

Animals degrade the biopolymer polyhydroxyalkanoate

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Supplementary Text

Supplementary Text 1 | *O. algarvensis* PHAD degrades short chain PHAs

Using AlphaFold2^{1,2}, we generated a model of the *O. algarvensis* PHAD by comparing it to the complete AlphaFold2 database. We then compared it to the crystal structure of a fungal homolog derived from *P. funiculosum* (pdb 2d81; predicted local distance difference test (pLDDT) = 94; Figure 1c; Supplementary Figure 2b)³. Although the protein sequence similarity between the animal PHAD and the fungal homolog was relatively low (31.6 %), the prediction of the enzyme indicated that the superposition between the proteins was strong (root-mean-square deviation (RMSD) of 0.736 Å based on 236 Cα; DALI Z = 45.8). Because the two enzymes demonstrated high levels of structural homology, we explored their structural similarities. Like the fungal PHAD, the animal protein is predicted to fold into a single-domain enzyme. Within the core of the enzyme, our prediction revealed that the *O. algarvensis* PHAD is composed of an active site that includes a catalytic triad consisting of S₂₇-D₁₂₈ and H₁₆₁ residues and an oxyanion hole made up of S₂₈ and C₂₅₇. The residues that make up the active site of the *O. algarvensis* protein aligned closely with those of the fungal homolog (RMSD = 0.047-0.127 Å), suggesting that the *O. algarvensis* enzyme, like microbial PHADs, cleaves PHA via a nucleophilic attack on the ester bond between the hydroxyalkanoate monomeric units^{3,4}. While the protein sequence of the substrate binding region of the *O. algarvensis* enzyme only shared only 28.6% sequence homology to the same region of the fungal homolog, the structure of the protein was highly conserved (RMSD =

0.081-0.393 Å). Specifically, the positions of the residue W₃₀₄, which stabilizes the polymer chain, and V₁₂₉ and P₂₉₈, which are located around the catalytic crevice and attract the substrate, were identical between the animal model and fungal homolog. Using SignalP⁵, we also identified a signal peptide forming the first 19 amino acids of the *O. algarvensis* PHAD primary structure. This signal peptide is predicted to export the animal PHAD to the symbiont region underneath the worm's cuticle following the *SEC1* pathway.

In addition to generating models for *O. algarvensis*, we also used AlphaFold2 to predict the structures of PHADs recovered from nine more gutless oligochaete species (pLDDT = 85-96; Supplementary Figure 5b). The resulting PHAD models from all other nine gutless oligochaetes revealed the same tertiary protein structure as described for *O. algarvensis*: Complete conservation of the active site and critical residues of the substrate binding domain, and the presence of a signal peptide for Sec1 secretion for some gutless oligochaete PHADs (Supplementary Figure 5a; Supplementary Table 1). Our results indicated that the PHAD from gutless oligochaetes likely bind denatured extracellular PHA and degrade it.

Based on our AlphaFold2 models, we sought to explore the substrate specificity of the *O. algarvensis* PHAD for symbiont-produced PHA. Our analysis revealed that *O. algarvensis* PHAD is missing a beta-sheet between D₂₆₅ and G₃₁₈ compared to the fungal homolog, and instead are predicted to have a loop between I₂₉₁ and A₂₉₂. This loop opens the catalytic pocket, allowing the substrate to enter through a tunnel in the protein structure. Within the catalytic crevice of the *O. algarvensis* PHAD, Y₄₂ is substituted by E₄₄, which enlarges the crevice. We observed the same substitutions for most other gutless oligochaete PHADs (22 from 32 individuals from 14 species). Based on these results, we propose that the catalytic site of gutless oligochaete PHADs is larger than the fungal homolog, and can therefore bind a broad range of PHAs, including polyhydroxybutyrate (PHB), polyhydroxyvalerate (PHV) and polyhydroxymethylvalerate (PHMV). Considering that *O. algarvensis* symbionts produce a copolymer of PHB, PHV and PHMV⁶, our modeling results across gutless oligochaete enzymes suggest that the PHADs of these hosts are adapted to the PHAs their symbionts produce.

Supplementary Text 2 | Higher metabolic flexibility

Bacteria that have multiple PHAD copies might be metabolically more flexible. Depending on the bacteria's PHAD activity, PHA degradation results in different metabolic end products.

For example, the Betaproteobacterium *Ralstonia eutropha* has nine different intracellular PHADs that either release Co-A-bound monomers^{7, 8}, or hydrolyze PHA into hydroxycarboxylic monomers or oligomers^{9, 10}. In our study, some animals also have multiple copies of the PHAD enzyme. Considering that by having multiple PHAD copies bacteria can either use different PHA sources or degrade PHA to different degradation products, we speculate that animals with multiple PHAD enzymes also have a higher degree of metabolic flexibility.

