therefore, *E. albidus* combines traits of both primary decomposers (primary saprophytophages) and secondary decomposers (sapro-microphytophages/microbivores) and can be defined as an intermediate decomposer. Based on assemblies of publicly available RNA-Seq reads, we found close homologs for these cellulases and i-type lysozymes in various clitellate taxa, including Crassiclitellata and Enchytraeidae.

Keywords: potworms; decomposers; transcriptome; cellulase; digestive lysozyme; COI-monohaplotype culture

Citation: Gajda, Ł.; Daszkowska-Golec, A.; Świątek, P. Trophic Position of the White Worm (*Enchytraeus albidus*) in the Context of Digestive Enzyme Genes Revealed by Transcriptomics Analysis. *Int. J. Mol. Sci.* **2024**, *25*, 4685. https://doi.org/10.3390/ijms25094685

Academic Editor: Alain Couvineau

Received: 21 March 2024
Revised: 20 April 2024
Accepted: 23 April 2024
Published: 25 April 2024

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1. Introduction

The 1975 article by J. M. Anderson, "The Enigma of Soil Animal Species Diversity", highlighted the high species richness found in soils and emphasized the unknown mechanisms contributing to this diversity [1]. Despite several new hypotheses, the mechanisms driving species richness in soils have remained largely elusive [2,3]. The relationship between ecosystem characteristics and the number of trophic levels in food webs has been debated, with some studies suggesting that the number of trophic levels increases with productivity and resource availability [4], while others propose that nutrient-poor systems have more trophic levels due to a large number of interactions between species [5]. Over the past decade, researchers have also hypothesized that the high species richness observed in small quantities of soil is related to the high heterogeneity found at very fine scales within the soil [2]. However, the enigma of how large numbers of soil animal species occupying the same trophic level, such as decomposers, can coexist in one food web remains an open question. Traditional research methods often provide only limited information on feeding, leaving the trophic status of many soil invertebrate groups uncertain or theoretical [6–8]. Feeding is a complex process that involves food choice, ingestion, digestion, assimilation, and retention. Traditional research methods, which include direct observation of feeding

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behavior, gut content analyses, enzymatic analyses of whole-body homogenates, cultivation on different nutrient sources, or choice tests, typically address only one or a few component processes and are unable to provide comprehensive information about the exact source and components digested and assimilated from the ingested food bolus [7,9]. Significant advancements in the understanding of the diets and trophic interactions of soil animals in recent years have been made possible through more sophisticated methods such as stable isotope analysis [9]. This technique provides estimates of the retention of atoms from basal food resources and allows for the indication of the trophic level of the analyzed group of animals in the food web. However, bulk natural stable isotopes provide only rough information about the basal resources used by the analyzed animals, rarely allowing for the reconstruction of species-specific feeding interactions in soil [7]. Distinguishing between bacterial and fungal feeding, as well as feeding on different taxa of microorganisms, is challenging and often impossible using stable isotope analysis alone [8,10]. Moreover, several ontogenetic, physiological, and biochemical factors can affect the isotopic composition of animal tissues [6,11]. Another challenge is the dietary flexibility exhibited by many soil animals, which can vary depending on available food sources and may result in these animals operating on more than one trophic level [8,12]. Furthermore, the contribution of microbial enzymatic apparatus to the invertebrate digestion process cannot be overlooked. The enzyme activity of microbiota or food-associated microorganisms can significantly affect the host's digestive capabilities [13,14]. Therefore, while stable isotope analysis is currently a leading method in trophic ecology studies, it should be used in conjunction with other complementary approaches, given its limitations. Recently, these combined multi-methodological approaches have successfully revealed the multidimensional trophic niche of springtails (Collembola) [7].

Among the major groups of soil invertebrates, Enchytraeidae, also known as potworms, are no exception when it comes to the uncertainty of their trophic position. Despite being a widely distributed family of small annelids, their trophic status within the soil food web remains unsolved, even after several studies have used stable isotope analysis [11,15–17]. It is still unclear whether they should be classified as primary or secondary decomposers. Detailed studies on the food preferences of enchytraeids have only been conducted in a few species [18]. The conclusions drawn from these findings are also limited by the high level of cryptic diversity within the family [19–21], as cryptic species may differ in their specific ecological and physiological properties [22,23].

Enchytraeids share the general body plan of oligochaetes and represent a relatively simple and uniform group [24,25]. There are no significant differences in the anatomy of the alimentary tract or highly specialized morphological structures that could clearly indicate the feeding strategy of most potworm species (but cf. *Aspidodrilus kelsalli* or *Pelmatodrilus planariformis* [26]; these two unique species with some flattened body regions are ectocommensals that have adapted to living on earthworms). Different food preferences could be explained by varying enzymatic activities among different enchytraeid species. However, this hypothesis requires support from genetic methods to investigate the endogenous expression of digestive enzyme genes and distinguish the contribution of the microbiota to this process. Traditional biochemical assays are not sufficient for this purpose, as it is challenging to separate enzyme activity originating from the animal itself from activity related to exogenous sources, such as microbiota or food-associated microorganisms [8].

An alternative and more sophisticated approach to traditional biochemical methods in trophic ecology studies, which complements stable isotope analysis, involves the use of RNA sequencing and transcriptome profiling. Transcriptomics provides access to transcriptome-wide gene expression data, enabling the characterization of an organism's limitations and capacities for various traits [27], including the repertoire of digestive enzyme genes. Although RNA-Seq is commonly used to predict the digestive capacity of economically important species of crustaceans [28–30], fish [31], and insects [32,33], this approach has not been widely adopted in trophic ecology studies. Despite using other molecular methods, such as molecular gut content or meta-barcoding microbiome analyses

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to understand better the trophic links between species and their diets in soil food webs, the potential of RNA-Seq in this field remains largely untapped.

The white worm, *Enchytraeus albidus*, is an economically and scientifically important species of Enchytraeidae. It can be found in both terrestrial and marine littoral habitats [20]. To date, *E. albidus* or any other member of the Enchytraeidae has not been the subject of molecular studies regarding its digestive capacity. In the last published review dedicated to the food preferences of enchytraeids [18], a classification of trophic types was proposed for the most commonly studied genera in relation to food preferences and feeding behavior. *Enchytraeus* spp., including *E. albidus*, were assigned to the secondary decomposer group. In the present study, we determined the trophic position of *E. albidus* based on RNA-Seq data. We obtained raw reads and performed de novo transcriptome assembly for the *E. albidus* PL-A strain originating from a COI-monohaplotype culture. We conducted a transcriptome screening, identified the expressed genes involved in digestive enzyme production in *E. albidus*, and performed in silico characterization of the sequences. Moreover, we compared and cross-checked the obtained data with transcriptomics data related to the freeze-tolerant German (G) and Greenlandic (N) strains of *E. albidus* [34]. Given that primary decomposers are species that primarily feed on litter material that is little colonized by microorganisms, while secondary decomposers mainly feed on microorganisms and/or plant residues that are partially degraded due to microbial activity, we tested the following two hypotheses: (1) *E. albidus* does not exhibit endogenous expression of the enzyme genes from the cellulase group, and (2) *E. albidus* exhibits endogenous expression of digestive enzyme genes involved in the digestion of bacteria or fungi (e.g., peptidoglycan hydrolases or chitinases). These hypotheses, consistent with the last review's postulation that *E. albidus* belongs to the secondary decomposer group, were confronted with the repertoire of digestive enzyme genes in this species, as revealed by transcriptomics data.

2. Results

2.1. RNA-Seq, Transcriptome Assembly, and Annotation Results

To decipher the genes expressed and responsible for digestive enzyme production, and given the absence of a reference genome for *E. albidus*, we conducted transcriptome sequencing using the RNA-Seq method and performed de novo transcriptome assembly. RNA sequencing was performed on a single sample, comprising four PL-A strain specimens of *E. albidus* originating from a single cocoon. A total of 118,210,442 reads were generated, resulting in a cumulative read base of 17.8 gigabases (Gb). The GC content of the raw data was determined to be 44.96%. Furthermore, quality assessment indicated that the percentage of bases with a Phred quality score $\geq$ 30 (Q30) was 93.42%, while the percentage of bases with a Phred quality score $\geq$ 20 (Q20) was 97.57%.

As a technical side note pertaining to the quality assessment of RNA samples designated for RNA-Seq, with potential relevance for readers, it should be mentioned that in *E. albidus* the 28S ribosomal RNA undergoes fragmentation into two subparts under heat-denaturing conditions due to a so-called hidden break. Consequently, when analyzing the integrity of rRNA, samples exhibited an atypical profile in the Bioanalyzer electropherogram, characterized by a nearly dominant peak at the 18S position and the absence of a typical peak at the 28S position, resulting in a low rRNA ratio (e.g., 0.1) (for more details, see [35,36]). Nonetheless, as described above, the generated reads were of good quality.

The BUSCO assessment of de novo-assembled transcriptomes for enchytraeid species revealed that *E. albidus* is currently the only species with a transcriptome in the Sequence Read Archive (SRA) that can be considered complete (see Table 1). A comparison of KEGG-annotated transcriptomes for available *E. albidus* strains (refer to Table 2) demonstrates that, despite a roughly 30% difference in the number of assembled sequences, our PL-A strain transcriptome, derived from a single run, exhibits a striking similarity in the count of assigned non-redundant KOs (KEGG Orthology identifiers) for metabolic enzymes when compared to the other transcriptomes. However, it should be noted that the lower number of assembled sequences can be attributed to the high genetic homogeneity of our sample, as

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it was derived from a pure COI-monohaplotype culture, as well as the much lower number of specimens used for RNA-Seq library preparation.

Table 1. Transcriptomes available in the SRA repository for enchytraeid species, and their completeness.

		BUSCO				
Species	**SRR Run(s) for Assembly**	**Complete Single-Copy**	**Complete Duplicated**	**Fragmented**	**Missing**	**BUSCO Groups Complete Representation**
Guaranidrilus sp.	SRR10997448	7.55%	18.66%	15.72%	58.07%	26.21%
Grania simonae	SRR10997449	12.40%	30.30%	11.0%	46.30%	42.70%
Mesenchytraeus armatus	SRR10997443	16.98%	43.08%	17.72%	22.22%	60.06%
Mesenchytraeus pedatus	SRR10997442	10.27%	64.99%	11.43%	13.31%	75.26%
Mesenchytraeus solifugus	SRR10997441	12.58%	64.99%	10.38%	12.05%	77.57%
Enchytraeus crypticus	SRR10997417	15.93%	44.97%	18.76%	20.34%	60.90%
Enchytraeus albidus German strain	SRR5633671, SRR5633673, SRR5633674, SRR5633678, SRR5633679, SRR5633680	7.86%	86.48%	3.98%	1.68%	94.34%
Enchytraeus albidus Nuuk strain	SRR5633669, SRR5633670, SRR5633672, SRR5633676, SRR5633677, SRR5633681	7.55%	87.95%	3.04%	1.47%	95.50%
Enchytraeus albidus PL-A strain (this study)	SRR24185061	29.45%	67.51%	1.47%	1.57%	96.96%

Table 2. Comparison of the KEGG-annotated transcriptomes of different *E. albidus* strains. The table summarizes the number of sequences (entries) and their classification into functional categories by GhostKOALA.

	PL-A	German	Nuuk
Raw dataset entries	84,423	125,364	113,553
Clean dataset entries (after decontamination)	72,044	103,077	96,427
Clean dataset annotated entries (after decontamination)	34,412 (47.8%)	50,473 (49.0%)	48,234 (50.0%)
Protein families: genetic information processing	7451	10,394	9995
Environmental information processing	4424	6026	5953
Genetic information processing	4321	7063	6701
Protein families: signaling and cellular processes	4049	5957	5348
Cellular processes	2784	4114	3872
Protein families: metabolism	2062	2887	2824
Organismal systems	1696	2399	2361
Carbohydrate metabolism	1190	2016	1984
Human diseases	1117	1460	1493
Lipid metabolism	1030	1567	1605
Unclassified: metabolism	747	1175	1094
Glycan biosynthesis and metabolism	723	1076	974
Amino acid metabolism	698	976	925
Nucleotide metabolism	488	717	651

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Table 2. *Cont.*

	PL-A	German	Nuuk
Unclassified: signaling and cellular processes	411	650	514
Energy metabolism	401	743	749
Metabolism of cofactors and vitamins	397	620	583
Metabolism of other amino acids	239	397	376
Metabolism of terpenoids and polyketides	86	92	111
Unclassified: genetic information processing	44	62	63
Xenobiotics biodegradation and metabolism	28	39	38
Number of assigned non-redundant KOs for metabolic enzymes	1948	1959	1962
Number of assigned redundant/non-redundant KOs for glycosidases (EC 3.2.1)	39/38	41/39	45/42

2.2. Integrative Annotation of Glycosidase Genes in E. albidus Strains

Using microbial-decontaminated data for our COI-monohaplotype PL-A specimens, and corroborated by information from two intraspecifically heterogeneous freeze-tolerant strains of *E. albidus*, we identified over 1900 functional orthologs for metabolic enzymes. From these orthologs, around 40 KO identifiers were assigned to glycosidases by KEGG-GhostKOALA. Additional glycoside hydrolase candidates (i.e., lysozyme and mannan endo-1,4-β-mannosidases) for digestive enzyme genes that were unannotated in the initial GhostKOALA dataset were identified through PANNZER2 and KofamKOALA annotations using adjusted thresholds. Collectively, we pinpointed 30 digestive gene candidates encoding glycosidases and assessed the number of unique sequence variants for them across *E. albidus* strains (Table 3). These selected expressed genes could be further roughly grouped into (1) starch- and glycogen-digesting enzymes (α-amylase I/II, maltase-glucoamylase, maltase-glucoamylase intestinal-like isoform/intestinal-like isoform X2), (2) cellulose- and lichenan-digesting enzymes (endo-β-1,4-glucanase I/II, endo-1,3(4)-β-glucanase), (3) chitin-digesting enzymes (chitinase I/II, di-N-acetylchitobiase/di-N-acetylchitobiase isoform X1, and formally lysozyme, which is mainly a peptidoglycan-degrading enzyme), (4) xylan-digesting enzymes (β-glucosidase/xylosidase I–V), and (5) other specific carbohydrate-digesting enzymes (β-galactosidase, β-glucuronidase, α-L-fucosidase I–IV, β-mannosidase, and mannan endo-1,4-β-mannosidase I–IV). It is worth mentioning that no endogenous sequence for α,α-trehalase (α,α-trehalose glucohydrolase, EC 3.2.1.28) was found (only microbial) in the transcriptomes of *E. albidus* and other enchytraeid species, which suggests that enchytraeids lack this enzyme, similar to earthworms [37,38].

Table 3. Digestive enzyme gene candidates identified among annotated glycosidases from transcriptomics data of *E. albidus*.

	Gene Name	Enzyme Commission (EC) Number	GH Family Classification	KEGG Orthology (KO) Identifier	Recovered in Strain			Total Number of Protein Variants	Predicted Localization	Notes
					PL-A	German	Nuuk			
1	α-Amylase I	3.2.1.1	GH13_24	K01176	+	+	+	6	Extracellular	Reported in [39].
2	α-Amylase II	3.2.1.1	GH13_24	K01176	+	+	+	6	Extracellular	Reported in [39]. It might exhibit additional transglycosylation activity (EC 2.4.1.25).
3	Maltase-glucoamylase, intestinal	3.2.1.3; 3.2.1.20	GH31_1	K12047	+	+	+	6	Cell membrane	Because maltase-glucoamylase and sucrase-isomaltase share a common ancestry and striking structural similarities, an alternative EC annotation with dbCAN3 indicates sucrase-isomaltase (EC 3.2.1.48; EC 3.2.1.10).
4	Maltase-glucoamylase, intestinal-like isoform	3.2.1.20	GH31_1	K01187	Partial	+	+	7	Extracellular	
5	Maltase-glucoamylase, intestinal-like isoform X2	3.2.1.20	GH31_1	K01187	+	+	+	5	Extracellular	
6	Lysosomal α-glucosidase	3.2.1.20	GH31	K12316	+	+	+	8	Extracellular	Analyses with DeepLoc 2.0 and BUSCA are in agreement regarding the extracellular localization of the protein.
7	Endo-β-1,4-glucanase I	3.2.1.4	GH9	K01179	+	+	+	7	Extracellular	Homologous endoglucanases were reported for the earthworms *Metaphire hilgendorfi* [40], *Eisenia fetida* [41], and *E. andrei* [42], as well as for the polychaetes *Perinereis brevicirris* [43] and *P. aibuhitensis* (Acc. ANR02619).
8	Endo-β-1,4-glucanase II	3.2.1.4	GH9	K01179	+	+	+	14	Extracellular	
9	Endo-1,3(4)-β-glucanase	3.2.1.6	GH81	K01180	+	+	+	6	Extracellular	Based on transcriptomics data, complete homologous sequences were recovered for *Eisenia andrei* (SRR11091733–SRR11091735), *Lumbricus castaneus* (SRR7287337), *L. rubellus* (SRR10752881), and *Hrabeiella periglandulata* (SRR10997424), while a partial sequence was found for *Enchytraeus crypticus* (SRR10997417).
10	β-Glucosidase/xylosidase I	3.2.1.21; 3.2.1.37	GH3	K05349	+	+	+	10	Extracellular	Analysis using dbCAN3 revealed additional EC assignments for these proteins, i.e., EC 3.2.1.55, EC 3.2.1.6, and EC 3.2.1.73.
11	β-Glucosidase/xylosidase II	3.2.1.21; 3.2.1.37	GH3	K05349	+	+	+	7	Extracellular	
12	β-Glucosidase/xylosidase III	3.2.1.21; 3.2.1.37	GH3	K05349	Partial	+	+	3	Extracellular	
13	β-Glucosidase/xylosidase IV	3.2.1.21; 3.2.1.37	GH3	K05349	+	+	+	5	Extracellular	
14	β-Glucosidase/xylosidase V	3.2.1.21; 3.2.1.37	GH3	K05349	+	+	+	7	Extracellular	
15	Chitinase I	3.2.1.14	GH18	K01183	+	+	+	6	Extracellular	This is a homolog to a novel digestive chitinase from *Eisenia andrei* [44] and *E. fetida* [45].
16	Chitinase II	3.2.1.14	GH18	K01183	+	+	+	3	Extracellular	This is a divergent paralog of chitinase I that possesses an additional catalytic domain.
17	Di-N-acetylchitobiase	3.2.1.-	GH18	K12310	+	+	+	6	Extracellular	-

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Table 3. *Cont.*

	Gene Name	Enzyme Commission (EC) Number	GH Family Classification	KEGG Orthology (KO) Identifier	Recovered in Strain			Total Number of Protein Variants	Predicted Localization	Notes
					PL-A	German	Nuuk			
18	Di-N-acetylchitobiase isoform X1	3.2.1.-	GH18	K12310	+	+	+	3	Extracellular	-
19	Lysozyme (i-type)	3.2.1.17	GH22i	N/A	+	+	Partial	7	Extracellular	This is a close homolog to a novel i-type digestive lysozyme from *Eisenia andrei* (Acc. QBC73604) reported in [46]. The annotation of the destabilase domain also indicates isopeptidase (EC 3.5.1.44) activity. GH classification was assessed based on the WebLogo sequence signature from [47].
20	β-Galactosidase	3.2.1.23	GH35	K12309	+	+	+	6	Extracellular	Nielsen [37] reported β-galactosidase activity in enchytraeids and earthworms.
21	β-Glucuronidase	3.2.1.31	GH2	K01195	+	+	+	7	Extracellular	-
22	α-L-fucosidase I	3.2.1.51	GH29	K01206	+	+	+	3	Extracellular	Putative homologous sequences were identified by BLASTp in other annelids (*Owenia fusiformis*, *Ridgeia piscesae*, *Capitella teleta*), as well as in some mollusk and echinoderm species.
23	α-L-fucosidase II	3.2.1.51	GH29	K01206	+	+	+	8	Extracellular	
24	α-L-fucosidase III	3.2.1.51	GH29	K01206	+	+	+	6	Extracellular	
25	α-L-fucosidase IV	3.2.1.51	GH29	K01206	+	+	+	13	Extracellular	-
26	β-Mannosidase	3.2.1.25	GH2	K01192	Partial	+	+	8	Extracellular	-
27	Mannan endo-1,4-β-mannosidase I	3.2.1.78	GH5_10	K19355	+	+	+	5	Extracellular	-
28	Mannan endo-1,4-β-mannosidase II	3.2.1.78	GH5_10	K19355	Partial	+	+	6	Extracellular	-
29	Mannan endo-1,4-β-mannosidase III	3.2.1.78	GH5_10	K19355	Partial	+	+	8	Extracellular	-
30	Mannan endo-1,4-β-mannosidase IV	3.2.1.78	GH5_10	K19355	+	+	+	7	Extracellular	This is a close homolog to endo-1,4-β-mannanase from *Eisenia fetida* (Acc. BBB35836), reported in [48].

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2.3. Integrative Annotation of Protease Genes in E. albidus Strains

Sequence analysis and annotation of *E. albidus* transcriptomics data revealed that enchytraeids do not have close homologs of classical trypsin and chymotrypsin enzymes, similar to earthworms [49]. Earthworms possess proteases with trypsin-like and chymotrypsin-like activities, which are involved in the digestion of protein and peptides in food and are mainly localized in the crop, gizzard, and anterior intestine [50–52]. These serine proteases, collectively known as lumbrokinases, exhibit fibrinolytic activity and relatively broad substrate specificities [50]. Some of these enzymes can be in glycosylated form [53].

Based on the transcriptomics data for *E. albidus*, we identified at least four fibrinolytic serine protease genes for which the transcripts were initially KEGG-annotated as trypsin, chymotrypsin, and elastase sequences (Table 4). These fibrinolytic serine proteases share significant identity and similarity with sequences of fibrinolytic enzymes from enchytraeid *Enchytraeus japonensis*, as well as earthworms' lumbrokinases, as cataloged in GenBank. In *E. albidus*, these serine proteases constitute a related protein cluster, presenting sequence and structural parallels that complicate precise BLASTp identification of the potential single closest homolog, at least outside the taxonomic family. Moreover, we identified ten different genes comprising a total of 52 unigenes for carboxypeptidase A/B-like (EC 3.4.17.1; EC 3.4.17.2), and three different genes comprising a total of 14 unigenes for aminopeptidase N (EC 3.4.11.2), shared across the *E. albidus* strains (Table 5).

2.4. Integrative Annotation of Digestive Lipases in E. albidus Strains

Before nutritional fat can be transported within the body for storage in adipose tissues or direct energy production, it must first undergo hydrolysis by lipolytic enzymes [54]. We identified four candidates for bile salt-stimulated lipase (CEL) genes and one distinct gene candidate for digestive secretory phospholipase A2. The identified lipolytic enzyme genes are presented in Table 6. Among the four expressed CEL genes identified in *E. albidus*, bile salt-stimulated lipase IV was not recovered from the assembled transcripts for the PL-A strain. Nonetheless, a BLASTn search on the raw reads (SRX19982531), using the German strain sequence as a query, confirmed the presence of a short fragment (70 amino acids) of identical C-terminal end of the lipase in the data. This indicates that the gene is indeed expressed in the PL-A strain but was not recovered in subsequent steps of transcriptome assembly and protein prediction.

2.5. Phylogenetic Analysis of Selected Digestive Enzymes

The collected data (Supplementary Data S1), including transcriptomics data with varying sequencing depths and BUSCO completeness, enabled us to conduct an analysis and construct phylogenetic trees for putative cellulase (endo-β-1,4-glucanase, EC 3.2.1.4) and digestive i-type lysozyme proteins identified in *E. albidus* and among other members of Clitellata. This robust dataset underpins our phylogenetic inferences, providing insights into the evolutionary relationships of these enzymes.

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Table 4. Putative digestive fibrinolytic proteases identified among trypsin-like and chymotrypsin-like sequences from transcriptomics data of *E. albidus* and their in silico characterization.

	GhostKOALA-KofamKOALA Annotation			All Data	SignalP 6.0	DeepTMHMM	DeepLoc 2.0	Swiss Model			BLASTp		SMART and InterPro
	Enzyme	**KO**	**Gene**	**Total Number of Protein Variants**	**Signal Peptide**	**Transmembrane Region and Topology Prediction**	**Subcellular Localization Prediction and Probability**	**Possible Template, Its Origin, and Accession Number**	**Identity [%]**	**GMQE**	**Hit and GenBank Accession Number**	**Identity [%]**	**Predicted Domain and Family**
1	Trypsin (EC 3.4.21.4)	K01312	*PRSS1/2/3*	9	Yes	Globular + signal peptide	Extracellular (0.94)	Fibrinolytic enzyme Ej-FEI-1; *Enchytraeus japonensis*; Uniprot ID H1A7B3	83–84	0.90	Fibrinolytic enzyme *Enchytraeus japonensis* Ej-FEI-2; BAL43183	84	Tryp_SPc; Peptidase S1A, chymotrypsin family
2	Chymotrypsin (EC 3.4.21.1)	K01310	*CTRB*	14	Yes	Globular + signal peptide	Extracellular (0.95)	Fibrinolytic enzyme component B; *Eisenia fetida*; PDB ID 1ym0.1.A	48–50	0.68–0.69	Fibrinolytic enzyme *Enchytraeus japonensis* Ej-FEIII-2b; BAL43192	49	
3	Trypsin (EC 3.4.21.4)	K01312	*PRSS1/2/3*	1	Yes	Globular + signal peptide	Extracellular (0.96)	Cationic trypsin; *Bos Taurus*; PDB ID 4xoj.1.A	29	0.61	Fibrinolytic protease 0 *Eisenia fetida*; ABG68022	53	
4	Pancreatic elastase 1/2 (EC 3.4.21.36) (EC 3.4.21.71)	K01326 K01346	*CELA1/ CELA2*	15	Yes	Globular + signal peptide	Extracellular (0.95)	Fibrinolytic enzyme Ej-FEI-1; *Enchytraeus japonensis*; Uniprot ID H1A7B3	74–86	0.87–0.90	Fibrinolytic enzymes *Enchytraeus japonensis* BAL43184, BAL43186, BAL43182, BAL43188	74–85	

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Table 5. Putative carboxypeptidase A/B-like and aminopeptidase N gene candidates annotated for *E. albidus* using transcriptomics data.

	Recovered in Strain			All Data	SignalP 6.0	DeepTMHMM	DeepLoc 2.0
Enzyme and (Pre-)Protein Length	**PL-A**	**German**	**Nuuk**	**Total Number of Protein Variants**	**Signal Peptide**	**Transmembrane Region and Topology Prediction**	**Subcellular Localization**
Carboxypeptidase A/B-like I (502)	+	+	+	5	Yes	Globular + signal peptide	Extracellular
Carboxypeptidase A/B-like II (505)	+	+	Partial	4	Yes	Globular + signal peptide	Extracellular
Carboxypeptidase A/B-like III (467)	+	+	+	5	Yes	Globular + signal peptide	Extracellular
Carboxypeptidase A/B-like IV (431)	+	+	+	1	Yes	Globular + signal peptide	Extracellular
Carboxypeptidase A/B-like V (424)	+	+	+	2	Yes	Globular + signal peptide	Extracellular
Carboxypeptidase A/B-like VI (425)	+	+	+	12	Yes	Globular + signal peptide	Extracellular
Carboxypeptidase A/B-like VII (432)	+	+	+	6	Yes	Globular + signal peptide	Extracellular
Carboxypeptidase A/B-like VIII (422)	+	+	+	4	Yes	Globular + signal peptide	Extracellular
Carboxypeptidase A/B-like IX (446)	+	+	Partial	6	Yes	Globular + signal peptide	Extracellular
Carboxypeptidase A/B-like X (429)	+	+	+	7	Yes	Globular + signal peptide	Extracellular
Aminopeptidase N I (968)	+	+	+	5	No	Alpha TM	Cell membrane
Aminopeptidase N II (968)	+	+	+	3	No	Alpha TM	Cell membrane
Aminopeptidase N III (1006)	+	+	+	6	No	Alpha TM	Cell membrane

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Table 6. Putative lipase gene candidates annotated for *E. albidus* from transcriptomics data.

GhostKOALA Annotation			Recovered in Strain			Data	SignalP 6.0	DeepTMHMM	DeepLoc 2.0	Panther	InterPro
Enzyme and Pre-Protein Length	**KO**	**Gene**	**PL-A**	**German**	**Nuuk**	**Total Number of Protein Variants**	**Signal Peptide**	**Transmembrane Region and Topology Prediction**	**Subcellular Localization**	**Panther Hit**	**Predicted Domain**
Bile salt-stimulated lipase I [EC 3.1.1.3 3.1.1.13] (631)	K12298	*CEL*	Partial	+	+	6	Yes	Globular + signal peptide	Extracellular	Bile salt-activated lipase	Carboxylesterase type B
Bile salt-stimulated lipase II [EC 3.1.1.3 3.1.1.13] (636; 638)	K12298	*CEL*	+	Partial	Partial	5	Yes	Globular + signal peptide	Extracellular	Bile salt-activated lipase	Carboxylesterase type B
Bile salt-stimulated lipase III [EC 3.1.1.3 3.1.1.13] (636)	K12298	*CEL*	Partial	+	+	5	Yes	Globular + signal peptide	Extracellular	Carboxylesterase	Carboxylesterase type B
Bile salt-stimulated lipase IV [EC 3.1.1.3 3.1.1.13] (638)	K12298	*CEL*	-	+	+	3	Yes	Globular + signal peptide	Extracellular	Bile salt-activated lipase	Carboxylesterase type B
Secretory phospholipase A2 [EC 3.1.1.4] (237; 236)	K01047	*PLA2G*, *SPLA2*	+	+	+	8	Yes	Globular + signal peptide	Extracellular	RH14732P	Phospholipase A2

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2.5.1. Phylogenetic Analysis of Cellulases (Endo-β-1,4-Glucanases)

Reciprocal BLASTp analyses of endo-β-1,4-glucanase I/II protein sequences primarily obtained from Clitellata revealed significant similarity to previously characterized and closely related cellulases (GH9 family) from earthworms such as *Metaphire hilgendorfi* [40] and *Eisenia* spp. [41,42]. All hits to these sequences had an E-value of 0, indicating a high-quality match (Supplementary Table S1). Additionally, we identified two closely homologous cellulases from the transcriptomics data of the terrestrial polychaete *Hrabeiella periglandulata*. This species is the sole representative of Hrabeiellidae, and along with the polychaete *Aeolosoma* is considered to form a sister group to Clitellata in the species phylogeny of Annelida [55]. After rooting the tree at *H. periglandulata*, our phylogenetic analysis of endo-β-1,4-glucanase I/II proteins (Figure 1) indicated that *Capilloventer australis* and Phreodrilidae sp. cellulases diverged from a shared ancestor. Notably, two distinct but homologous cellulase genes have been identified in *C. australis*. Furthermore, the *Capilloventer*–Phreodrilidae cluster was found to be a sister to the second paralogous protein variants from *C. australis* plus all remaining clitellate sequences. Within the remaining Clitellata, two main clades were recovered with high support. The first one contains a single sequence from the lumbriculid *Lumbriculus variegatus*. In the second, a single representative of Naididae in our analysis—*Pristina leydyi*—was recovered as a sister to Crassiclitellata (represented by earthworm species) plus Enchytraeidae with *Randiella*. The Crassiclitellata cluster was highly resolved, but its evolutionary history appears complex, as indicated by an independent endoglucanase duplication event in the most basally placed *Metaphire guillelmi*. This paralogous copy was recovered as a sister to all other earthworm endoglucanases, including the remaining *Metaphire* sequences.

The second main clade comprised all Enchytraeidae proteins sister to a single cellulase from *Randiella*, with high support. The Enchytraeidae cluster was mostly well resolved. Within Enchytraeidae, two subclusters were recovered. In the first, sequences from *Grania*, *Guaranidrilus*, and *Mesenchytraeus* spp. were grouped. In the second, proteins from *Enchytraeus albidus* and *E. crypticus* were grouped together in a manner discordant with species phylogeny. A paralogous sequence from *E. crypticus* was recovered as a sister to all other proteins from *E. albidus* and *E. crypticus*. The remaining *E. crypticus* sequences were recovered as nested within *E. albidus*. This may suggest incomplete lineage sorting, as vertical gene flow between those species is unlikely due to physical reproductive barriers, although horizontal gene transfer for *E. crypticus* was reported [56].

2.5.2. Phylogenetic Analysis of Digestive i-Type Lysozyme

Phylogenetic analysis of the putative digestive i-type lysozyme proteins found across Clitellata reveals that the tree (Figure 2) bifurcates into two distinct clades when rooted at *C. australis*. The first clade is composed of a single sequence from a member of the family Phreodrilidae. The second clade, which is almost maximally supported, encompasses the remaining sequences from all other analyzed clitellate families. This clade is further divided into two clusters, each receiving very high support. The first cluster contains all Enchytraeidae, with two members of Naididae (*Pristina* and *Paranais*) nested within it. These Naididae members were recovered as a sister group to *Mesenchytraeus* spp., although this relationship within Enchytraeidae is supported with low confidence. Interestingly, in a highly resolved subclade containing *E. crypticus* and *E. albidus*, the sequences were not sorted in a species-specific manner.

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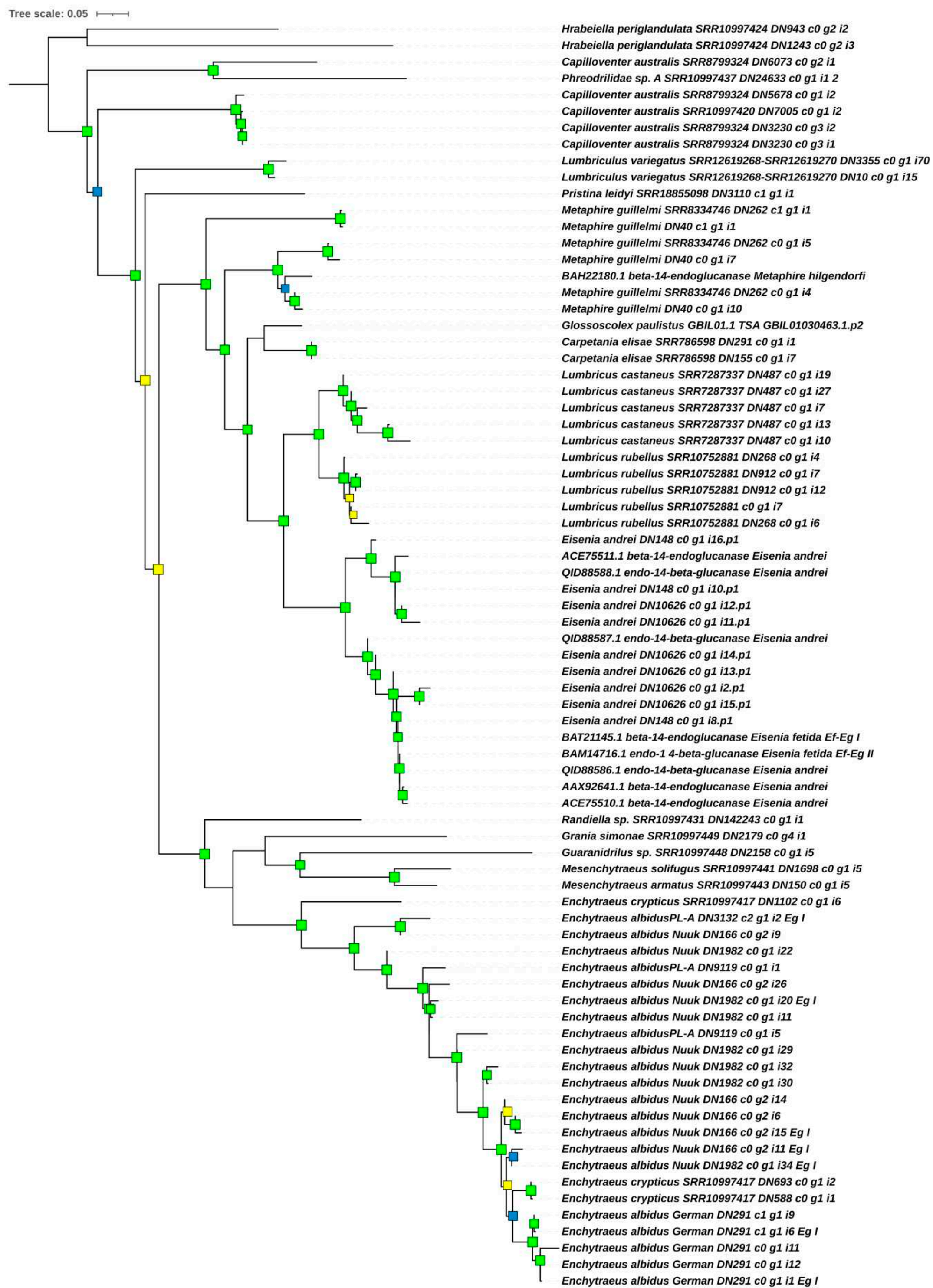

Figure 1. Best-scoring maximum likelihood tree (lnL = −11,983.127) resulting from the analysis of mature amino acid sequences of the putative endo-β-1,4-glucanase I/II proteins in Clitellata. Most sequences used in the analysis were retrieved from SRA transcriptomics data assembled in Trinity. Sequences with identifiers prefixed by an accession number were sourced from GenBank database. Details of the other sources, used for acquiring additional transcriptomics data and included in the phylogenetic analysis, can be found in Supplementary Table S3. Green squares denote branches with both SH-aLRT and UFBoot values (if ≥70) at the respective nodes. Yellow squares indicate support values (if ≥70) only for UFBoot, while blue squares indicate support values (if ≥70) only for SH-aLRT. The tree was rooted at the terrestrial polychaete *Hrabeiella periglandulata*.