To explore the hypothesis that a diverse repertoire of PHAD enzymes bind different types of PHAs and allow the individual to produce different degradation products, we chose to model four PHADs from the water flea *Daphnia magna* using AlphaFold2. We used the models to explore structural similarities across the four homologs. In comparison to the fungal homolog, the catalytic triad, oxyanion hole and W_{302/317/336} residue that holds the polymer chain in place during degradation were fully conserved in all four PHADs from *D. magna*, suggesting that all four enzymes degrade PHA. Like the gutless oligochaete PHADs, the main differences across *D. magna*'s PHAD homologues are located at the substrate binding site. Specifically, each of the PHAD homologues has a single amino acid substitution that likely impacts the type of PHA it can bind. For example, the two amino acids of the substrate binding site that follow the catalytic D_{139/123/157} are substituted by either SV₁₄₀₋₁₄₁ or ST_{124-125/158-159}. The variation in the substrate binding region shifts the size of the catalytic triad from 3.8 x 2.5 x 7.0 Å to 4.7 x 4.4 x 8.5 Å (Supplementary Figure 16).

Supplementary Text 3 | Gutless oligochaetes fully degrade PHA to generate energy

After *O. algarvensis*'s PHAD breaks down the symbiont's PHA into oligomers and monomers, additional enzymes are required to further catabolize the breakdown products to produce carbon and energy molecules. Bacteria use a hydroxybutyrate-dimer hydrolase (EC 3.1.1.22) to break down PHA-derived oligomers to monomers, and a beta-hydroxybutyrate dehydrogenase (BHBD; EC 1.1.1.30) to transform the monomers into acetoacetate. Acetoacetate can then be oxidized to acetyl coenzyme A (acetyl-CoA), which is fed into the citric acid cycle for energy gain (Supplementary Figure 17)¹¹⁻¹⁹. In animals, there are no known homologs for the hydroxybutyrate-dimer hydrolase, but they do have BHBDs that would allow them to degrade PHA-derived monomers to acetate²⁰. We used the same method as described for the PHAD identification and expression analysis to identify the three genes that further degrade PHA for

energy generation. In *O. algarvensis*, we were unable to find a hydroxybutyrate-dimer hydrolase but the BHBD gene was present in the host-enriched transcriptomic libraries obtained from *O. algarvensis* individuals (Supplementary Figure 18). This enzyme was expressed at similar levels as the animal's PHAD and other enzymes *O. algarvensis* uses to digest its symbionts in the epidermis. For the other gutless oligochaete species, we looked for homologs of the BHBD and recovered transcripts from eight of the ten species we investigated. Like *O. algarvensis*, BHBD of the other gutless oligochaetes was expressed at similar levels as their PHAD (Supplementary Figure 18). The expression of PHADs in all, and of BHBDs in most species investigated indicates that the expression of these genes is widespread across the gutless oligochaetes.

Supplementary Text 4 | Protist PHADs

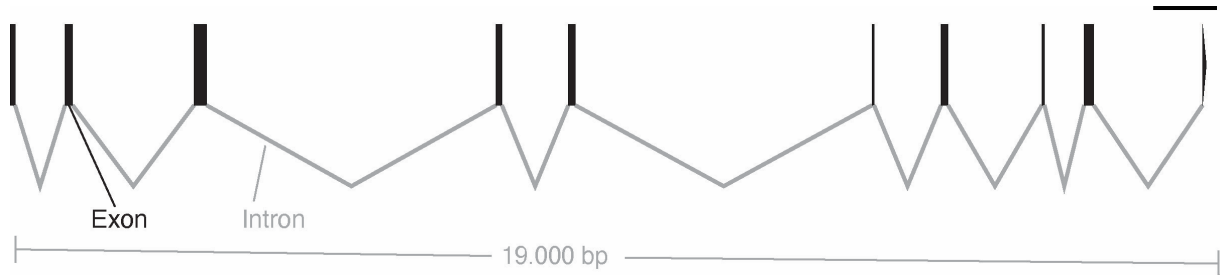
Adding to our discovery that PHADs are widespread in animals, we also found homologs in 21 protist species from five protist supergroups (Supplementary Table 5). Previously, only the Amoebozoa species, *Acanthamoeba castellanii*, and the Evosea species, *Dictyostelium discoideum* were thought to encode PHADs²¹. Our analysis revealed that PHADs were also present in Choanoflagellates, Euglenozoa, Ciliophora, and Heterolobosea. PHADs from Ciliophora formed a sister clade to Evosea PHADs and to a clade containing sequences from Evosea, Choanoflagellate and Euglenozoa (Supplementary Figure 14). The phylogeny of protist PHADs did not reflect the current protist phylogeny²²: the Choanoflagellata PHADs were not closely related to metazoan PHADs and instead formed a sister clade to the PHAD from *A. castellanii* (Amoebozoa), while the metazoan PHADs clustered with PHADs from Heterolobosea. This incongruence could have been caused by the under sampling of protist PHADs, the paraphyletic nature of major protist groups²², or the poor resolution of the PHAD tree at deep-branching nodes.