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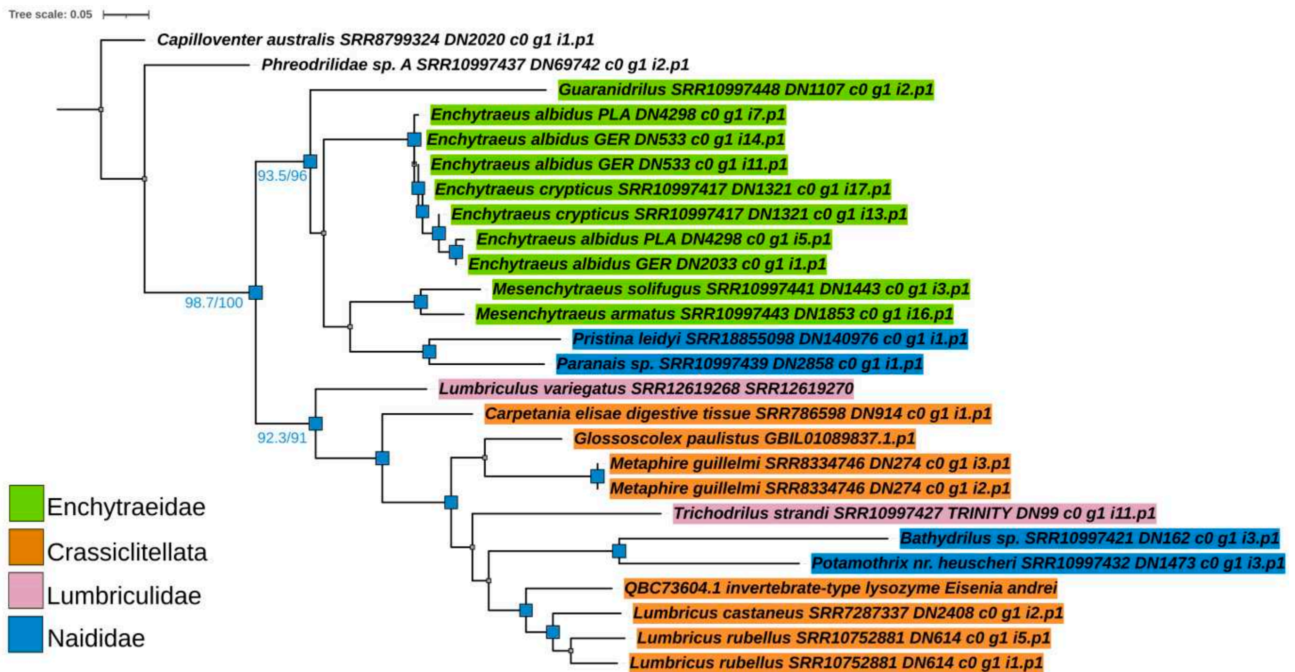

Figure 2. The best-scoring maximum likelihood tree (lnL = −3611.212) resulting from the analysis of mature amino acid sequences of the putative digestive i-type lysozyme in Clitellata. Only closely homologous sequences, distinct from those of other i-type lysozymes/destabilases, were used in the analysis. These sequences were retrieved from SRA raw transcriptomics data assembled in Trinity. A reciprocal BLASTp search for queries (Supplementary Table S2) revealed a match with the digestive i-type lysozyme from *Eisenia andrei* (Acc. QBC73604), with an E-value lower than 2×10^{-90}. This sequence was used as the reference. Blue squares denote branches with both SH-aLRT and UFBoot values (if ≥70) at the respective nodes. The exact values for the selected branches are given below. The tree was rooted at *Capilloventer australis*.

The second cluster consists of members of Lumbriculidae, Crassiclitellata, and two members of Naididae (*Bathydrilus* and *Potamothrix*), which are grouped mostly in a non-family-specific and highly discordant manner. Interestingly, these two naidid species belong to the subfamilies Phallodrilinae and Tubificinae, and are therefore grouped separately from members of the same family, *Paranais* and *Pristina*, which belong to the subfamilies Naidinae and Pristininae (the latter was previously included in Naidinae). Incongruent positioning of the lysozyme from the lumbriculid *Trichodrilus strandi* within the Crassiclitellata proteins, and not with *Lumbriculus variegatus*, might be attributed to low sampling of the Lumbriculidae. In contrast, the separate grouping of lysozyme sequences from different members of Naididae suggests a rather complex evolutionary history of lysozyme proteins in the family.

Based on the performed phylogenetic analysis, digestive i-type lysozyme proteins in Clitellata can be divided into three groups: (1) Capilloventridae–Phreodrilidae (as sequences from these two families were grouped together before rooting the tree), (2) Enchytraeidae–Naididae I, and (3) Crassiclitellata–Lumbriculidae–Naididae II.

2.6. Sequence Analysis, Domain Architecture, and Three-Dimensional Models of Selected Glycosidases

To elucidate the functional implications of the phylogenetic relationships, we performed a detailed structural analysis of the glycosidases, focusing on endo-β-1,4-glucanases (EC 3.2.1.4) and the digestive i-type lysozyme (3.2.1.17).

2.6.1. Digestive i-Type Lysozyme (Ealb-iLys)

Our examination of digestive i-type lysozyme from *E. albidus* (referred to here as Ealb-iLys) using InterProScan and SMART uncovered the presence of an invertebrate-type lysozyme domain, commonly referred to as destabilase [57]. The classification of this protein into the subfamily GH_22i was based on the InterProScan search and the identification of the signature sequence (L/D/Y/N)SCGPYQIK, as reported by Wohlkönig and co-workers [47]. Destabilase-lysozyme proteins (i-type lysozymes) are known to have both muramidase and isopeptidase activities. The muramidase activity, typical of lysozyme, involves hydrolysis of the glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan layer of bacterial cell walls. Its function as a destabilase, an endo-ε(γ-Glu)-Lys isopeptidase, is related to the specific hydrolysis of isopeptide bonds between the γ-carboxamide group of glutamine and the ε-amino group of lysine (i.e., bonds between the side-chains of Glu and Lys) [58]. The predictive analysis identified a signal peptide of 19 amino acids, MQAAVLFVFLSV(T/A)LPAALA, with the cleavage site ALA-DIT. All pre-protein variants of Ealb-iLys were 230 amino acids long, resulting in 211 residues for the mature protein. The domain architecture of Ealb-iLys was found to encompass the destabilase-lysozyme domain and the SH3b domain (Figure 3A), the latter being easily distinguishable in the tertiary structure model as densely packed anti-parallel beta-sheets and situated upstream of the destabilase domain (Figure 3C). These two domains are linked by a short region with low compositional complexity (linker). The SH3b domain in Ealb-iLys consists of seven tightly packed beta-strands arranged as a β-barrel-like fold. The last strand is interrupted by a turn of the 3_{10} helix (η1) located between the β6 and β7 strands. The SH3b domain is zipped by the α1-helix positioned toward the C-terminal end and contains a cysteine residue that forms a potential disulfide bridge with another cysteine residue of the β1-strand (Figures 3 and 4). The destabilase-lysozyme domain of Ealb-iLys consists of two parts, which can be roughly distinguished. The first part, called a subdomain, is formed by an α-helix (α2), two anti-parallel β-strands (β8 and β9) forming a β-sheet, and two relatively short α-helices (α3 and α4). This part is interconnected with another part by a long α-helix, leading to a second α-helix-based subdomain formed by two α-helices (α5 and α6) interrupted by two 3_{10}-helices. Both parts of the destabilase-lysozyme domain form an active site cleft. In the destabilase from the leech *Hirudo medicinalis* (UniProt ID: Q25091), which lacks the SH3b domain in the enzyme structure, an additional 3_{10}-helix is located after the first α-helix, while the β-sheet is formed by three anti-parallel β-strands, rather than two.

The conserved amino acids in Ealb-iLys for muramidase activity, glutamic acid, and aspartic acid [57] are located in the first subdomain; more precisely, Glu103 is located in the α2-helix and Asp115 in the β8-strand. In a study dedicated to a closely homologous i-type lysozyme from the earthworm *E. andrei* by Yu et al. [46], the authors mistakenly proposed a nearby serine (Ser118 in Ealb-iLys) as an additional third residue contributing to this activity. In fact, this serine is considered to be a primary candidate for the nucleophile in isopeptidase activity but not muramidase activity. Furthermore, in i-type lysozymes from mollusks, alanine often replaces a serine residue corresponding to residue 151 in Ealb-iLys [59]. This substitution is also observed in several clitellate species, including *E. albidus*, as we have demonstrated (Figure 5). The serine residue at this site was initially considered to be a candidate for the isopeptidase active site. However, research by Marin and co-workers [57] revealed that this residue is deeply buried within the protein core and lacks access to any protein cavities, contradicting its proposed role in isopeptidase activity.

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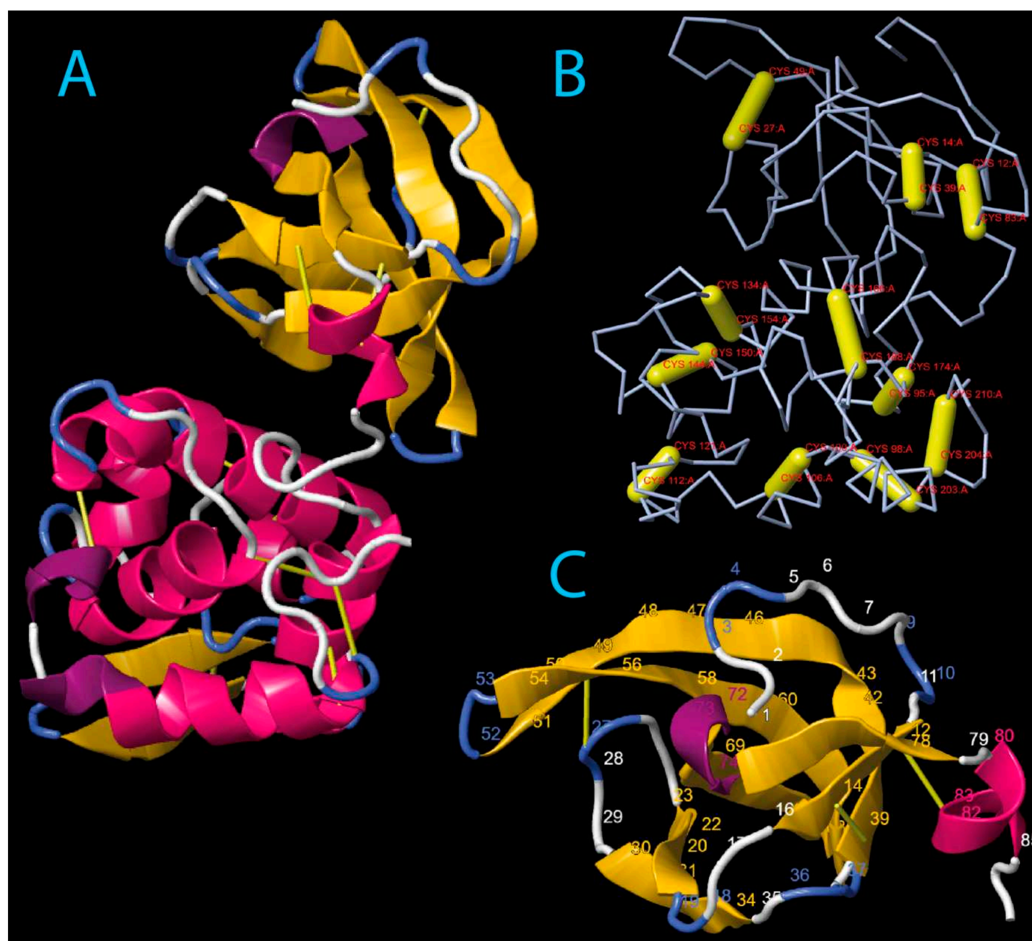

Figure 3. Three-dimensional model of mature digestive i-type lysozyme Ealb-iLys from the *E. albidus* PL-A strain (pLDDT = 91.386): (**A**) The tertiary structure of Ealb-iLys predicted by AlphaFold2/DeepMind v0.2, with secondary structure elements visualized using the First Glance in Jmol tool (version 4.1) and the DSSP 2.0 algorithm. β-Strands are shown in yellow, α-helices in pink, 3_{10}-helices in magenta, turns in blue, and regions without a defined structure in white. Disulfide bridges are indicated by thick or thin yellow rods. (**B**) The spatial location of predicted disulfide bridges within the protein backbone of Ealb-iLys. The amino acid positions that form each bond were specified. (**C**) SH3b domain isolated from the rest of the Ealb-iLys protein for clarity. The selected residue numbers were labeled for reference.

The structural model of Ealb-iLys indicates the presence of twenty-two cysteine residues that potentially form eleven disulfide bridges (Figure 3B). Within the SH3b domain, three disulfide bridges are expected to be formed. On the other hand, the destabilase domain is predicted to contain eight bridges, which is one more (an extra one at the C-terminal end) than in the *H. medicinalis* destabilase. Comparative analyses with homologs of Ealb-iLys from other clitellate species (see Figure 5) spotlight two additional conserved cysteines (positions 192 and 194) in a majority of these species. This includes the Ea-iLys sequence from *Eisenia andrei*. Homology-based modeling of Ea-iLys with the AlphaFold-predicted Ealb-iLys model as a template revealed that these two cysteines can form an additional, twelfth disulfide bridge. However, the formation of this bond was the only one not favored by Disulfide by Design 2.0 analysis [60]. Nevertheless, as with many other lysozymes [59,61], the results suggest that all twenty-two cysteine residues in Ealb-iLys are involved in the formation of disulfide bonds.

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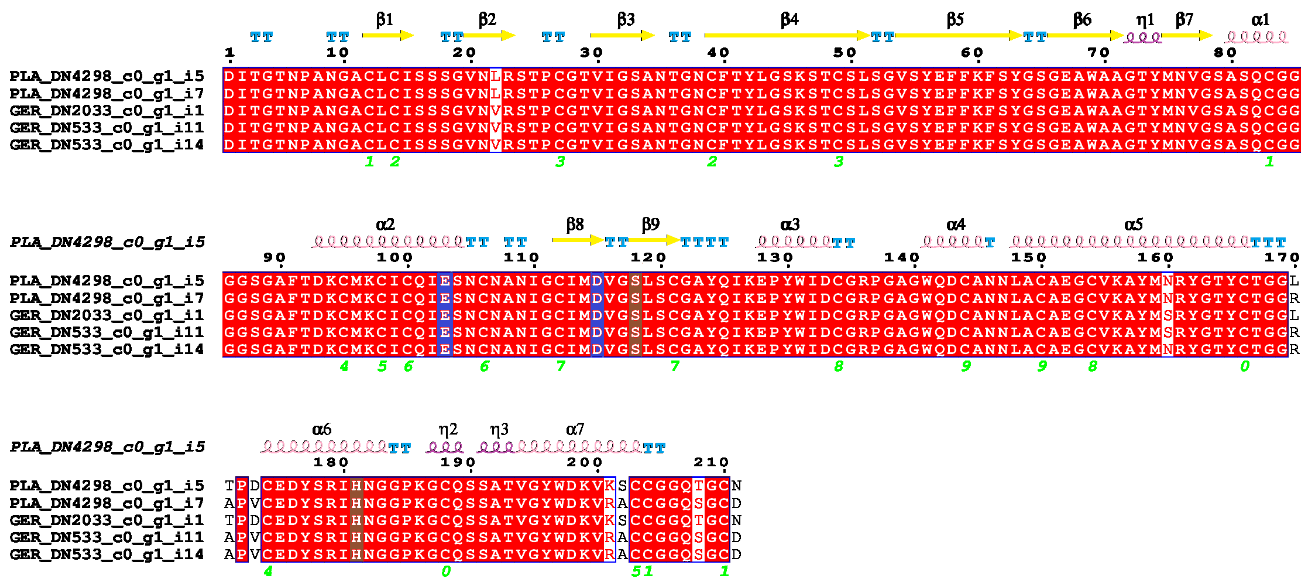

Figure 4. Sequence alignment and secondary structure element consensus of mature digestive i-type lysozyme Ealb-iLys allozymes from *E. albidus*. Secondary structure elements were predicted and marked according to Jmol using DSSP v2.0. β-Strands are marked as arrows. The α-helices and 3_{10}-helices are displayed as higher and lower squiggles, respectively. The symbol η refers to the 3_{10}-helix. Turns are marked as "TT" letters above the sequence. One-residue "T" segments indicate that the β-turn overlaps a structure of higher priority (e.g., a helix). The positions of potential disulfide bridges are marked as pairs of green digits below the alignment. Catalytic residues for muramidase activity are highlighted in blue, whereas those for isopeptidase activity are highlighted in brown. Strictly identical residues are shown as white characters boxed in red, while similar residues within a group are shown as red characters.

2.6.2. Endo-β-1,4-Glucanase I/II

Both identified endoglucanases (EC 3.2.1.4), referred to here as Ealb-Eg I and Ealb-Eg II, have been classified as members of GH family 9. We initially distinguished between these two putative genes based on their signal peptide sequences and distinct cleavage sites. However, this distinction might be somewhat oversimplified, as we identified groups of transcripts with three different open reading frame (ORF) lengths (1371, 1368, and 1353 bp), and there are no available supportive genomics data for *E. albidus*. Notably, the Ealb-Eg I variants from the N-strain exhibited a unique deletion of a single amino acid in the sequence, in addition to substitutions. Despite these differences, all Ealb-Eg proteins share a relatively high level of amino acid identity and possess conserved amino acid stretches that are common across variants of both genes. The pairwise sequence divergence between Ealb-Eg I and Ealb-Eg II was estimated to range from 4.1% to 30.1% (Table 7). It is also worth mentioning that the original TransDecoder-predicted longest open reading frame (ORF) for Ef-Eg I contains two additional start codons within the same frame as the coding sequence, i.e., upstream start codons within an upstream open reading frame. The proper codon site within the longest ORF was identified based on the Kozak sequence (AAC**ATG**A) variant for Annelida, as reported by Satake and coworkers [62]. This identification was further confirmed through signal peptide sequence analysis in SignalP 6.0. Notably, this Kozak sequence variant is also found in previously characterized *E. albidus* α-amylases [39]. Conversely, a slightly distinct ATG flanking motif (AAT**ATG**A) was identified in Ef-Eg I from the German strain.

Figure 5. Multiple sequence alignment of i-type lysozymes containing the SH3b domain, found in Clitellata. Mature sequences of Ealb-iLys homologs were aligned. Catalytic residues for muramidase activity are marked with red arrows, whereas those for isopeptidase activity are marked with brown arrows. The crossed-out arrow indicates a semi-conserved serine previously thought to be involved in isopeptidase activity but disproven by a recent study by Marin and co-workers [57].

Table 7. Estimates of evolutionary divergence between sequences of Ealb-Eg I and Ealb-Eg II pre-proteins. The number of amino acid substitutions per site is shown. Standard error estimates are shown above the diagonal and were obtained by a bootstrap procedure (500 replicates). Analysis was conducted in MEGA7 using the Poisson correction model and involved 21 amino acid sequences. All ambiguous positions were removed for each sequence pair. There were a total of 456 positions in the final dataset.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
G_DN291_c0_g1_i1_EG_I	1		0.005	0.023	0.012	0.011	0.017	0.011	0.012	0.009	0.011	0.021	0.018	0.015	0.019	0.015	0.026	0.020	0.023	0.018	0.015	0.015
G_DN291_c1_g1_i6_EG_I	2	0.013		0.023	0.012	0.011	0.017	0.010	0.013	0.011	0.009	0.021	0.017	0.014	0.019	0.014	0.026	0.019	0.022	0.017	0.014	0.015
PL-A_DN3132_c2_g1_i2_EG_I	3	0.234	0.228		0.022	0.023	0.021	0.023	0.025	0.026	0.026	0.023	0.025	0.026	0.024	0.026	0.014	0.024	0.021	0.026	0.027	0.027
N_DN166_c0_g2_i11_EG_I	4	0.066	0.061	0.226		0.009	0.017	0.006	0.017	0.015	0.015	0.020	0.018	0.011	0.017	0.013	0.025	0.020	0.023	0.017	0.015	0.016
N_DN166_c0_g2_i15_EG_I	5	0.054	0.054	0.243	0.034		0.017	0.009	0.016	0.014	0.014	0.021	0.018	0.012	0.017	0.010	0.025	0.019	0.022	0.017	0.015	0.014
N_DN1982_c0_g1_i20_EG_I	6	0.131	0.121	0.196	0.116	0.129		0.015	0.018	0.020	0.019	0.014	0.016	0.019	0.014	0.020	0.024	0.010	0.016	0.014	0.018	0.018
N_DN1982_c0_g1_i34_EG_I	7	0.054	0.045	0.229	0.016	0.036	0.099		0.015	0.014	0.013	0.021	0.018	0.011	0.017	0.013	0.025	0.019	0.022	0.016	0.013	0.015
G_DN291_c0_g1_i11_EG_II	8	0.069	0.083	0.283	0.120	0.118	0.148	0.108		0.008	0.009	0.019	0.019	0.013	0.015	0.014	0.023	0.015	0.020	0.016	0.013	0.014
G_DN291_c0_g1_i12_EG_II	9	0.041	0.055	0.289	0.105	0.093	0.174	0.093	0.027		0.005	0.021	0.017	0.012	0.017	0.011	0.024	0.017	0.020	0.015	0.011	0.012
G_DN291_c1_g1_i9_EG_II	10	0.055	0.041	0.283	0.100	0.093	0.164	0.083	0.041	0.013		0.020	0.016	0.011	0.016	0.011	0.024	0.016	0.020	0.014	0.010	0.011
PL-A_DN9119_c0_g1_i1_EG_II	11	0.201	0.196	0.237	0.193	0.204	0.086	0.196	0.156	0.182	0.177		0.011	0.019	0.013	0.019	0.022	0.012	0.015	0.014	0.019	0.018
PL-A_DN9119_c0_g1_i5_EG_II	12	0.143	0.133	0.269	0.148	0.153	0.128	0.151	0.156	0.125	0.115	0.055		0.016	0.016	0.016	0.024	0.015	0.018	0.011	0.016	0.015
N_DN166_c0_g2_i14_EG_II	13	0.100	0.091	0.289	0.060	0.062	0.164	0.057	0.081	0.067	0.057	0.164	0.120		0.013	0.006	0.022	0.017	0.019	0.012	0.010	0.011
N_DN166_c0_g2_i26_EG_II	14	0.161	0.151	0.260	0.141	0.133	0.081	0.133	0.100	0.125	0.115	0.081	0.123	0.076		0.014	0.020	0.009	0.014	0.013	0.013	0.013
N_DN166_c0_g2_i6_EG_II	15	0.098	0.093	0.286	0.076	0.045	0.172	0.074	0.088	0.064	0.060	0.172	0.123	0.016	0.083		0.022	0.017	0.019	0.013	0.011	0.009
N_DN166_c0_g2_i9_EG_II	16	0.277	0.272	0.081	0.277	0.260	0.234	0.269	0.229	0.237	0.232	0.209	0.243	0.204	0.177	0.204		0.020	0.015	0.021	0.024	0.023
N_DN1982_c0_g1_i11_EG_II	17	0.172	0.161	0.254	0.172	0.164	0.052	0.159	0.100	0.125	0.115	0.067	0.108	0.120	0.041	0.128	0.188		0.012	0.011	0.014	0.015
N_DN1982_c0_g1_i22_EG_II	18	0.223	0.209	0.193	0.223	0.212	0.113	0.209	0.177	0.185	0.172	0.100	0.141	0.159	0.091	0.159	0.108	0.071		0.014	0.018	0.017
N_DN1982_c0_g1_i29_EG_II	19	0.138	0.123	0.274	0.123	0.130	0.093	0.110	0.118	0.103	0.088	0.100	0.060	0.064	0.071	0.081	0.196	0.052	0.088		0.010	0.011
N_DN1982_c0_g1_i30_EG_II	20	0.108	0.093	0.301	0.093	0.096	0.138	0.086	0.079	0.064	0.050	0.153	0.110	0.045	0.071	0.062	0.232	0.081	0.143	0.050		0.007
N_DN1982_c0_g1_i32_EG_II	21	0.105	0.096	0.298	0.110	0.079	0.146	0.103	0.096	0.071	0.062	0.153	0.105	0.052	0.069	0.036	0.220	0.098	0.133	0.057	0.025	

103:8651185770

Because our phylogenetic analysis found that Ealb-Eg I and Ealb-Eg II proteins form a highly resolved single clade rather than separate gene-specific clusters (Figure 1), we calculated the omega (dN/dS) ratio collectively for all mature sequences of Ealb-Eg as if for a single gene. We estimated the ratio to be 0.21620, indicating that endo-β-1,4-glucanases in *E. albidus* are under purifying selection. Therefore, changes in their coding sequences could be detrimental.

The domain arrangements of Ealb-Eg I and Ealb-Eg II were typical of other known GH9 endo-β-1,4-glucanases. The catalytic domain structure of Ealb-Eg proteins consists of 12 α-helices that form the $(\alpha/\alpha)_6$-barrel fold, with six internal and six external α-helices. Additionally, the overall structure includes four extra α-helices and three conserved 3_{10}-helices. Furthermore, Ealb-Eg II, similar to Ef-EG2 from the earthworm *Eisenia fetida* [63], contains five β-strands arranged as a conserved β-sheet and β-hairpin. In contrast, Ealb-Eg I lacks a β-hairpin in its structure (Figures 6 and 7). The significance of this modification of the structure is not known. In both modeled Ealb-Eg proteins, a single π-helix was predicted to be located at the end of the longer α13-helix. Nonetheless, Ealb-Eg I/II proteins were found to be very similar in structure to Ef-EG2, which allowed for homology-based modeling and the generation of high-quality models.

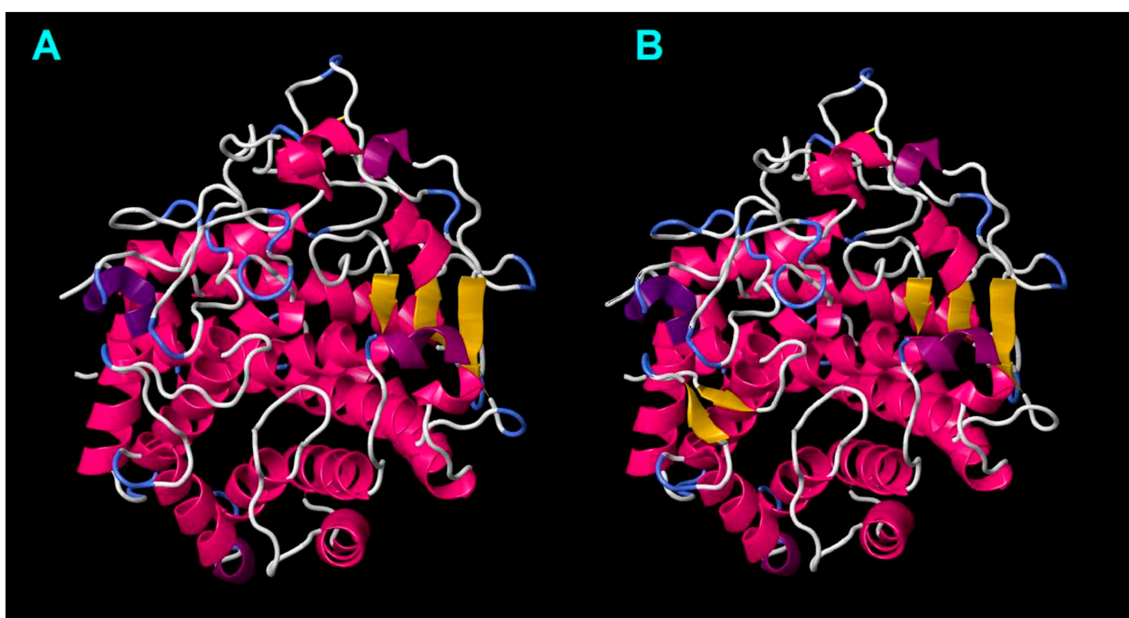

Figure 6. Three-dimensional models of mature endo-β-1,4-glucanases from the *E. albidus* PL-A strain generated by SWISS-MODEL: (**A**) Tertiary structure of Ealb-Eg I. (**B**) Tertiary structure of Ealb-Eg II. β-Strands are shown in yellow, α-helices in pink, 3_{10}-helices in magenta, π-helices in purple, turns in blue, and regions without a defined structure in white. Disulfide bridges are indicated by thin yellow rods. The quality of the generated models for Ealb-Eg I/II was high, with a Global Model Quality Estimate (GMQE) of 0.92/0.93 and a QMEANDisCo global score of 0.89.

The catalytic domains of Ealb-Eg proteins, consistent with other GH9 endo-β-1,4-glucanases, have two catalytic Asp residues within the conserved motif Asp-Ala-Gly-Asp (DAGD; here corrigendum for [64]) and one Glu residue within the semi-conserved motif Asn-Glu-Val [64], adjacent to the highly conserved Asp-Tyr-Asn-Ala (DYNA) motif of the α16-helix (see Figure 7). The study of the crystal structure of Ef-EG2 from *E. fetida* [63] underpins that there are binding sites for calcium and sodium ions. These sites exhibit limited conservation in *E. albidus* Ealb-Eg I/II and hint at a nuanced evolutionary adaptation of Ealb-Eg enzymes in ion binding, potentially reflecting distinct environmental contexts. This was observed in other GH9 endo-β-1,4-glucanases, including the enzyme from the higher termite *Nasutitermes takasagoensis* [65] (see also Supplementary Figure S1).

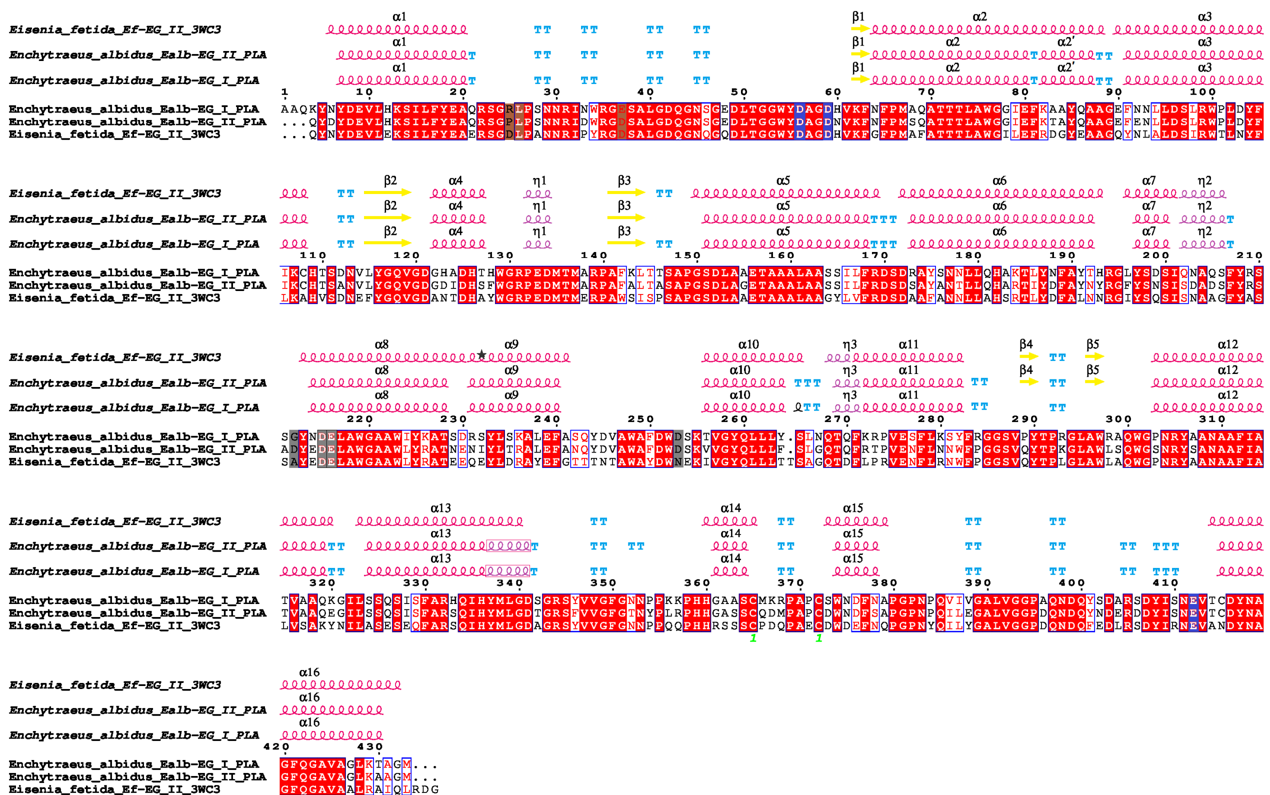

Figure 7. Secondary structure alignment of mature endo-β-1,4-glucanases: Ealb-Eg I/II from *Enchytraeus albidus* PL-A and Ef-EG2 from the earthworm *Eisenia fetida*. The secondary structure elements of Ealb-Eg I/II were predicted and marked according to Jmol with the implementation of the DSSP v2.0 algorithm. The secondary structure elements of Ef-EG2 were annotated according to the updated model (version 1.2) of the crystal structure of endo-1,4-beta-glucanase (PDB ID: 3WC3) from *E. fetida*. β-Strands are marked as arrows. The α-helices and 3_{10}-helices are displayed as higher and lower squiggles, respectively. The η symbol refers to a 3_{10}-helix. The boxed squiggle refers to the π-helix. Turns are marked with the letters "TT" above the sequence. One-residue "T" segments indicate that the β-turn overlaps a structure of higher priority (e.g., a helix). The position of a potential disulfide bridge is marked as a pair of green digits below the alignment. A selected residue with an alternate conformation is marked above with a black star on top of the secondary structure element annotation. Catalytic residues for cellulase activity are highlighted in blue. Residues involved in binding calcium are highlighted in gray, and those potentially involved in binding sodium are highlighted in brown. Strictly identical residues are shown as white characters boxed in red, while similar residues within a group are shown as red characters.

3. Discussion

3.1. General Considerations Regarding Digestive Enzyme Gene Candidates in E. albidus

In animals, most digestive enzymes belong to hydrolases [33,66–68]. Their primary function is to break down larger molecules from food into a form that can be absorbed by the organism [69]. These enzymes can be secreted into the lumen of the alimentary tract or bound to the microvilli [70]. Secreted proteins generally require a signal peptide sequence for proper targeting and secretion, whereas enzymes in microvilli have transmembrane domains that bind them to the plasma membrane or are clustered on the cell surface, requiring specific signals for proper localization and GPI anchoring [70–73]. These facts seem to have been overlooked by other authors when predicting digestive capacity based on transcriptomics data and functional annotation (see [29,30]). To distinguish intracellular metabolic and lysosomal enzymes from extracellular-acting digestive enzymes [74] in our datasets, we thoroughly analyzed the sequence features mentioned above, along with other features, in the recovered candidates for digestive enzyme genes. Among the hy-

drolytic enzymes, glycosidases play a crucial role in the digestion of saccharides. They are responsible for breaking down common biopolymers such as cellulose, chitin, and starch, which are abundant in nature. Glycosidases are significant in assessing trophic positions, as they define the digestive capabilities of animals by participating in the degradation of plant, fungal, or bacterial materials, including cell wall components, within the decomposer system. Moreover, glycosidases appear to be the best-characterized digestive enzymes in Annelida [41,45,46,48,63,75,76]. Although we identified candidates for proteolytic and lipolytic enzyme genes in *E. albidus*, the scope of the present study is somewhat limited, as we restricted our analysis to only the best annotated and orthologously supported candidates. Nevertheless, our findings provide initial insights into the genetics of enchytraeid digestive enzymes, which can be further expanded upon. While fibrinolytic proteases such as lumbrokinases are currently gathering some scientific attention, mainly for potential medical applications [77], digestive lipases remain very challenging to study not only in Enchytraeidae but also in the wider Annelida, as they are still a largely genetically unexplored group of enzymes. Recently, the hormone-sensitive lipase gene, which is an intracellular metabolic neutral lipase, was cloned and its expression was analyzed in the leech *Whitmania pigra* [78]. However, to the best of our knowledge, no dedicated molecular studies have focused on the typical digestive lipases in members of Annelida. Studies on potential digestive lipases in this taxon are often limited to biochemical enzyme assays. Indeed, the general activity patterns of hydrolytic enzymes, including lipases, in the digestive systems of representatives of Acanthobdellida, Branchiobdellida, and Hirudinida were studied using API ZYM tests by one of the co-authors of the present study [66].

3.2. Endogenous Expression of GH9 Cellulase Genes in E. albidus and Other Clitellates

In a review [18], the first author and colleagues proposed a classification of the trophic types of enchytraeids from the most commonly studied genera based on food preferences and feeding behavior reported in the available literature. According to the definition, primary decomposers in the soil food web consume plant litter prior to substantial microbial degradation [17,79]. Thus, it is presumed that primary decomposers need to produce enzymes involved in breaking down major plant cell wall components. In contrast, secondary decomposers rely on plant residues initially degraded by microflora or on microorganisms as food sources. *Enchytraeus* spp. were assigned to the secondary decomposer group, as no definitive evidence of endogenous cellulolytic capability has been provided before. Although some cellulase activity has been detected in a few studies on *Enchytraeus* spp., there has been no attempt to determine whether these cellulases originate from the potworms themselves or from microorganisms. Moreover, the results obtained by different authors using biochemical techniques were not always consistent [37,80–82]. For example, Nielsen [37], using enzymatic assays and chromatographic analyses, found no cellulolytic activity in *E. albidus*, nor in three other enchytraeid species. In contrast, Urbášek and Chalupský [81] detected very low to low cellulolytic activities in four species, including *E. albidus*. However, these authors clearly stated that there was no attempt to differentiate the origin of the detected enzymes. Similarly, Dash et al. [80] reported low-to-moderate cellulolytic activity in homogenates of entire specimens of *E. berhampurosus* and in two other tropical enchytraeid species. In addition to enzymatic assays, some ecohistological studies have been performed on *Enchytraeus* species. Reichert et al. [83] investigated the feeding behavior of *E. coronatus* on agar plates with air-dried *Sambucus nigra* leaves and observed signs of leaf tissue damage and consumption. They suggested that *E. coronatus* exhibited significant cellulolytic activity to pre-digest the leaves externally before ingestion. However, Gajda et al. [18] strongly disagreed with this conclusion. They performed similar experiments but included proper controls (plates with leaves but no animals), which were lacking in the study of Reichert et al. [83], and demonstrated clearly that the contribution of microbial activity to the maceration of the plant material on the experimental plate could not be disregarded as a possible explanation.