Supplementary Text 5 | Binning of the symbiont genomes

In order to generate the reference “*Ca. Thiosymbion*” genome bins used in this study, we used the metagenomic data published under the accession PRJEB76586²³. The raw metagenomic reads were trimmed from each dataset that contained sequencing adaptors, were shorter than 51 bp, had quality scores that dropped to zero, and had kmers less than 11 bp long at the sequence ends using BBduk (v38.90; <https://jgi.doe.gov/data-and-tools/software->

tools/bbtools/). We error corrected the reads using bayeshammer (SPAdes v3.12.0.; only error correction)²⁴. We then assessed the quality of the trimmed reads using FastQC²⁵ and generated the taxonomic summary of the samples using phyloFLASH (v3.4)²⁶. The quality-controlled reads were assembled using SPAdes (v3.12.0)²⁷ with the option for metagenomes, a specific kmer length of either 99 or 127 and a PHRED quality offset of 33. The assembled reads were mapped to a custom database of the symbionts found in gutless oligochaetes using BMap (v39.00)²⁸. Finally, we binned the assembled reads with a minimum contig size of 1500 bp in to metagenomically assembled genomes (MAGs) using MetaBAT (v2.0)²⁹. The resulting MAGs were used in our analyses if they were >50% complete with <10% contamination as determined by checkM (v2.0)³⁰ and if we were able to recover a 16S rRNA gene sequence from the bin using barnap (<https://github.com/tseemann/barnap>).

Supplementary Figures

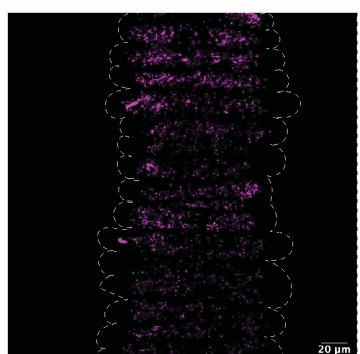
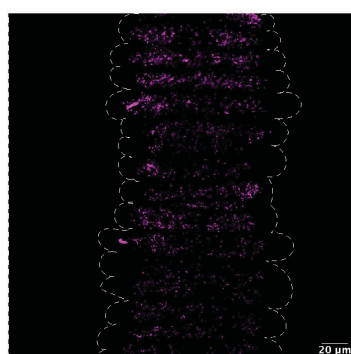
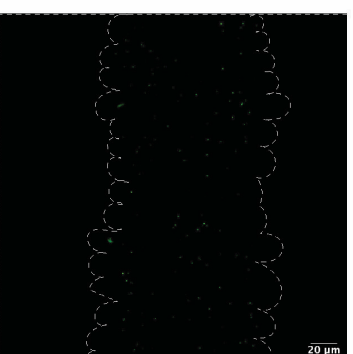
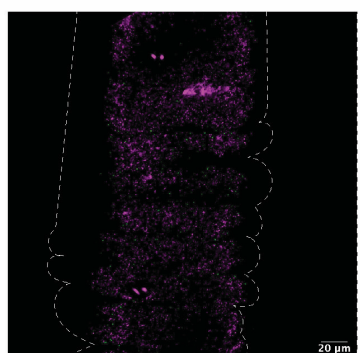
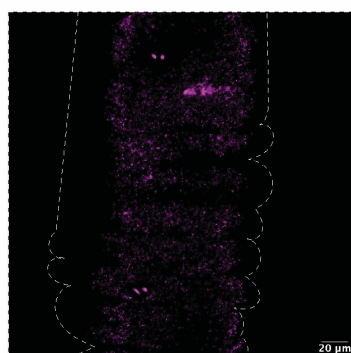
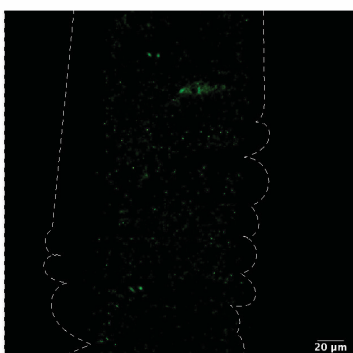
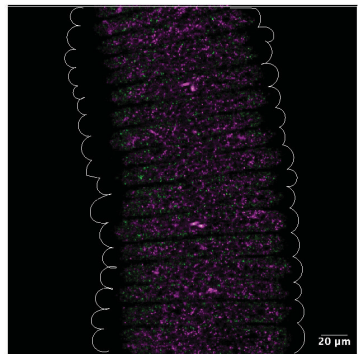
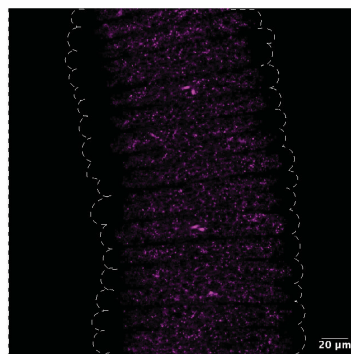
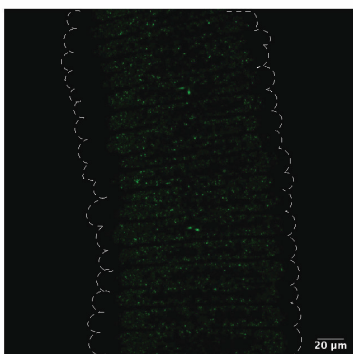
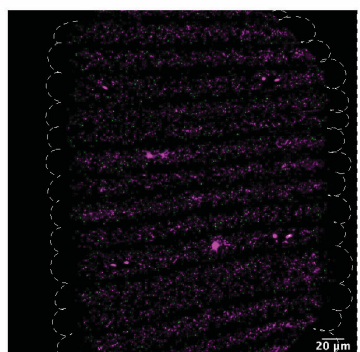
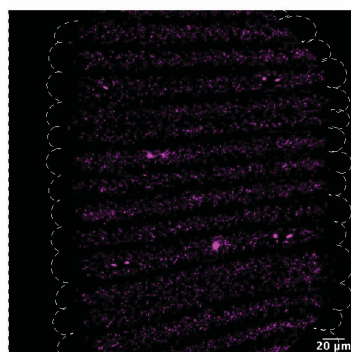
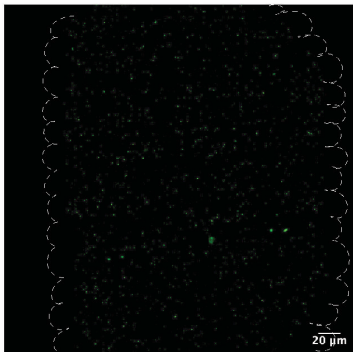
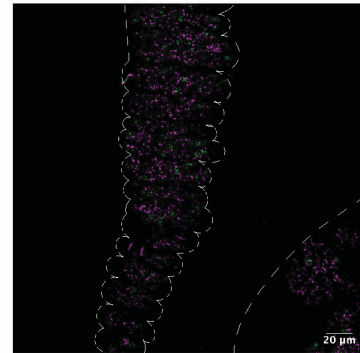
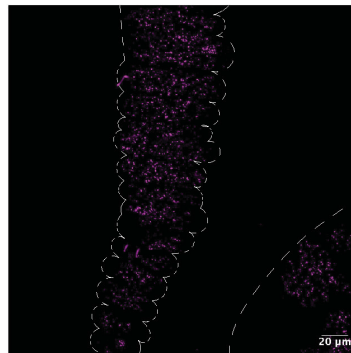
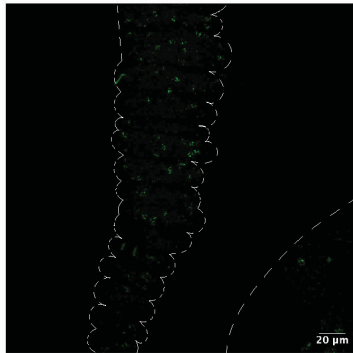