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Considering all of the information recapitulated above, we proposed the following research hypothesis (1): *E. albidus* does not exhibit endogenous expression of enzyme genes from the cellulase group. However, our transcriptomics data analysis in this study identified 30 digestive gene candidates encoding glycosidases, among which we annotated cellulolytic enzymes—endo-β-1,4-glucanases (EC 3.2.1.4). Therefore, this hypothesis was rejected. Phylogenetic and in silico structural analyses revealed that *E. albidus* endo-β-1,4-glucanases are homologous to a few previously described endo-β-1,4-glucanases (cellulases) from earthworm species such as *Metaphire hilgendorfi*, *Eisenia fetida*, and *E. andrei* [40–42]. Moreover, transcriptomics data derived from other clitellate species and integrated into phylogenetic analysis demonstrated that, in addition to the aforementioned earthworm species, which provided initial evidence for endogenous cellulase production in clitellates, GH9 endo-β-1,4-glucanases are present in other members of Clitellata, including Capilloventridae, Phreodrilidae, Naididae, Lumbriculidae, and Randiellidae. Endo-β-1,4-glucanases were found to be especially widespread in members of Enchytraeidae and Crassiclitellata (i.e., earthworms). However, as a side note, it should be mentioned here that the recovered sequence for Randiellidae should be treated with caution, as the only available raw RNA-Seq reads for *Randiella* seem to be contaminated, at least to some degree, by other annelid sequences, as noted in our paper related to amylases (for details, see [39]), and this might be further indicated by the unusual result that we noticed in another study using the same transcriptomics data (please note the extraordinarily high number of linker genes in *Randiella* across all analyzed species for hexagonal bilayer hemoglobin in [84]). Apart from clitellates, we also recovered a closely homologous endo-β-1,4-glucanase from the terrestrial polychaete *Hrabeiella periglandulata*. Orthologous sequences for other polychaetes are available for the nereids *Perinereis brevicirris* and *Perinereis aibuhitensis*. Generally, all of these GH9 endo-β-1,4-glucanases from both Clitellata and Polychaeta share high similarity (≥68%) and a similar length of mature protein sequences (>420 amino acids; see also [64]). In light of this, we question the short sequence for *E. andrei* "cellulase 2" reported by Kim et al. [85], as the provided sequence lacks a signal peptide, an α1-helix in its structure and, importantly, the two catalytic Asp residues in the DAGD motif, which are essential for cellulase activity. The provided sequence for "cellulase 2" represents a 5′ partial ORF recovered from RNA-Seq data. This also underscores the importance of basic structural modeling in similar studies.

Endo-β-1,4-glucanases belonging to glycosyl hydrolase family 9 are present among diverse invertebrate lineages, demonstrating varied feeding strategies [64]. Unlike their counterparts in microbes and plants, where these cellulases often possess catalytic domains linked to carbohydrate-binding modules (CBMs) enabling crystalline cellulose breakdown [86], many GH9 animal cellulases lack such CBMs (but cf. [87–89]). As a result, these enzymes exhibit limited or no activity against crystalline cellulose but break down the amorphous fraction of the polysaccharide. Consequently, Linton [64] posited that the capacity to hydrolyze crystalline cellulose efficiently should serve as a proper indicator for assessing cellulases, suggesting that cellulolytic enzymes solely capable of breaking down carboxymethylcellulose (CMC) should not be considered genuine cellulases but, rather, enzymes digesting β-1,4-glucans. Additionally, it was raised that endo-β-1,4-glucanases in some animals can cleave lichenan or mixed-linkage β-D-glucans at comparable or even greater rates compared to CMC. While Linton has a point in their postulation, it is rather not universally accepted by other authors. However, based on research on other polysaccharides, it could also be argued that, for example, different amylose forms (e.g., amylose A and B) can be digested by α-amylases with extremely different efficiencies [75]. Furthermore, concerning Linton's discussion on deriving the amounts of metabolizable sugars from cellulosic material in non-primarily herbivorous invertebrates, research on *E. fetida* demonstrated that a single amino acid substitution in the sequence can dramatically change the catalytic activity and the stability of Ef-EG2 endoglucanase mutants, impacting the amount of hydrolysis products released from CMC [76]. Moreover, screening of Clitellata transcriptomes in our study revealed that the endo-β-1,4-glucanases in the enchytraeid *E.*

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albidus and the earthworms *Lumbricus* spp., *Eisenia andrei*, and *Metaphire guillelmi* are highly polymorphic. Notably, Ef-EG1 and Ef-EG2 endo-β-1,4-glucanases in *E. fetida* [41,90] were originally identified as distinct genes based on cloned ORFs. However, it is likely that they actually represent allelic variants, as the differences are only related to a single nucleotide resulting in a single amino acid substitution. Support from genomics data analysis could be a solution to address this issue. Despite these minimal sequence variations, purified Ef-EG1 and Ef-EG2 proteins from the *Eisenia fetida* Waki strain [90] demonstrate significant biochemical differences between each other in terms of activity and substrate specificity, which is in agreement with the above-mentioned study of Ef-EG2 mutants [76]. In *Enchytraeus*, copy variants of endo-β-1,4-glucanases are more divergent than those in *Eisenia* spp. (see Figure 1). The adaptive significance of endo-β-1,4-glucanase polymorphisms in Clitellata could be related to broader substrate specificity; however, further molecular and biochemical studies are needed to confirm this in *E. albidus*.

3.3. Endogenous Expression of Digestive i-Type Lysozyme Gene in E. albidus and Other Clitellates

Apart from hypothesis (1), related to the absence of cellulases, we postulated hypothesis (2): that *E. albidus* demonstrates endogenous expression of enzyme genes engaged in the digestion of microorganisms. Thus, it was presumed that secondary decomposers, which at least partially utilize the microbial material, need to produce enzymes involved in breaking down major bacterial and fungal cell wall components, such as peptidoglycan hydrolases or chitinases. To the best of our knowledge, no studies have clearly demonstrated microphytophagous (i.e., microbivorous) behavior in *E. albidus* related to bacteria and fungi. However, some reports are available for other *Enchytraeus* species. The first report related to the genus was probably by Dougherty and Solberg [91], who partially succeeded in maintaining *Enchytraeus fragmentosus* under monoxenic conditions with *Escherichia coli* growing on a nutrient agar medium, but the growth of the animal was suboptimal. Subsequently, Brockmeyer et al. [92] demonstrated the use of microbial protein from radiolabeled ^{35}S-enriched *Bacillus cereus* and the yeast *Saccharomyces cerevisiae* for *Enchytraeus* cf. *globuliferus* and *E. christenseni* (syn. *E. minutus*). In relation to this, Reichert et al. [83] reported that *E. coronatus* fed with *B. cereus* was in good condition, but its reproduction rate was lower than when fed with rolled oats. The most explicit microphytophagous behavior related to bacteria and fungi has been reported for *Enchytraeus crypticus* [13,93–98]. In general, this species can use certain species of *Streptomyces* bacteria and microscopic fungi as its sole nutrient source [13,94,96]. Moreover, it can preferably consume and utilize particular species of cyanobacteria and eukaryotic microalgae [95,97].

Based on the transcriptomics data analysis of *E. albidus*, we identified an endogenous novel digestive i-type lysozyme, named Ealb-iLys (GH22i family; EC 3.2.1.17), and two chitinases (GH18 family; EC 3.2.1.14), referred to here as Ealb-Chit I and Ealb-Chit II. The latter enzymes will be addressed in detail elsewhere, in a separate paper. Consequently, hypothesis (2), regarding the production of enzymes involved in breaking down major bacterial and fungal cell wall components in this enchytraeid species, was supported. In a previous review, several hypotheses were proposed concerning the capacity of Enchytraeidae to utilize various bacterial strains as a nutrient source [18]. Notably, the presence of β-N-acetylglucosaminidase in the intestinal epithelium of the enchytraeid *Lumbricillus lineatus*, as reported by Gelder [99], raised speculation about the potential role of this enzyme and other murein hydrolases in breaking down bacterial cell walls in the alimentary tract of enchytraeids. Indeed, some studies on invertebrates suggest that β-N-acetylglucosaminidase may be involved in digestion [67,100,101]. However, to the best of our knowledge, no contribution of typical digestive β-N-acetylglucosaminidase to microbial cell lysis has been described to date in invertebrates, at least in Annelida. Conversely, complete coding sequences for endo-β-N-acetylglucosaminidases (EC 3.2.1.96) recovered from transcriptomics data of *E. albidus* lack signal peptides, and putative proteins were predicted to be localized in the cytoplasm. Therefore, these are not secretory digestive enzymes released into the gut lumen that can contribute to trophic digestion in

enchytraeids, despite our initial assumption based on Gelder's results [18,99]. Another obvious, yet at the time of review [18] rather theoretical, candidate for the enzyme involved in microbes' digestion in enchytraeids was lysozyme. A pivotal study that significantly contributed to considering this enzyme was the identification and histolocalization of a novel digestive lysozyme, Ea-iLys, from *E. andrei* by Yu and co-workers [46]. The annotation of a homologous sequence in *E. albidus* posed challenges owing to the absence of a functional ortholog for this lysozyme in the KEGG database. Therefore, we initially recovered the homologous sequences based on the presence of a signature sequence for the i-type lysozymes. Additionally, the assignment of the Ealb-iLys sequence as a lysozyme had a low positive predictive value (PPV) from the PANNZER2 annotation, highlighting the significance of annotating data using diverse methods and tools. Animal lysozymes containing the SH3b domain, such as Ealb-iLys, have rarely been identified. The i-type lysozyme, which contains a destabilase with the SH3b domain, was reported as HcLyso4 in the triangle-shell pearl mussel (*Hyriopsis cumingii*), while the SH3b domain was also noted after alignment in the sequence of MGL-2 lysozyme (Acc. AB298451) from the Mediterranean mussel (*Mytilus galloprovincialis*) [61]. Additionally, we identified this domain in the above-mentioned Ea-iLys from *E. andrei* [46], as it was not initially annotated in the original study. Moreover, we recovered closely homologous (orthologous) sequences to *Enchytraeus*–*Eisenia*-type lysozyme containing the SH3b domain from transcriptomics data related to several clitellates, including other enchytraeid species (*Enchytraeus crypticus*, *Mesenchytraeus solifugus*, *M. armatus*, and *Guranidrilus* sp.). Notably, the RNA-Seq reads (SRR786598) associated with the earthworm *Carpetania elisae* (now *C. matritensis*) [102], where we also found this novel i-type lysozyme, originated from a sample consisting of isolated digestive tissues. This finding aligns with the observation that Ea-iLys is highly expressed in the gut epithelium [46]. The possible role of the SH3b domain in this type of lysozyme may be related to peptidoglycan recognition and bacterial cell wall binding [103]; however, further studies are required to confirm this hypothesis. Based on the findings presented, we propose orthologs of *Enchytraeus*–*Eisenia*-type SH3b-domain-containing i-type lysozymes (i.e., Ealb-iLys and Ea-iLys) as potential molecular markers of bacterivory in clitellates.

3.4. Trophic Position of E. albidus as an Intermediate Decomposer and the Status of Other Clitellates

Considering the tested research hypotheses related to the trophic position of *Enchytraeus albidus*, we found that this enchytraeid species expresses genes for both cellulases and enzymes involved in the digestion of microbial cell walls, including a specialized digestive type of lysozyme. Therefore, *E. albidus* combines traits of both primary and secondary decomposers and can be defined as an intermediate type of decomposer. The term "intermediate decomposers" was originally coined by Eisenhauer and Schädler [104] to roughly define the position of enchytraeids and highlight the uncertain trophic position of this taxon, which could represent a functional gradient ranging from primary to secondary decomposers. Our transcriptomics approach, novel to trophic ecology studies, in which we determined *E. albidus* as an intermediate decomposer, corresponds well with the newest findings related to *E. albidus* sensu lato by Korobushkin et al. [105] using stable isotope analysis. In that most recent study (note: published when our manuscript was under review), where trophic niches of 16 common terrestrial enchytraeid species were determined, the analysis found them to act as primary and secondary decomposers within three trophic guilds (epigeic, epi-endogeic, and endogeic), depending on species. Korobushkin et al. [105] assigned epigeic enchytraeids, including *E. albidus* sensu lato (identified based on morphology only), among primary decomposers feeding on litter. However, the wide ranges of Δ^{15}N values obtained in the study also indicated the co-ingestion of microorganisms. Thus, the revealed trophic niche of *E. albidus* matches with intermediate decomposers. Furthermore, Korobushkin et al. [105] expressed the view that the classification of individual enchytraeid species as primary or secondary decomposers requires

further experimental intervention, incorporating multiple metrics instead of solely relying on stable isotopic signatures. We believe that the presented transcriptomics approach could provide a solution to this challenge.

The composition of digestive enzyme genes in *E. albidus* revealed by transcriptomics analysis is in general agreement with the results of the study by Urbášek and Chalupský [81], who analyzed enzymatic profiles from the whole-body homogenates of enchytraeids. *Enchytraeus albidus* was characterized there by moderate activity of α-amylase, β-xylanase, laminarinase, and lichenase, and low to very low activity of proteases (pH = 6.0), Cx-cellulase (endo-1,4-β-D-glucanase, EC 3.2.1.4), and the cellulase complex (a mixture of exo- and endo-1,4-β-D-glucanases). Moreover, our study revealed homologous sequences for conserved cellulases and digestive i-type lysozymes in the transcriptomics data of other clitellates, particularly for enchytraeid and earthworm species, suggesting a similar trophic position of these animals. However, recent work by Korobushkin et al. [106] using stable isotopes demonstrated that the trophic position of enchytraeids and earthworms can differ based on available food sources. In their microcosm experiment, they observed that enchytraeids (a mixture of littoral species, *E. albidus* sensu lato, and *Lumbricillus* spp.) were preconditioning the macroalgal material, while probably grazing on bacteria as well, making it suitable for the earthworm *Eisenia fetida*, which lacked direct feeding activity on non-conditioned macroalgae. This indicates that marine littoral enchytraeids can act as primary/intermediate decomposers, while *E. fetida* serves as a typical secondary decomposer in this specific scenario, depending on food availability. The results of the study by Korobushkin et al. [106] are in contrast to other research that considered earthworm species such as *Lumbricus terrestris* as primary decomposers in soil microcosm experiments while assigning enchytraeids to a higher trophic level [107]. Interestingly, it was demonstrated that *L. terrestris* can also function as a granivore and seedling herbivore [107]. Concerning this species, we found that *L. terrestris* congeners possess both cellulases and digestive lysozyme, similar to enchytraeids and other earthworms. The use of an enchytraeid species mixture by Korobushkin et al. [106] prevents drawing conclusions strictly for *E. albidus*; however, our study demonstrated that this enchytraeid species expresses several enzymes (e.g., EC 3.2.1.6, EC 3.2.1.51, and EC 3.2.1.78) that could be potentially engaged in the digestion of macroalgal material [108,109], which could be expected from typical marine littoral species. Dietary flexibility, which is a known challenge in trophic ecology studies, could be analyzed by a comparative study of enzymes of both enchytraeid and earthworm species, as in the above example, but this requires sufficiently deep sequenced transcriptomes for all species of interest and general molecular and biochemical knowledge of digestive enzymes. In general, much work remains to be conducted on the trophic position of Enchytraeidae, as well as other clitellates and their digestive capacities. A natural progression in research would involve studying food-dependent gene expression, molecular cloning, and the utilization of expression vectors to further investigate the biochemical properties of the identified digestive enzymes. Next, a more than 60-year-old dilemma related to the feeding mechanism and exact mode of digestion in enchytraeids (pre-oral digestion or internal digestion?) [18,24,83,93,110–114], for which there is no consensus among researchers to date, can be analyzed by histolocalization of transcripts of selected digestive enzyme genes. Furthermore, bacterivory in deep molecular details was recently studied in the model nematode *Caenorhabditis elegans*. This includes the fate of various bacterial strains ingested, chemical cues stimulating feeding and digestion, specific lysozyme expression, signaling pathways regulating digestion of bacteria, and recognition of palatable and unpalatable food ([115–117]; see also [118], preprint). These studies shed new light on somewhat forgotten yet crucial preliminary studies conducted by Krištůfek et al. [94], which relate, among other things, to chemoattraction in enchytraeid–bacteria interactions and primarily demonstrated that bacteria can serve as an important source of food for enchytraeids. Finally, more advanced enchytraeid and earthworm molecular studies require support from annotated genomics datasets. The first step in this direction was performed by Amorim and co-workers [56], who provided raw but high-quality genomics data for *Enchytraeus crypticus*

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isolate CE2183. We hope that more genomics and monohaplotype-derived transcriptomics data will be generated for enchytraeid and earthworm species in the near future. This will significantly enhance the advancement of molecular research on the trophic ecology of these groups of clitellates.

4. Materials and Methods

4.1. Animal Material

The initial culture of *Enchytraeus albidus* was established from a stock culture purchased on the e-commerce platform Allegro from a commercial seller, Bodzio-1234. The animals were kept at room temperature in a plastic box with defaunized garden soil and fed fish flakes twice weekly. Random specimens from the initial culture underwent DNA barcoding (Acc. MK044803–MK044805) and were analyzed using PCR-RF-SSCP (PCR–restriction fragments–single-strand conformation polymorphism) [119] of the Folmer fragment (Supplementary Figure S2). A COI-monohaplotype culture (PL-A strain; Acc. MK044803) was obtained from a single cocoon transferred and hatched on a 1% molecular grade agarose plate. Juvenile specimens were then relocated to defaunized soil and maintained as described earlier. The genetic purity of the established culture was confirmed by amplifying and sequencing the COI gene fragment.

4.2. RNA-Seq Data Generation for the E. albidus PL-A Strain

In the preliminary study, the number of *E. albidus* specimens required for obtaining an optimal amount of RNA was experimentally determined by extracting RNA from one to five specimens per sample using the GeneMATRIX Universal RNA Purification Kit (EURx, Gdańsk, Poland), following the manufacturer's protocol. The concentration and quality of the isolated RNA were assessed using a NanoDrop 2000 (NanoDrop Technologies, Wilmington, DE, USA). Additionally, cDNA was synthesized by reverse-transcribing half a microgram of RNA, primed with oligo(dT)$_{20}$, according to the instructions provided with the NG dART RT kit (EURx). Control PCR was conducted for proper nucleic acid purification, targeting the coding sequence of α-amylase I from *E. albidus* (Acc. OQ830662; [39]). Each PCR mixture, with a total volume of 50 µL, consisted of EURx Color OptiTaq PCR Master Mix (2×) (final concentration: 1.25 U OptiTaq DNA Polymerase, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP), 0.2 µM forward AmyStrF (5′-ATGCTGTCACTGATTGTGTTTTGTC-3′) and reverse AmyEndR (5′-TCAGACATGTAGAGCAATCATGG-3′) primers, and 1 µL of cDNA as the template. The amplification thermal profile was set as follows: an initial denaturation at 95 °C for 260 s, followed by 35 cycles of denaturation at 95 °C for 40 s, annealing at 45 °C for 45 s, and extension at 72 °C for 60 s, with a final extension at 72 °C for 120 s. To confirm amplification, the PCR products were run on a 1.2% agarose gel in TBE buffer with the addition of SimplySafe (EURx).

Adult specimens of the *E. albidus* PL-A strain in live form, on agarose plates, were dispatched to A&A Biotechnology (Gdańsk, Poland) for the extraction of RNA. The extraction procedure involved the use of the Total RNA Mini Kit with DNase treatment (A&A Biotechnology) and was conducted on a pooled sample of four adult specimens. The quality/concentration of extracted RNA was analyzed by agarose gel electrophoresis and by the NanoDrop 2000. To generate RNA-Seq reads, RNA samples were sent to Macrogen Europe (Amsterdam, The Netherlands) via A&A Biotechnology. The cDNA library was prepared using the TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA, USA). Subsequently, paired-end sequencing was performed on the Illumina platform (NovaSeq 6000; 2 × 151 bp reads).

4.3. Transcriptome De Novo Assembly and Data Annotation

Sequence quality control of all raw reads was performed using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/, accessed on 21 March 2024). The removal of adapters and quality trimming were executed using the BBDuk plugin in Geneious Prime version 2023.2.1. The settings used were as follows: adapter trimming (default settings),

partial adapter trimming from ends with a kmer length of 11, low-quality trimming at both ends with a minimum quality of 20, and adapter trimming based on paired read overhangs with a minimum overlap of 24. It is important to note that we experimented with two quality values for trimming low-quality ends, specifically, scores of 20 or 24 using Phred33. However, we found that a quality value of 24 was overly restrictive, consequently hindering the assembly's effectiveness in recovering some of the digestive enzyme gene transcripts.

In addition to the generated *E. albidus* PL-A strain transcriptomics data, we retrieved raw reads data (Illumina HiSeq 2500 runs) related to the freeze-tolerant German (G) and Greenlandic (N) strains of the same species from the NCBI Sequence Read Archive (SRA: SRP108369). Moreover, we assembled and assessed transcriptomics data available in the Sequence Read Archive (SRA) repository for other clitellates, with special emphasis on enchytraeid species.

Each transcriptome was assembled separately using Trinity RNA-Seq [120,121] integrated in the OmicsBox suite version 3.0.30 using the default k-mer length settings. Assembled transcriptomes were tested for completeness using Benchmarking Universal Single-Copy Orthologs (BUSCO) [122] analysis against the metazoan database, using a Blast e-value threshold of 1×10^{-5}. Transcriptomes were further processed using TransDecoder (http://transdecoder.github.io, accessed on 21 March 2024) with default settings to detect coding regions. TransDecoder-predicted ORFs were translated into amino acid sequences of at least 100 amino acids in length and annotated using a combination of the GhostKOALA/KofamKOALA automatic annotation and KEGG mapping service [123,124] and PANNZER2 (http://ekhidna2.biocenter.helsinki.fi, accessed on 21 March 2024) [125]. The functional annotation included KO (KEGG Orthology) assignment, KEGG pathway mapping, and prediction of gene ontology (GO) terms. Transcriptome decontamination was carried out by removing non-animal-originating KEGG-annotated sequences with the use of the QIIME filter fasta script [126] on the Galaxy platform [127]. The obtained clean data were screened for hydrolases—more specifically, glycosidases, peptidases, and lipases.

4.4. In Silico Analysis of Annotated Data

The annotated sequences were analyzed by several bioinformatics tools. Sequence similarity searches were conducted using BLASTp [128]. Prediction of signal peptides was performed with SignalP 6.0 [129]. The potential subcellular localization was carried out by DeepLoc 2.0 [130] and BUSCA (Bologna Unified Subcellular Component Annotator) [131]. Transmembrane domains were predicted using DeepTMHMM [132]. Glycosylphosphatidylinositol anchoring was predicted by NetGPI 1.1 [133]. Furthermore, protein domain architectures were predicted using InterProScan [134] and SMART [135]. For glycoside hydrolase (GH) family assignment, especially in complex cases, the web server for dbCAN3, an automated carbohydrate-active enzyme and substrate annotation tool (https://bcb.unl.edu/dbCAN2/index.php, accessed on 21 March 2024), was used with at least three available run tools [136]. For lipases, an additional HMMs search in the PANTHER [137] library version 18.0 was performed.

4.5. Additional Data and Phylogenetic Analyses

Sequences recovered from *E. albidus* were supplemented with sequences obtained from the GenBank database and the SRA repository. For the latter, additional transcriptomics data were assembled de novo for other annelids, encompassing all enchytraeid species referenced in Table 1. Sequencing run IDs (SRR) used for the additional data assembly are provided in Supplementary Table S3.

Homologous sequences in GenBank were identified through a BLASTp search. The protein sequences were aligned using either MAFFT 7 [138] with an automatic assignment of the alignment strategy or MUSCLE [139], depending on the dataset. The resulting alignments were visually inspected for accuracy. A web server version of IQ-TREE was employed to estimate the best-fitting model of amino acid evolution and subsequently construct a maximum likelihood tree. All trees were built using the model suggested by

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IQ-TREE, with 1000 replications. Ultrafast Bootstrap (UFBoot) and SH-like Approximate Likelihood Ratio Test (SH-aLRT) support values were calculated using 1000 replicates with default settings. The generated trees were rooted according to the previously proposed phylogenetic hypothesis for Clitellata [55] and visualized using iTOL [140].

4.6. Sequence Analysis, Protein Modeling, Structural Alignment, and Visualization

Evolutionary divergence between sequences was assessed through the pairwise distance method with the Poisson correction model in MEGA7 [141]. The ratio of nonsynonymous to synonymous substitutions (dN/dS) was computed using the CodeML program in the PAML 4.9 package [142], on the Galaxy platform [127]. The 3D structure of the proteins of interest was modeled using AlphaFold2/DeepMindv0.2 [143] on the Superbio.ai platform (https://www.superbio.ai, accessed on 21 March 2024) or via homology-based modeling using SWISS-MODEL [144]. The quality of the models was evaluated using pLDDT confidence scores and SWISS-MODEL structure assessment methods (GMQE, QMEANDisCo, and QMEAN Z-scores), respectively. Secondary structure alignments were initially created using ESPript [145] and modified according to the predicted structure by implementing the DSSP 2.0 algorithm in Jmol within FirstGlance in Jmol version 4.1 (http://firstglance.jmol.org, accessed on 21 March 2024). Figures of the tertiary structure of proteins were rendered with the same tool.

5. Conclusions

Based on RNA-Seq data, we identified cellulolytic enzymes (endo-β-1,4-glucanases) and enzymes engaged in the digestion of microorganisms (i-type lysozymes and two chitinases) in *Enchytraeus albidus*. Thus, *E. albidus* combines traits of both primary and secondary decomposers and is defined as an intermediate type of decomposer. Through phylogenetic and bioinformatic analyses, it was determined that the endo-β-1,4-glucanases in *E. albidus* share homology with those previously described in a few species of earthworms. These GH9 cellulases were also found in transcriptomics data of other clitellates, predominantly enchytraeids and earthworms. Closely homologous sequences to *Enchytraeus*–*Eisenia*-type destabilase-lysozyme, which contains the SH3b domain, were identified in transcriptomics data from other clitellates as well. The presence of close orthologs of the *Enchytraeus*–*Eisenia*-type SH3b-domain-containing i-type lysozyme is a potential molecular marker of bacterivory in clitellates. Our study demonstrates that RNA-Seq, even with a single sample but with sufficiently deep sequencing and taxonomically well-characterized input, could be a powerful and cost-effective tool, yet it is surprisingly rarely used in trophic ecology studies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms25094685/s1.

Author Contributions: Conceptualization, Ł.G.; Methodology, Ł.G.; Software, Ł.G. and A.D.-G.; Validation, Ł.G.; Formal analysis, Ł.G.; Investigation, Ł.G.; Resources, P.Ś.; Data curation, Ł.G.; Writing—original draft, Ł.G.; Writing—review & editing, Ł.G., A.D.-G. and P.Ś.; Visualization, Ł.G.; Supervision, A.D.-G. and P.Ś. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding, and was carried out as part of the statutory activities of the University of Silesia in Katowice.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data underlying this article are available in Supplementary Materials. Raw RNA-Seq reads generated for a series of studies, including this study, were deposited in the Sequence Read Archive (SRA) at NCBI under accession number SRR24185061. Details are available under BioProject PRJNA956247. Endo-β-1,4-glucanase and destabilase-lysozyme coding sequences predicted for the *E. albidus* PL-A strain from RNA-Seq data were deposited in the GenBank database

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under accession numbers PP480665 (Ef-Eg I), PP484683-PP484684 (Ef-Eg II), and PP488544-PP488545 (Ealb-iLys).

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Anderson, J.M. The Enigma of Soil Animal Species Diversity. In *Progress in Soil Zoology*; Vaněk, J., Ed.; Springer: Dordrecht, The Netherlands, 1975; pp. 51–58. ISBN 978-94-010-1935-4.
2. Nielsen, U.N.; Osler, G.H.R.; Campbell, C.D.; Neilson, R.; Burslem, D.F.R.P.; Van Der Wal, R. The Enigma of Soil Animal Species Diversity Revisited: The Role of Small-Scale Heterogeneity. *PLoS ONE* **2010**, *5*, e11567. [CrossRef]
3. Thakur, M.P.; Phillips, H.R.P.; Brose, U.; De Vries, F.T.; Lavelle, P.; Loreau, M.; Mathieu, J.; Mulder, C.; Van Der Putten, W.H.; Rillig, M.C.; et al. Towards an Integrative Understanding of Soil Biodiversity. *Biol. Rev.* **2020**, *95*, 350–364. [CrossRef] [PubMed]
4. Persson, L.; Diehl, S.; Johansson, L.; Andersson, G.; Hamrin, S.F. Trophic Interactions in Temperate Lake Ecosystems: A Test of Food Chain Theory. *Am. Nat.* **1992**, *140*, 59–84. [CrossRef]
5. Vander Zanden, M.J.; Shuter, B.J.; Lester, N.; Rasmussen, J.B. Patterns of Food Chain Length in Lakes: A Stable Isotope Study. *Am. Nat.* **1999**, *154*, 406–416. [CrossRef]
6. Briones, M.J.I. Soil Fauna and Soil Functions: A Jigsaw Puzzle. *Front. Environ. Sci.* **2014**, *2*, 7. [CrossRef]
7. Potapov, A.M.; Pollierer, M.M.; Salmon, S.; Šustr, V.; Chen, T. Multidimensional Trophic Niche Revealed by Complementary Approaches: Gut Content, Digestive Enzymes, Fatty Acids and Stable Isotopes in Collembola. *J. Anim. Ecol.* **2021**, *90*, 1919–1933. [CrossRef] [PubMed]
8. Maraun, M.; Thomas, T.; Fast, E.; Treibert, N.; Caruso, T.; Schaefer, I.; Lu, J.-Z.; Scheu, S. New Perspectives on Soil Animal Trophic Ecology through the Lens of C and N Stable Isotope Ratios of Oribatid Mites. *Soil Biol. Biochem.* **2023**, *177*, 108890. [CrossRef]
9. Potapov, A.M.; Beaulieu, F.; Birkhofer, K.; Bluhm, S.L.; Degtyarev, M.I.; Devetter, M.; Goncharov, A.A.; Gongalsky, K.B.; Klarner, B.; Korobushkin, D.I.; et al. Feeding Habits and Multifunctional Classification of Soil-associated Consumers from Protists to Vertebrates. *Biol. Rev.* **2022**, *97*, 1057–1117. [CrossRef]
10. Uhlik, O.; Leewis, M.-C.; Strejcek, M.; Musilova, L.; Mackova, M.; Leigh, M.B.; Macek, T. Stable Isotope Probing in the Metagenomics Era: A Bridge towards Improved Bioremediation. *Biotechnol. Adv.* **2013**, *31*, 154–165. [CrossRef]
11. Schmidt, O.; Curry, J.P.; Dyckmans, J.; Rota, E.; Scrimgeour, C.M. Dual Stable Isotope Analysis (δ^{13}C and δ^{15}N) of Soil Invertebrates and Their Food Sources. *Pedobiologia* **2004**, *48*, 171–180. [CrossRef]
12. Scheu, S. The Soil Food Web: Structure and Perspectives. *Eur. J. Soil Biol.* **2002**, *38*, 11–20. [CrossRef]
13. Krištůfek, V.; Fischer, S.; Bührmann, J.; Zeltins, A.; Schrempf, H. In Situ Monitoring of Chitin Degradation by *Streptomyces lividans* pCHIO12 within *Enchytraeus crypticus* (Oligochaeta) Feeding on *Aspergillus proliferans*. *FEMS Microbiol. Ecol.* **1999**, *28*, 41–48. [CrossRef]
14. Herrera, L.M.; García-Laviña, C.X.; Marizcurrena, J.J.; Volonterio, O.; De León, R.P.; Castro-Sowinski, S. Hydrolytic Enzyme-Producing Microbes in the Antarctic Oligochaete *Grania* sp. (Annelida). *Polar Biol.* **2017**, *40*, 947–953. [CrossRef]
15. Scheu, S.; Falca, M. The Soil Food Web of Two Beech Forests (*Fagus sylvatica*) of Contrasting Humus Type: Stable Isotope Analysis of a Macro- and a Mesofauna-Dominated Community. *Oecologia* **2000**, *123*, 285–296. [CrossRef]
16. Crotty, F.V.; Blackshaw, R.P.; Murray, P.J. Tracking the Flow of Bacterially Derived ^{13}C and ^{15}N through Soil Faunal Feeding Channels. *Rapid Commun. Mass Spectrom.* **2011**, *25*, 1503–1513. [CrossRef]
17. Puppe, D.; Schrader, S.; Giesemann, A.; Gebauer, G. Isotopic Labelling of Enchytraeids under FACE Conditions: A Possible Way to Analyse the Residue-Enchytraeid-Soil System Considering Elevated Atmospheric CO_2 Concentrations. *Landbauforsch. VTI Agric. For. Res. Spec. Issue* **2012**, *357*, 21–26.
18. Gajda, Ł.; Gorgoń, S.; Urbisz, A.Z. Food Preferences of Enchytraeids. *Pedobiologia* **2017**, *63*, 19–36. [CrossRef]
19. Schmelz, R.M.; Beylich, A.; Boros, G.; Dózsa-Farkas, K.; Graefe, U.; Hong, Y.; Römbke, J.; Schlaghamersky, J.; Martinsson, S. How to Deal with Cryptic Species in Enchytraeidae, with Recommendations on Taxonomical Descriptions. *Opusc. Zool.* **2017**, *48*, 45–51. [CrossRef]
20. Erséus, C.; Klinth, M.J.; Rota, E.; De Wit, P.; Gustafsson, D.R.; Martinsson, S. The Popular Model Annelid *Enchytraeus albidus* Is Only One Species in a Complex of Seashore White Worms (Clitellata, Enchytraeidae). *Org. Divers. Evol.* **2019**, *19*, 105–133. [CrossRef]
21. Nagy, H.; Dózsa-Farkas, K.; Felföldi, T. New Insights into the *Enchytraeus albidus* Complex (Annelida, Enchytraeidae), with the Description of Three New Species from Seashores in Italy and Croatia. *Eur. J. Taxon.* **2023**, *870*, 107–145. [CrossRef]
22. Cabrol, J.; Winkler, G.; Tremblay, R. Physiological Condition and Differential Feeding Behaviour in the Cryptic Species Complex *Eurytemora affinis* in the St Lawrence Estuary. *J. Plankton Res.* **2015**, *37*, 372–387. [CrossRef]
23. Martinsson, S.; Rota, E.; Erséus, C. Revision of *Cognettia* (Clitellata, Enchytraeidae): Re-Establishment of *Chamaedrilus* and Description of Cryptic Species in the *sphagnetorum* Complex. *Syst. Biodivers.* **2015**, *13*, 257–277. [CrossRef]
24. O'Connor, F.B. The Enchytraeidae. In *Soil Biology*; Elsevier: Amsterdam, The Netherlands, 1967; pp. 213–257. ISBN 978-0-12-395699-6.
25. Schmelz, R.M.; Collado, R. A Guide to European Terrestrial and Freshwater Species of Enchytraeidae (Oligochaeta). *Soil Org.* **2010**, *82*, 1–176.