Supplementary Figure 1 | We identified a *O. algarvensis phaZ* gene that encodes for a PHAD. The *phaZ* gene from the *O. algarvensis*' draft genome spans 19,000 bp and was split into 10 different exons (black bars). Scale bar=1000bp.

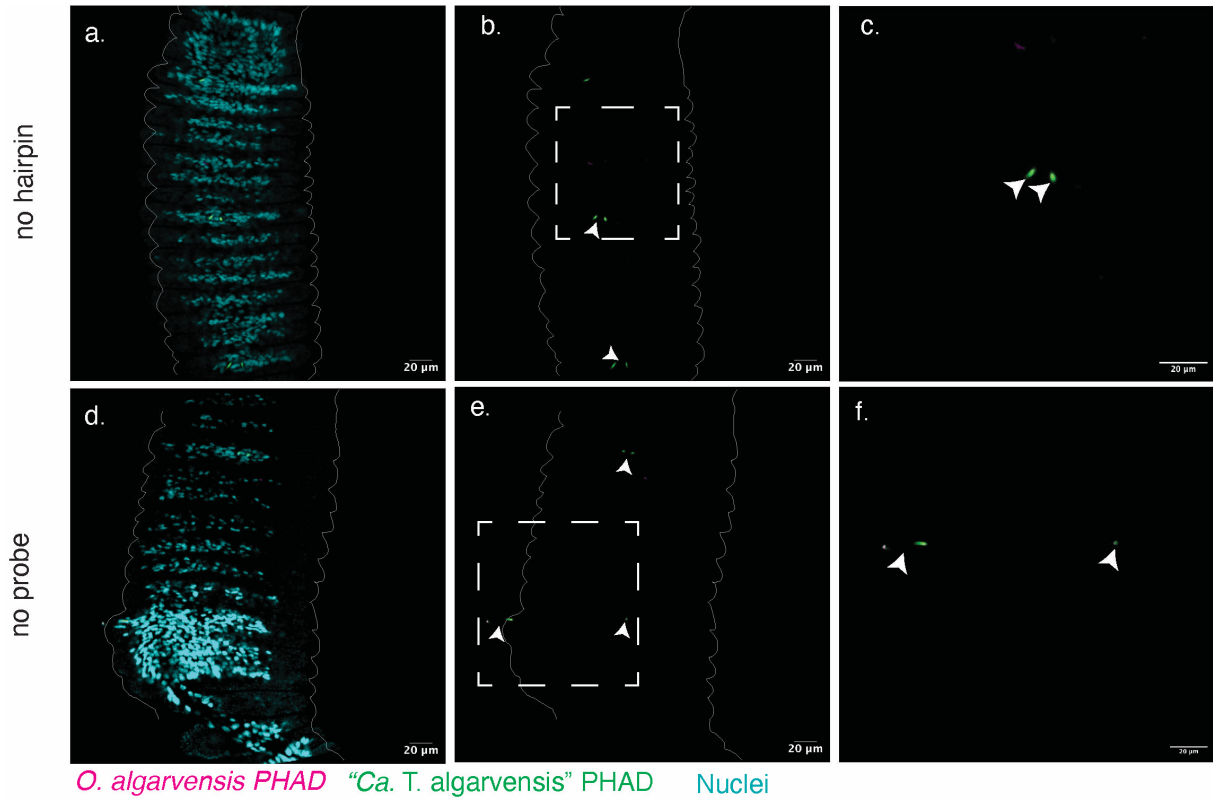
“*Ca. Thiosymbion algarvensis*”
PHAD

host PHAD

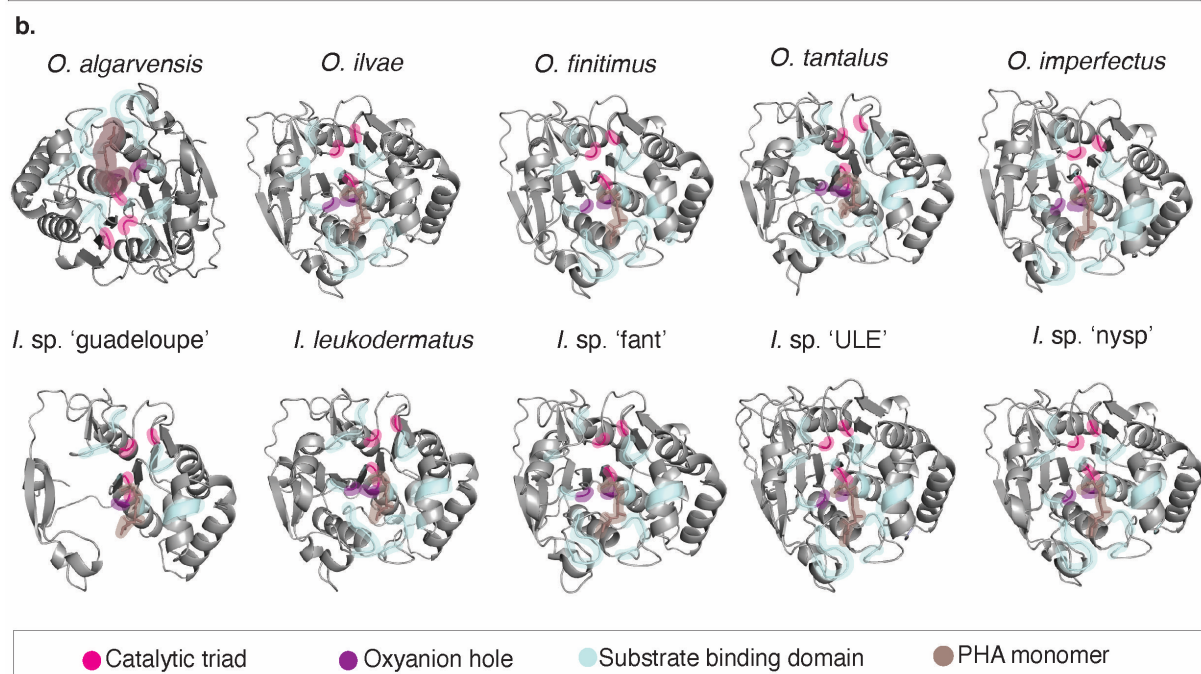
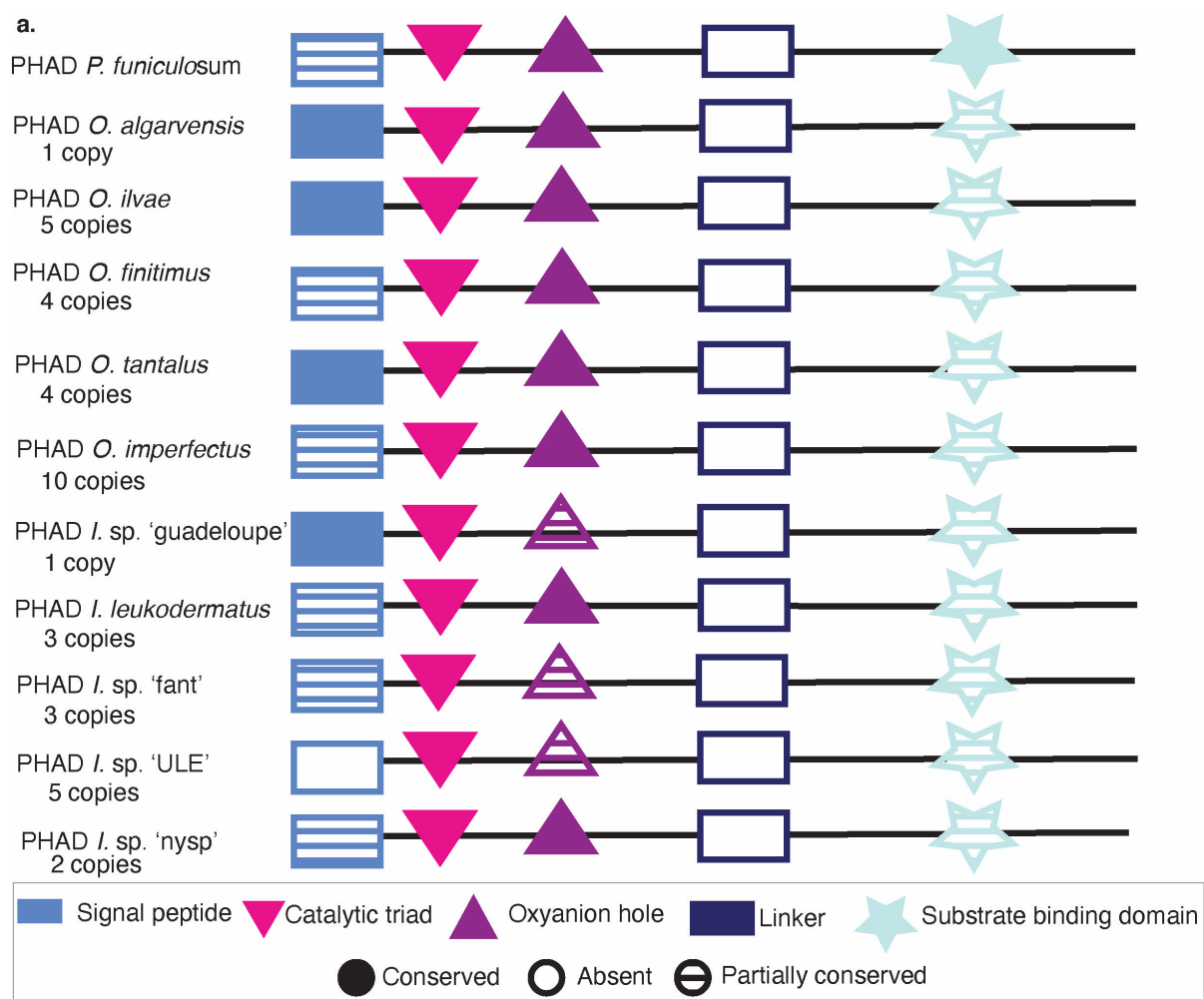
merged host & symbiont
PHAD