26. Coates, K.A. Redescriptions of *Aspidodrilus* and *Pelmatodrilus*, Enchytraeids (Annelida, Oligochaeta) Ectocommensal on Earthworms. *Can. J. Zool.* **1990**, *68*, 498–505. [CrossRef]
27. Page, T.M.; Lawley, J.W. The Next Generation Is Here: A Review of Transcriptomic Approaches in Marine Ecology. *Front. Mar. Sci.* **2022**, *9*, 757921. [CrossRef]
28. Dammannagoda, L.K.; Pavasovic, A.; Prentis, P.J.; Hurwood, D.A.; Mather, P.B. Expression and Characterization of Digestive Enzyme Genes from Hepatopancreatic Transcripts from Redclaw Crayfish (*Cherax quadricarinatus*). *Aquac. Nutr.* **2015**, *21*, 868–880. [CrossRef]
29. Wei, J.; Zhang, X.; Yu, Y.; Li, F.; Xiang, J. RNA-Seq Reveals the Dynamic and Diverse Features of Digestive Enzymes during Early Development of Pacific White Shrimp *Litopenaeus vannamei*. *Comp. Biochem. Physiol. Part D Genom. Proteom.* **2014**, *11*, 37–44. [CrossRef]
30. Li, Y.; Xue, H.; Li, X. Transcriptome Analysis of the Chinese Grass Shrimp *Palaemonetes sinensis* (Sollaud 1911) and Its Predicted Feeding Habit. *J. Oceanol. Limnol.* **2018**, *36*, 1778–1787. [CrossRef]
31. Heras, J.; Chakraborty, M.; Emerson, J.J.; German, D.P. Genomic and Biochemical Evidence of Dietary Adaptation in a Marine Herbivorous Fish. *Proc. R. Soc. B Biol. Sci.* **2020**, *287*, 20192327. [CrossRef]
32. Van Lommel, J.; Holtof, M.; Tilleman, L.; Cools, D.; Vansteenkiste, S.; Polgun, D.; Verdonck, R.; Van Nieuwerburgh, F.; Vanden Broeck, J. Post-Feeding Transcriptomics Reveals Essential Genes Expressed in the Midgut of the Desert Locust. *Front. Physiol.* **2023**, *14*, 1232545. [CrossRef]
33. Geng, A.; Cheng, Y.; Wang, Y.; Zhu, D.; Le, Y.; Wu, J.; Xie, R.; Yuan, J.S.; Sun, J. Transcriptome Analysis of the Digestive System of a Wood-Feeding Termite (*Coptotermes formosanus*) Revealed a Unique Mechanism for Effective Biomass Degradation. *Biotechnol. Biofuels* **2018**, *11*, 24. [CrossRef] [PubMed]
34. de Boer, T.E.; Roelofs, D.; Vooijs, R.; Holmstrup, M.; Amorim, M.J. Population-Specific Transcriptional Differences Associated with Freeze Tolerance in a Terrestrial Worm. *Ecol. Evol.* **2018**, *8*, 3774–3786. [CrossRef]
35. McCarthy, S.D.; Dugon, M.M.; Power, A.M. 'Degraded' RNA Profiles in Arthropoda and Beyond. *PeerJ* **2015**, *3*, e1436. [CrossRef]
36. Natsidis, P.; Schiffer, P.H.; Salvador-Martínez, I.; Telford, M.J. Computational Discovery of Hidden Breaks in 28S Ribosomal RNAs across Eukaryotes and Consequences for RNA Integrity Numbers. *Sci. Rep.* **2019**, *9*, 19477. [CrossRef]
37. Nielsen, C.O. Carbohydrases in Soil and Litter Invertebrates. *Oikos* **1962**, *13*, 200. [CrossRef]
38. Prentø, P. Blood Sugar, Sugar Metabolism and Related Enzymes in the Earthworm, *Lumbricus terrestris* L. *Comp. Biochem. Physiol. Part B Comp. Biochem.* **1987**, *86*, 333–341. [CrossRef]
39. Gajda, Ł.; Daszkowska-Golec, A.; Świątek, P. Discovery and Characterization of the α-Amylases cDNAs from *Enchytraeus albidus* Shed Light on the Evolution of "*Enchytraeus-Eisenia* Type" Amy Homologs in Annelida. *Biochimie* **2024**, *221*, 38–59. [CrossRef]
40. Nozaki, M.; Miura, C.; Tozawa, Y.; Miura, T. The Contribution of Endogenous Cellulase to the Cellulose Digestion in the Gut of Earthworm (*Pheretima hilgendorfi*: Megascolecidae). *Soil Biol. Biochem.* **2009**, *41*, 762–769. [CrossRef]
41. Ueda, M.; Ito, A.; Nakazawa, M.; Miyatake, K.; Sakaguchi, M.; Inouye, K. Cloning and Expression of the Cold-Adapted Endo-1,4-β-Glucanase Gene from *Eisenia fetida*. *Carbohydr. Polym.* **2014**, *101*, 511–516. [CrossRef]
42. Park, I.Y.; Cha, J.R.; Ok, S.-M.; Shin, C.; Kim, J.-S.; Kwak, H.-J.; Yu, Y.-S.; Kim, Y.-K.; Medina, B.; Cho, S.-J.; et al. A New Earthworm Cellulase and Its Possible Role in the Innate Immunity. *Dev. Comp. Immunol.* **2017**, *67*, 476–480. [CrossRef]
43. Ito, K.; Nozaki, M.; Ohta, T.; Miura, C.; Tozawa, Y.; Miura, T. Differences of Two Polychaete Species Reflected in Enzyme Activities. *Mar. Biol.* **2011**, *158*, 1211–1221. [CrossRef]
44. Kim, D.; Kim, J.-S.; Park, I.-Y.; Kwak, H.-J.; Lee, D.H.; Cho, S.-J.; Park, S.C. A Novel Chitinase from the Earthworm, *Eisenia andrei*. *Anim. Cells Syst.* **2016**, *20*, 48–51. [CrossRef]
45. Ueda, M.; Shioyama, T.; Nakadoi, K.; Nakazawa, M.; Sakamoto, T.; Iwamoto, T.; Sakaguchi, M. Cloning and Expression of a Chitinase Gene from *Eisenia fetida*. *Int. J. Biol. Macromol.* **2017**, *104*, 1648–1655. [CrossRef]
46. Yu, Y.-S.; Lee, J.-Y.; Woo, J.-W.; Kim, J.-S.; Bae, Y.-H.; Cho, S.-J.; Park, S.C. Identification and Expression Pattern of a New Digestive Invertebrate-Type Lysozyme from the Earthworm. *Genes Genom.* **2019**, *41*, 367–371. [CrossRef]
47. Wohlkönig, A.; Huet, J.; Looze, Y.; Wintjens, R. Structural Relationships in the Lysozyme Superfamily: Significant Evidence for Glycoside Hydrolase Signature Motifs. *PLoS ONE* **2010**, *5*, e15388. [CrossRef]
48. Ueda, M.; Hirano, Y.; Fukuhara, H.; Naka, Y.; Nakazawa, M.; Sakamoto, T.; Ogata, Y.; Tamada, T. Gene Cloning, Expression, and X-Ray Crystallographic Analysis of a β-Mannanase from *Eisenia fetida*. *Enzyme Microb. Technol.* **2018**, *117*, 15–22. [CrossRef]
49. Nakajima, N.; Sugimoto, M.; Ishihara, K. Earthworm-Serine Protease: Characterization, Molecular Cloning, and Application of the Catalytic Functions. *J. Mol. Catal. B Enzym.* **2003**, *23*, 191–212. [CrossRef]
50. Pan, R.; Zhang, Z.-J.; He, R.-Q. Earthworm Protease. *Appl. Environ. Soil Sci.* **2010**, *2010*, 294258. [CrossRef]
51. Ordoñez-Arévalo, B.; Guillén-Navarro, K.; Huerta, E.; Cuevas, R.; Calixto-Romo, M.A. Enzymatic Dynamics into the *Eisenia fetida* (Savigny, 1826) Gut during Vermicomposting of Coffee Husk and Market Waste in a Tropical Environment. *Environ. Sci. Pollut. Res.* **2018**, *25*, 1576–1586. [CrossRef]
52. Wang, K.Y.; Tull, L.; Cooper, E.; Wang, N.; Liu, D. Recombinant Protein Production of Earthworm Lumbrokinase for Potential Antithrombotic Application. *Evid. Based Complement. Alternat. Med.* **2013**, *2013*, 783971. [CrossRef]
53. Wu, J.X.; Zhao, X.Y.; Pan, R.; He, R.Q. Glycosylated Trypsin-like Proteases from Earthworm *Eisenia fetida*. *Int. J. Biol. Macromol.* **2007**, *40*, 399–406. [CrossRef]

115:4094442388

54. Kulminskaya, N.; Oberer, M. Protein-Protein Interactions Regulate the Activity of Adipose Triglyceride Lipase in Intracellular Lipolysis. *Biochimie* **2020**, *169*, 62–68. [CrossRef]
55. Erséus, C.; Williams, B.W.; Horn, K.M.; Halanych, K.M.; Santos, S.R.; James, S.W.; Creuzé des Châtelliers, M.; Anderson, F.E. Phylogenomic Analyses Reveal a Palaeozoic Radiation and Support a Freshwater Origin for Clitellate Annelids. *Zool. Scr.* **2020**, *49*, 614–640. [CrossRef]
56. Amorim, M.J.; Gansemans, Y.; Gomes, S.I.; Van Nieuwerburgh, F.; Scott-Fordsmand, J.J. Annelid Genomes: *Enchytraeus crypticus*, a Soil Model for the Innate (and Primed) Immune System. *Lab Anim.* **2021**, *50*, 285–294. [CrossRef]
57. Marin, E.; Kornilov, D.A.; Bukhdruker, S.S.; Aleksenko, V.A.; Manuvera, V.A.; Zinovev, E.V.; Kovalev, K.V.; Shevtsov, M.B.; Talyzina, A.A.; Bobrovsky, P.A.; et al. Structural Insights into Thrombolytic Activity of Destabilase from Medicinal Leech. *Sci. Rep.* **2023**, *13*, 6641. [CrossRef]
58. Callewaert, L.; Michiels, C.W. Lysozymes in the Animal Kingdom. *J. Biosci.* **2010**, *35*, 127–160. [CrossRef]
59. Van Herreweghe, J.M.; Michiels, C.W. Invertebrate Lysozymes: Diversity and Distribution, Molecular Mechanism and in Vivo Function. *J. Biosci.* **2012**, *37*, 327–348. [CrossRef]
60. Craig, D.B.; Dombkowski, A.A. Disulfide by Design 2.0: A Web-Based Tool for Disulfide Engineering in Proteins. *BMC Bioinform.* **2013**, *14*, 346. [CrossRef]
61. Ren, Q.; Qi, Y.-L.; Hui, K.-M.; Zhang, Z.; Zhang, C.-Y.; Wang, W. Four Invertebrate-Type Lysozyme Genes from Triangle-Shell Pearl Mussel (*Hyriopsis cumingii*). *Fish Shellfish Immunol.* **2012**, *33*, 909–915. [CrossRef] [PubMed]
62. Satake, H.; Takuwa, K.; Minakata, H.; Matsushima, O. Evidence for Conservation of the Vasopressin/Oxytocin Superfamily in Annelida. *J. Biol. Chem.* **1999**, *274*, 5605–5611. [CrossRef] [PubMed]
63. Arimori, T.; Ito, A.; Nakazawa, M.; Ueda, M.; Tamada, T. Crystal Structure of Endo-1,4-β-Glucanase from *Eisenia fetida*. *J. Synchrotron Radiat.* **2013**, *20*, 884–889. [CrossRef] [PubMed]
64. Linton, S.M. Review: The Structure and Function of Cellulase (Endo-β-1,4-Glucanase) and Hemicellulase (β-1,3-Glucanase and Endo-β-1,4-Mannase) Enzymes in Invertebrates That Consume Materials Ranging from Microbes, Algae to Leaf Litter. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **2020**, *240*, 110354. [CrossRef] [PubMed]
65. Khademi, S.; Guarino, L.A.; Watanabe, H.; Tokuda, G.; Meyer, E.F. Structure of an Endoglucanase from Termite, *Nasutitermes takasagoensis*. *Acta Crystallogr. D Biol. Crystallogr.* **2002**, *58*, 653–659. [CrossRef] [PubMed]
66. Cichocka, J.M.; Bielecki, A.; Świątek, P.; Jabłońska-Barna, I.; Kobak, J.; Hildebrand, J.; Dmitryjuk, M.; Strużyński, W.; Rost-Roszkowska, M. The Activity of Hydrolytic Enzymes in the Digestive System of Acanthobdellida, Branchiobdellida and Hirudinida (Annelida, Clitellata)—Considerations on Similarity and Phylogeny. *Eur. Zool. J.* **2021**, *88*, 26–43. [CrossRef]
67. Terra, W.R.; Ferreira, C. Biochemistry and Molecular Biology of Digestion. In *Insect Molecular Biology and Biochemistry*; Elsevier: Amsterdam, The Netherlands, 2012; pp. 365–418. ISBN 978-0-12-384747-8.
68. Chen, J.; Li, Z.; Lin, B.; Liao, J.; Zhuo, K. A *Meloidogyne graminicola* Pectate Lyase Is Involved in Virulence and Activation of Host Defense Responses. *Front. Plant Sci.* **2021**, *12*, 651627. [CrossRef] [PubMed]
69. Steinmetz, P.R.H. A Non-Bilaterian Perspective on the Development and Evolution of Animal Digestive Systems. *Cell Tissue Res.* **2019**, *377*, 321–339. [CrossRef] [PubMed]
70. Espinoza-Fuentes, F.P.; Ribeiro, A.F.; Terra, W.R. Microvillar and Secreted Digestive Enzymes from *Musca domestica* Larvae. Subcellular Fractionation of Midgut Cells with Electron Microscopy Monitoring. *Insect Biochem.* **1987**, *17*, 819–827. [CrossRef]
71. Lyons, P.J.; Fricker, L.D. Carboxypeptidase O Is a Glycosylphosphatidylinositol-Anchored Intestinal Peptidase with Acidic Amino Acid Specificity. *J. Biol. Chem.* **2011**, *286*, 39023–39032. [CrossRef] [PubMed]
72. Danielsen, E.M.; Hansen, G.H. Lipid Rafts in Epithelial Brush Borders: Atypical Membrane Microdomains with Specialized Functions. *Biochim. Biophys. Acta BBA—Biomembr.* **2003**, *1617*, 1–9. [CrossRef] [PubMed]
73. Hooton, D.; Lentle, R.; Monro, J.; Wickham, M.; Simpson, R. The Secretion and Action of Brush Border Enzymes in the Mammalian Small Intestine. In *Reviews of Physiology, Biochemistry and Pharmacology*; Nilius, B., Gudermann, T., Jahn, R., Lill, R., Petersen, O.H., De Tombe, P.P., Eds.; Springer International Publishing: Cham, Switzerland, 2015; Volume 168, pp. 59–118. ISBN 978-3-319-22502-9.
74. Lobo-da-Cunha, A.; Casartelli, M.; Tettamanti, G. Editorial: Molecular Physiology of Invertebrate Digestive System. *Front. Physiol.* **2023**, *14*, 1304915. [CrossRef]
75. Tsukamoto, K.; Ariki, S.; Nakazawa, M.; Sakamoto, T.; Ueda, M. Novel Cold-Adapted Raw-Starch Digesting α-Amylases from *Eisenia fetida*: Gene Cloning, Expression, and Characterization. *Biotechnol. Rep.* **2021**, *31*, e00662. [CrossRef] [PubMed]
76. Kuroki, C.; Hirano, Y.; Nakazawa, M.; Sakamoto, T.; Tamada, T.; Ueda, M. A Single Mutation Asp43Arg Was Increased 2.5-Fold the Catalytic Activity and Maintained the Stability of Cold-Adapted Endo-1,4-Beta Glucanase (Ef-EG2) from *Eisenia fetida*. *Curr. Res. Biotechnol.* **2023**, *5*, 100126. [CrossRef]
77. Chen, Y.; Liu, Y.; Zhang, J.; Zhou, K.; Zhang, X.; Dai, H.; Yang, B.; Shang, H. Efficacy and Safety of Lumbrokinase plus Aspirin versus Aspirin Alone for Acute Ischemic Stroke (LUCENT): Study Protocol for a Multicenter Randomized Controlled Trial. *Trials* **2022**, *23*, 285. [CrossRef]
78. Guo, L.-Y.; Guo, Q.-S.; Shi, H.-Z.; Yang, F.; Miao, Y.-X. Cloning and Expression Analysis of the *HSL* Gene in *Whitmania pigra* (Annelida: Hirudinea). *Invertebr. Reprod. Dev.* **2022**, *66*, 59–66. [CrossRef]
79. Illig, J.; Langel, R.; Norton, R.A.; Scheu, S.; Maraun, M. Where Are the Decomposers? Uncovering the Soil Food Web of a Tropical Montane Rain Forest in Southern Ecuador Using Stable Isotopes (^{15}N). *J. Trop. Ecol.* **2005**, *21*, 589–593. [CrossRef]

116:8364664301

80. Dash, M.C.; Nanda, B.; Mishra, P.C. Digestive Enzymes in Three Species of Enchytraeidae (Oligochaeta). *Oikos* **1981**, *36*, 316. [CrossRef]
81. Urbasek, F.; Chalupsky Jr, J. Activity of Digestive Enzymes in 4 Species of Enchytraeidae (Oligochaeta). *Rev. Ecol. Biol. Sol* **1991**, *28*, 145–154.
82. Sustr, V.; Chalupsky, J. Activity of Digestive Enzymes in Two Species of Potworms (Oligochaeta, Enchytraeidae). *Pedobiologia* **1996**, *40*, 255–259. [CrossRef]
83. Reichert, A.; Mothes-Wagner, U.; Seitz, K.-A. Ecohistological Investigation of the Feeding Behaviour of the Enchytraeid *Enchytraeus coronatus* (Annelida, Oligochaeta). *Pedobiologia* **1996**, *40*, 118–133. [CrossRef]
84. Belato, F.A.; Schrago, C.G.; Coates, C.J.; Halanych, K.M.; Costa-Paiva, E.M. Newly Discovered Occurrences and Gene Tree of the Extracellular Globins and Linker Chains from the Giant Hexagonal Bilayer Hemoglobin in Metazoans. *Genome Biol. Evol.* **2019**, *11*, 597–612. [CrossRef]
85. Kim, S.; Jeon, D.; Lee, J.-Y.; Cho, S.-J.; Lim, Y.; Eyun, S.; Park, S.C.; Seo, Y.-J. Upregulation of Cellulase Activity and mRNA Levels by Bacterial Challenge in the Earthworm *Eisenia andrei*, Supporting the Involvement of Cellulases in Innate Immunity. *Biochem. Biophys. Res. Commun.* **2020**, *521*, 15–18. [CrossRef] [PubMed]
86. Smant, G.; Stokkermans, J.P.W.G.; Yan, Y.; De Boer, J.M.; Baum, T.J.; Wang, X.; Hussey, R.S.; Gommers, F.J.; Henrissat, B.; Davis, E.L.; et al. Endogenous Cellulases in Animals: Isolation of β-1,4-Endoglucanase Genes from Two Species of Plant-Parasitic Cyst Nematodes. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 4906–4911. [CrossRef] [PubMed]
87. Suzuki, K.; Ojima, T.; Nishita, K. Purification and cDNA Cloning of a Cellulase from Abalone *Haliotis discus hannai*. *Eur. J. Biochem.* **2003**, *270*, 771–778. [CrossRef] [PubMed]
88. Sakamoto, K.; Touhata, K.; Yamashita, M.; Kasai, A.; Toyohara, H. Cellulose Digestion by Common Japanese Freshwater Clam *Corbicula japonica*. *Fish. Sci.* **2007**, *73*, 675–683. [CrossRef]
89. Sakamoto, K.; Uji, S.; Kurokawa, T.; Toyohara, H. Immunohistochemical, in Situ Hybridization and Biochemical Studies on Endogenous Cellulase of *Corbicula japonica*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **2008**, *150*, 216–221. [CrossRef] [PubMed]
90. Akazawa, S.; Ikarashi, Y.; Yarimizu, J.; Yokoyama, K.; Kobayashi, T.; Nakazawa, H.; Ogasawara, W.; Morikawa, Y. Characterization of Two Endoglucanases for the Classification of the Earthworm, *Eisenia fetida* Waki. *Biosci. Biotechnol. Biochem.* **2016**, *80*, 55–66. [CrossRef] [PubMed]
91. Dougherty, E.C.; Solberg, B. Monoxenic Cultivation of an Enchytraeid Annelid. *Nature* **1960**, *186*, 1067–1068. [CrossRef]
92. Brockmeyer, V.; Schmid, R.; Westheide, W. Quantitative Investigations of the Food of Two Terrestrial Enchytraeid Species (Oligochaeta). *Pedobiologia* **1990**, *34*, 151–156. [CrossRef]
93. Larsen, T.; Ventura, M.; Maraldo, K.; Triadó-Margarit, X.; Casamayor, E.O.; Wang, Y.V.; Andersen, N.; O'Brien, D.M. The Dominant Detritus-feeding Invertebrate in Arctic Peat Soils Derives Its Essential Amino Acids from Gut Symbionts. *J. Anim. Ecol.* **2016**, *85*, 1275–1285. [CrossRef] [PubMed]
94. Krištůfek, V.; Hallmann, M.; Westheide, W.; Schrempf, H. Selection of Various *Streptomyces* Species by *Enchytraeus crypticus* (Oligochaeta). *Pedobiologia* **1995**, *39*, 547–554. [CrossRef]
95. Krištůfek, V.; Lukešová, A.; Nováková, A. Soil Microorganisms as Source of Food for Enchytraeids (Annelida, Enchytraeidae). In *Life in Soil*; Czechoslovak Society for Microbiology, Institute of Microbiology SAS Bratislava: Bratislava, Slovak Republic, 1997; pp. 41–42.
96. Krištůfek, V.; Nováková, A.; Pižl, V. Coprophilous Streptomycetes and Fungi—Food Sources for Enchytraeid Worms (Enchytraeidae). *Folia Microbiol.* **2001**, *46*, 555–558. [CrossRef] [PubMed]
97. Lukešová, A.; Frouz, J. Soil and Freshwater Micro-Algae as a Food Source for Invertebrates in Extreme Environments. In *Algae and Cyanobacteria in Extreme Environments*; Seckbach, J., Ed.; Cellular Origin, Life in Extreme Habitats and Astrobiology; Springer: Dordrecht, The Netherlands, 2007; Volume 11, pp. 265–284. ISBN 978-1-4020-6111-0.
98. Jaffee, B.A.; Muldoon, A.E.; Didden, W.A.M. Enchytraeids and Nematophagous Fungi in Soil Microcosms. *Biol. Fertil. Soils* **1997**, *25*, 382–388. [CrossRef]
99. Gelder, S.R. Diet and Histophysiology of the Alimentary Canal of *Lumbricillus lineatus* (Oligochaeta, Enchytraeidae). *Hydrobiologia* **1984**, *115*, 71–81. [CrossRef]
100. Moreti, R.; Perrella, N.N.; Lopes, A.R. Carbohydrate Digestion in Ticks and a Digestive α-L-Fucosidase. *J. Insect Physiol.* **2013**, *59*, 1069–1075. [CrossRef] [PubMed]
101. Moraes, C.S.; Lucena, S.A.; Moreira, B.H.S.; Brazil, R.P.; Gontijo, N.F.; Genta, F.A. Relationship between Digestive Enzymes and Food Habit of *Lutzomyia longipalpis* (Diptera: Psychodidae) Larvae: Characterization of Carbohydrases and Digestion of Microorganisms. *J. Insect Physiol.* **2012**, *58*, 1136–1145. [CrossRef] [PubMed]
102. Novo, M.; Riesgo, A.; Fernández-Guerra, A.; Giribet, G. Pheromone Evolution, Reproductive Genes, and Comparative Transcriptomics in Mediterranean Earthworms (Annelida, Oligochaeta, Hormogastridae). *Mol. Biol. Evol.* **2013**, *30*, 1614–1629. [CrossRef] [PubMed]
103. Moroz, O.V.; Blagova, E.; Lebedev, A.A.; Skov, L.K.; Pache, R.A.; Schnorr, K.M.; Kiemer, L.; Friis, E.P.; Nymand-Grarup, S.; Ming, L.; et al. Module Walking Using an SH3-like Cell-Wall-Binding Domain Leads to a New GH184 Family of Muramidases. *Acta Crystallogr. Sect. Struct. Biol.* **2023**, *79*, 706–720. [CrossRef] [PubMed]
104. Eisenhauer, N.; Schädler, M. Inconsistent Impacts of Decomposer Diversity on the Stability of Aboveground and Belowground Ecosystem Functions. *Oecologia* **2011**, *165*, 403–415. [CrossRef] [PubMed]