Supplementary Figure 3 | The PHAD of *O. algarvensis* is expressed in its epidermal cells.
Replicates of the whole mount HCR-FISH of the PHAD mRNA of the host and symbiont.
The probes specific to PHAD transcripts of the symbiont "*Ca. T. algarvensis*" (green) and the
host *O. algarvensis* (pink) are distinct from each other and are expressed throughout the
worm's epidermis. (n=6)



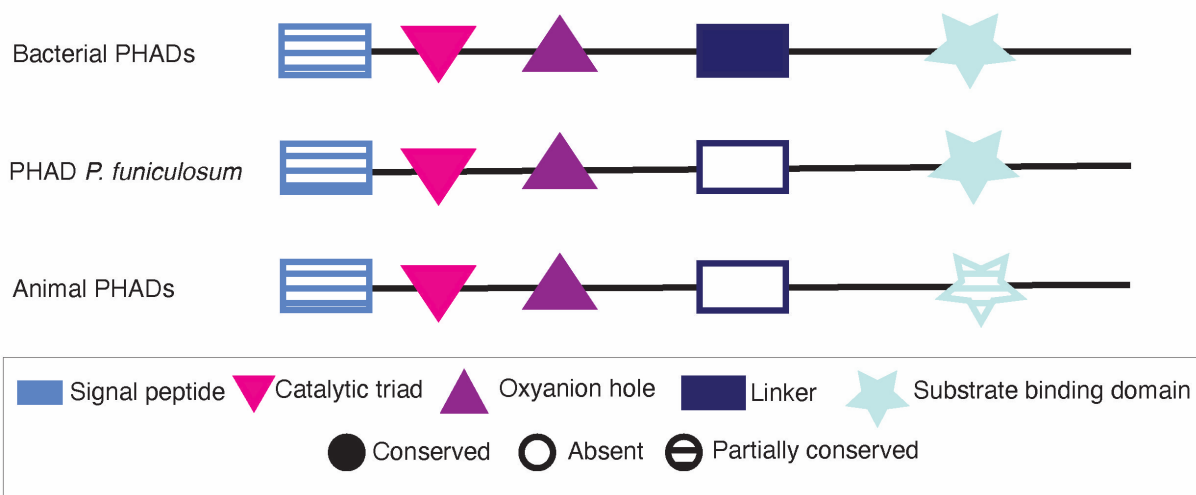
Supplementary Figure 4 | HCR-FISH controls. Whole mount HCR-FISH images of controls without a hairpin (a,b,c) and without the probe (d,e,f) showed that the HCR-FISH signals in Figure 2 are specific to the mRNA transcripts. The white triangles in c,b,e and f show the autofluorescence of the worm's seta. The worm's cuticle is outlined in white. Cyan = nuclei, magenta = *O. algarvensis* PHAD, green = "*Ca. Thiosymbion algarvensis*" PHAD



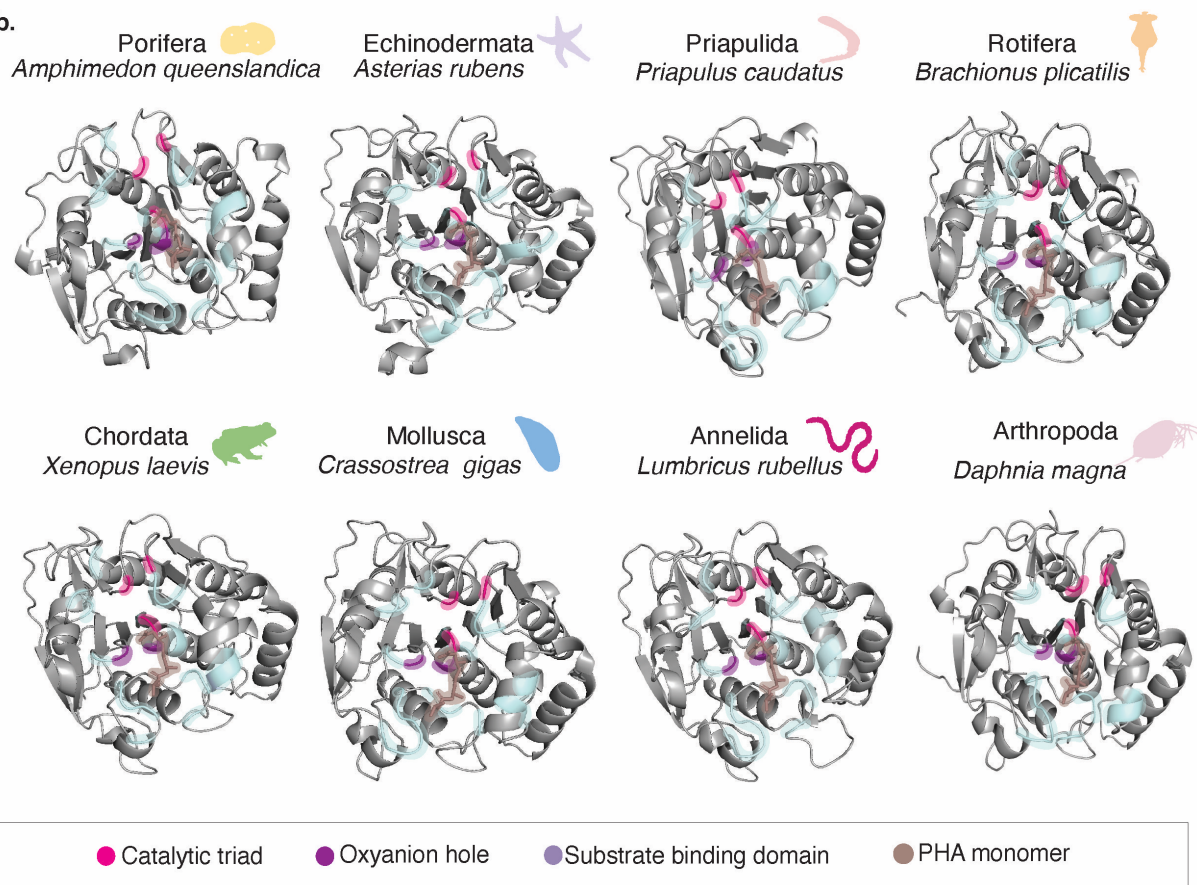
Supplementary Figure 5 | Predicted PHAD enzymes from ten different gutless oligochaete species. **a**, The primary structure of the *phaZ* gene of 10 gutless oligochaetes species included in this study encoded for a protein between 211 - 367 amino acids. A MAFFT alignment to the predicted homolog of *P. funiculosum* (pdb 2d81) showed 100% conservation of the catalytic triad and 70% of the oxyanion hole across all PHAD sequences (pink and purple triangles). 19 out of 40 predicted PHADs had a signal peptide (blue squares; Supplementary Table 1). Across the entire primary structure, the gutless oligochaete enzymes shared 14.5% - 33.8% identity and 65.9% - 95.3% coverage to *P. funiculosum*'s PHAD. Symbols represent different regions of the protein that are either conserved (filled), partially conserved (stripped) or absent (open). **b**, AlphaFold2 structures of the gutless oligochaete PHADs aligned to the PHAD crystal structure from *P. funiculosum*'s (pdb 2d81; RMSD = 0.720 - 2.740 Å), suggesting that the predicted enzymes have a similar function as the fungal homolog. The catalytic triad (pink), oxyanion hole (purple), the residue that holds the polymer in place (cyan) and substrate binding site (cyan) showed strong overlap between the fungal homolog and each of the gutless oligochaete PHADs. A PHB monomer (brown) is predicted to fit within the catalytic pocket of each of the enzymes (pLDDT = 85 – 96).