117:4571054542

105. Korobushkin, D.I.; Guseva, P.A.; Gongalsky, K.B.; Saifutdinov, R.A.; Zaitsev, A.S.; Degtyarev, M.I. Are There Different Trophic Niches of Enchytraeids? A Stable Isotopic (δ^{13}C, δ^{15}N) Evidence. *Soil Biol. Biochem.* **2024**, *194*, 109422. [CrossRef]
106. Korobushkin, D.I.; Zaitsev, A.S.; Degtyarev, M.I.; Danilova, M.A.; Filimonova, Z.V.; Guseva, P.A.; Pelgunova, L.A.; Pronina, N.A.; Tsurikov, S.M.; Vecherskii, M.V.; et al. Littoral Enchytraeids and *Eisenia fetida* Earthworms Facilitate Utilization of Marine Macroalgae as Biofertilizers. *Appl. Soil Ecol.* **2023**, *188*, 104882. [CrossRef]
107. Eisenhauer, N.; Butenschoen, O.; Radsick, S.; Scheu, S. Earthworms as Seedling Predators: Importance of Seeds and Seedlings for Earthworm Nutrition. *Soil Biol. Biochem.* **2010**, *42*, 1245–1252. [CrossRef]
108. Berteau, O.; McCort, I.; Goasdoue, N.; Tissot, B.; Daniel, R. Characterization of a New α-L-Fucosidase Isolated from the Marine Mollusk *Pecten maximus* That Catalyzes the Hydrolysis of α-L-Fucose from Algal Fucoidan (*Ascophyllum nodosum*). *Glycobiology* **2002**, *12*, 273–282. [CrossRef] [PubMed]
109. Bäumgen, M.; Dutschei, T.; Bornscheuer, U.T. Marine Polysaccharides: Occurrence, Enzymatic Degradation and Utilization. *ChemBioChem* **2021**, *22*, 2247–2256. [CrossRef]
110. Ude, J. Licht- Und Elektronenmikroskopische Untersuchung Des Septaldrüsensystems von *Pachydrilus lineatus* (Annelida-Oligochaeta). *Zool. Jb. Physiol. Bd.* **1977**, *81*, 42–82.
111. Mothes-Wagner, U.; Reichert, A.; Seitz, K.A. Functional Histology of the Enchytraeid *Enchytraeus coronatus* (Oligochaeta) Digestive Epithelium. *Pedobiologia* **1996**, *40*, 328–341. [CrossRef]
112. Ponge, J.F. Food Resources and Diets of Soil Animals in a Small Area of Scots Pine Litter. *Geoderma* **1991**, *49*, 33–62. [CrossRef]
113. Didden, W.A.M. Ecology of Terrestrial Enchytraeidae. *Pedobiologia* **1993**, *37*, 2–29. [CrossRef]
114. Mellin, A. Quantitative Feeding Activity of *Mesenchytraeus glandulosus* (Annelida, Oligochaeta). *Ökol. Naturschutz Im Agrar.* **1990**, *19*, 192–199.
115. Gravato-Nobre, M.J.; Vaz, F.; Filipe, S.; Chalmers, R.; Hodgkin, J. The Invertebrate Lysozyme Effector ILYS-3 Is Systemically Activated in Response to Danger Signals and Confers Antimicrobial Protection in *C. elegans*. *PLoS Pathog.* **2016**, *12*, e1005826. [CrossRef]
116. Liu, H.; Qi, B. Protocol for Investigating the Effect of Food Digestion in *C. elegans* on Development by Feeding the Inedible Bacteria *Staphylococcus saprophyticus*. *STAR Protoc.* **2023**, *4*, 101990. [CrossRef]
117. Geng, S.; Li, Q.; Zhou, X.; Zheng, J.; Liu, H.; Zeng, J.; Yang, R.; Fu, H.; Hao, F.; Feng, Q.; et al. Gut Commensal *E. coli* Outer Membrane Proteins Activate the Host Food Digestive System through Neural-Immune Communication. *Cell Host Microbe* **2022**, *30*, 1401–1416.e8. [CrossRef]
118. Hao, F.; Liu, H.; Qi, B. Bacterial Peptidoglycan as a Food Digestive Signal in the Nematode That Facilitates Adaptation of Animals in Nature. *bioRxiv*, 2023; *preprint*. [CrossRef]
119. Rakus, K.; Wiegertjes, G.; Adamek, M.; Bekh, V.; Stet, R.; Irnazarow, I. Application of PCR-RF-SSCP to Study Major Histocompatibility Class II B Polymorphism in Common Carp (*Cyprinus carpio* L.). *Fish Shellfish Immunol.* **2008**, *24*, 734–744. [CrossRef]
120. Grabherr, M.G.; Haas, B.J.; Yassour, M.; Levin, J.Z.; Thompson, D.A.; Amit, I.; Adiconis, X.; Fan, L.; Raychowdhury, R.; Zeng, Q.; et al. Full-Length Transcriptome Assembly from RNA-Seq Data without a Reference Genome. *Nat. Biotechnol.* **2011**, *29*, 644–652. [CrossRef]
121. Haas, B.J.; Papanicolaou, A.; Yassour, M.; Grabherr, M.; Blood, P.D.; Bowden, J.; Couger, M.B.; Eccles, D.; Li, B.; Lieber, M.; et al. De Novo Transcript Sequence Reconstruction from RNA-Seq Using the Trinity Platform for Reference Generation and Analysis. *Nat. Protoc.* **2013**, *8*, 1494–1512. [CrossRef]
122. Seppey, M.; Manni, M.; Zdobnov, E.M. BUSCO: Assessing Genome Assembly and Annotation Completeness. In *Gene Prediction*; Kollmar, M., Ed.; Methods in Molecular Biology; Springer: New York, NY, USA, 2019; Volume 1962, pp. 227–245. ISBN 978-1-4939-9172-3.
123. Kanehisa, M.; Sato, Y.; Morishima, K. BlastKOALA and GhostKOALA: KEGG Tools for Functional Characterization of Genome and Metagenome Sequences. *J. Mol. Biol.* **2016**, *428*, 726–731. [CrossRef]
124. Aramaki, T.; Blanc-Mathieu, R.; Endo, H.; Ohkubo, K.; Kanehisa, M.; Goto, S.; Ogata, H. KofamKOALA: KEGG Ortholog Assignment Based on Profile HMM and Adaptive Score Threshold. *Bioinformatics* **2020**, *36*, 2251–2252. [CrossRef]
125. Törönen, P.; Holm, L. PANNZER —A Practical Tool for Protein Function Prediction. *Protein Sci.* **2022**, *31*, 118–128. [CrossRef]
126. Caporaso, J.G.; Kuczynski, J.; Stombaugh, J.; Bittinger, K.; Bushman, F.D.; Costello, E.K.; Fierer, N.; Peña, A.G.; Goodrich, J.K.; Gordon, J.I.; et al. QIIME Allows Analysis of High-Throughput Community Sequencing Data. *Nat. Methods* **2010**, *7*, 335–336. [CrossRef] [PubMed]
127. The Galaxy Community; Afgan, E.; Nekrutenko, A.; Grüning, B.A.; Blankenberg, D.; Goecks, J.; Schatz, M.C.; Ostrovsky, A.E.; Mahmoud, A.; Lonie, A.J.; et al. The Galaxy Platform for Accessible, Reproducible and Collaborative Biomedical Analyses: 2022 Update. *Nucleic Acids Res.* **2022**, *50*, W345–W351. [CrossRef]
128. Camacho, C.; Coulouris, G.; Avagyan, V.; Ma, N.; Papadopoulos, J.; Bealer, K.; Madden, T.L. BLAST+: Architecture and Applications. *BMC Bioinform.* **2009**, *10*, 421. [CrossRef] [PubMed]
129. Teufel, F.; Almagro Armenteros, J.J.; Johansen, A.R.; Gíslason, M.H.; Pihl, S.I.; Tsirigos, K.D.; Winther, O.; Brunak, S.; von Heijne, G.; Nielsen, H. SignalP 6.0 Predicts All Five Types of Signal Peptides Using Protein Language Models. *Nat. Biotechnol.* **2022**, *40*, 1023–1025. [CrossRef] [PubMed]
130. Thumuluri, V.; Almagro Armenteros, J.J.; Johansen, A.R.; Nielsen, H.; Winther, O. DeepLoc 2.0: Multi-Label Subcellular Localization Prediction Using Protein Language Models. *Nucleic Acids Res.* **2022**, *50*, W228–W234. [CrossRef] [PubMed]

118:2083741198

131. Savojardo, C.; Martelli, P.L.; Fariselli, P.; Profiti, G.; Casadio, R. BUSCA: An Integrative Web Server to Predict Subcellular Localization of Proteins. *Nucleic Acids Res.* **2018**, *46*, W459–W466. [CrossRef]
132. Hallgren, J.; Tsirigos, K.D.; Pedersen, M.D.; Almagro Armenteros, J.J.; Marcatili, P.; Nielsen, H.; Krogh, A.; Winther, O. DeepTMHMM Predicts Alpha and Beta Transmembrane Proteins Using Deep Neural Networks. *bioRxiv*, 2022; *preprint*. [CrossRef]
133. Gíslason, M.H.; Nielsen, H.; Almagro Armenteros, J.J.; Johansen, A.R. Prediction of GPI-Anchored Proteins with Pointer Neural Networks. *Curr. Res. Biotechnol.* **2021**, *3*, 6–13. [CrossRef]
134. Paysan-Lafosse, T.; Blum, M.; Chuguransky, S.; Grego, T.; Pinto, B.L.; Salazar, G.A.; Bileschi, M.L.; Bork, P.; Bridge, A.; Colwell, L.; et al. InterPro in 2022. *Nucleic Acids Res.* **2023**, *51*, D418–D427. [CrossRef] [PubMed]
135. Letunic, I.; Khedkar, S.; Bork, P. SMART: Recent Updates, New Developments and Status in 2020. *Nucleic Acids Res.* **2021**, *49*, D458–D460. [CrossRef]
136. Zheng, J.; Ge, Q.; Yan, Y.; Zhang, X.; Huang, L.; Yin, Y. dbCAN3: Automated Carbohydrate-Active Enzyme and Substrate Annotation. *Nucleic Acids Res.* **2023**, *51*, W115–W121. [CrossRef]
137. Thomas, P.D.; Ebert, D.; Muruganujan, A.; Mushayahama, T.; Albou, L.; Mi, H. PANTHER: Making Genome-scale Phylogenetics Accessible to All. *Protein Sci.* **2022**, *31*, 8–22. [CrossRef]
138. Katoh, K.; Rozewicki, J.; Yamada, K.D. MAFFT Online Service: Multiple Sequence Alignment, Interactive Sequence Choice and Visualization. *Brief. Bioinform.* **2019**, *20*, 1160–1166. [CrossRef]
139. Edgar, R.C. MUSCLE: Multiple Sequence Alignment with High Accuracy and High Throughput. *Nucleic Acids Res.* **2004**, *32*, 1792–1797. [CrossRef]
140. Letunic, I.; Bork, P. Interactive Tree Of Life (iTOL): An Online Tool for Phylogenetic Tree Display and Annotation. *Bioinforma. Oxf. Engl.* **2007**, *23*, 127–128. [CrossRef]
141. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.* **2016**, *33*, 1870–1874. [CrossRef] [PubMed]
142. Yang, Z. PAML 4: Phylogenetic Analysis by Maximum Likelihood. *Mol. Biol. Evol.* **2007**, *24*, 1586–1591. [CrossRef]
143. Jumper, J.; Evans, R.; Pritzel, A.; Green, T.; Figurnov, M.; Ronneberger, O.; Tunyasuvunakool, K.; Bates, R.; Žídek, A.; Potapenko, A.; et al. Highly Accurate Protein Structure Prediction with AlphaFold. *Nature* **2021**, *596*, 583–589. [CrossRef]
144. Waterhouse, A.; Bertoni, M.; Bienert, S.; Studer, G.; Tauriello, G.; Gumienny, R.; Heer, F.T.; de Beer, T.A.P.; Rempfer, C.; Bordoli, L.; et al. SWISS-MODEL: Homology Modelling of Protein Structures and Complexes. *Nucleic Acids Res.* **2018**, *46*, W296–W303. [CrossRef] [PubMed]
145. Robert, X.; Gouet, P. Deciphering Key Features in Protein Structures with the New ENDscript Server. *Nucleic Acids Res.* **2014**, *42*, W320–W324. [CrossRef] [PubMed]

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III. OŚWIADCZENIA WSPÓŁAUTORÓW PUBLIKACJI

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OŚWIADCZENIE KANDYDATA/WSPÓŁAUTORA

O WKŁADZIE PRACY

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Food Preferences of Enchytraeids, Pedobiologia 63, 2017, 19–36
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imiona i nazwiska autorów publikacji

Niniejszym oświadczam, że w ww. pracy mój udział polegał na:

ustaleniu koncepcji pracy, przygotowaniu oryginalnego manuskryptu, korekcie oryginalnego i końcowego manuskryptu, zbieraniu i analizie danych literaturowych, wykonaniu eksperymentów z udziałem zwierząt na płytkach agarowych i jego dokumentacji, wykonaniu tabel oraz części figur, sformułowaniu odpowiedzi dla recenzentów na wszystkich etapach recenzji.

szczegółowy opis wkładu współautora w powstaniu pracy (np. koncepcja pracy, zbieranie/analiza danych, wykonanie analiz laboratoryjnych, przygotowanie publikacji, korekta manuskryptu etc.)

podpis

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współprzygotowaniu jednego z rozdziałów publikacji, korekcie oryginalnego manuskryptu, analizie materiału w mikroskopie świetlnym, udziale w eksperymencie ze zwierzętami dotyczącym dekompozycji materiału roślinnego, wykonaniu części fotografii, przygotowaniu tablic z figurami oraz na zdobywaniu rzadkich pozycji literaturowych

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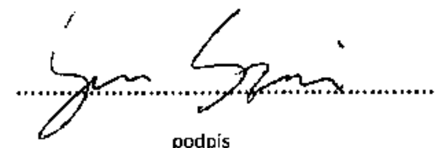

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OŚWIADCZENIE KANDYDATA/WSPÓŁAUTORA

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Food Preferences of Enchytraeids, Pedobiologia 63, 2017, 19–36
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OŚWIADCZENIE KANDYDATA/WSPÓŁAUTORA

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Discovery and characterization of the α-amylases cDNAs from *Enchytraeus albidus* shed light on the Evolution of "*Enchytraeus-Eisenia* type" Amy homologs in Annelida, Biochimie 221, 2024, 38–59

tytuł publikacji, czasopismo, rok wydania, strony

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Niniejszym oświadczam, że w ww. pracy mój udział polegał na:

ustaleniu koncepcji pracy, doborze metodologii, analizach laboratoryjnych i bioinformatycznych, analizie formalnej, zebraniu danych, udziale w doborze oprogramowania i walidacji wyników, przygotowaniu oryginalnego manuskryptu, korekcie oryginalnego i końcowego manuskryptu, przygotowaniu figur oraz tabel, sformułowaniu odpowiedzi na wszystkich etapach recenzji.

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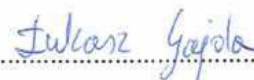

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podpis

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Discovery and characterization of the α-amylases cDNAs from *Enchytraeus albidus* shed light on the Evolution of "*Enchytraeus-Eisenia* type" Amy homologs in Annelida, Biochimie 221, 2024, 38–59
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Niniejszym oświadczam, że w ww. pracy mój udział polegał na:

doborze części oprogramowania i walidacji wyników, korekcie oryginalnego manuskryptu, nadzorze nad realizacją projektu badawczego

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Signed by / Podpisano przez:

Agata Jolanta Daszkowska-Golec Uniwersytet Śląski w Katowicach

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OŚWIADCZENIE KANDYDATA/WSPÓŁAUTORA

O WKŁADZIE PRACY

Katowice, 10-06-2024
miejscowość, data

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Discovery and characterization of the α-amylases cDNAs from *Enchytraeus albidus* shed light on the Evolution of "*Enchytraeus-Eisenia* type" Amy homologs in Annelida, Biochimie 221, 2024, 38–59
tytuł publikacji, czasopismo, rok wydania, strony

Łukasz Gajda, Agata Daszkowska-Golec, **Piotr Świątek**
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Niniejszym oświadczam, że w ww. pracy mój udział polegał na:

pozyskaniu środków na badania, korekcie oryginalnego manuskryptu, nadzorze nad realizacją projektu badawczego

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OŚWIADCZENIE KANDYDATA/WSPÓŁAUTORA

O WKŁADZIE PRACY

Ustroń, 10.06.2024
miejscowość, data

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Trophic position of the white worm (*Enchytraeus albidus*) in the context of digestive enzyme genes revealed by transcriptomics Analysis, International Journal of Molecular Sciences 25, 2024, 4685

tytuł publikacji, czasopismo, rok wydania, strony

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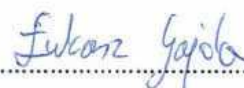

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OŚWIADCZENIE KANDYDATA/WSPÓŁAUTORA

O WKŁADZIE PRACY

Katowice, 12.06.2024
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Trophic position of the white worm (*Enchytraeus albidus*) in the context of digestive enzyme genes revealed by transcriptomics Analysis, International Journal of Molecular Sciences 25, 2024, 4685

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miejscowość, data

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