Supplementary Figure 6 | Gutless oligochaete PHADs clustered with extracellular PHADs of domain type 2, forming a separate cluster from their symbiont enzymes. The unrooted maximum likelihood tree (IQ TREE, ultrafast bootstraps) of all known PHADs of the PHAD engineering database shows that the animal enzyme is distinct from that of its bacterial symbiont, “*Ca. Thiosymbion*”. While the gutless oligochaetes PHADs clustered within the extracellular PHADs degrading short chain PHA of domain type 2, the “*Ca. Thiosymbion*” PHADs clustered with domain type 1 PHADs assumed to degrade short chain PHAs.

a.



b.



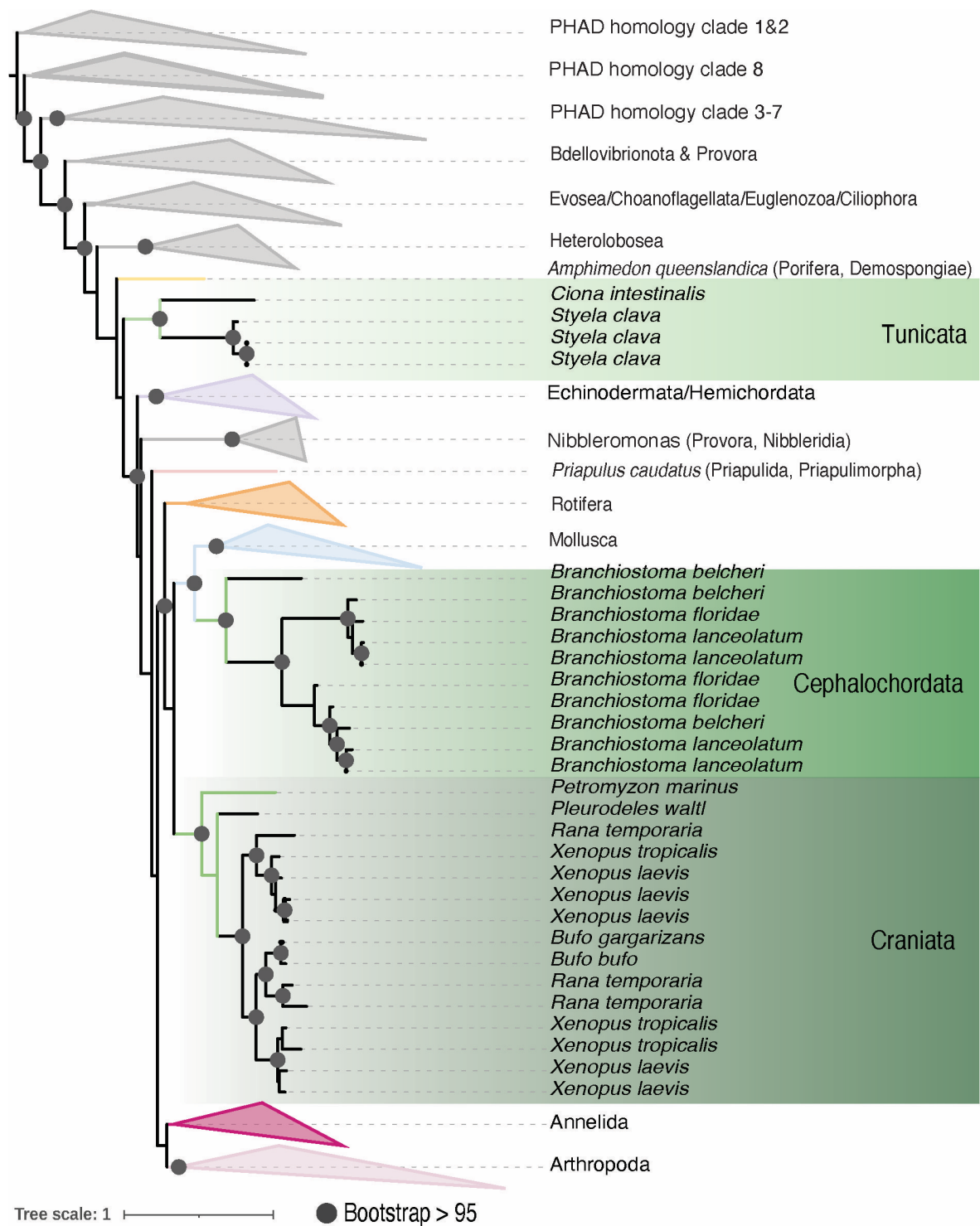
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Supplementary Figure 7 | PHADs from different metazoan phyla share a similar domain architecture with the fungal homolog. a, A schematic of a primary structure analysis showed that animal PHADs have a more similar structure to the fungal homolog than PHADs found in bacteria. Animal PHADs encode for a protein consisting of 190 - 1166 amino acids (Supplementary Table 1). A MAFFT alignment revealed that each of the metazoan enzymes shared between of 17.9% - 36.3% identity to the fungal PHAD (pdb 2d81). The catalytic site (pink triangles) and oxyanion hole (purple triangles) was 100% conserved for all metazoan PHADs and 75% of the enzymes had a signal peptide (blue square; Supplementary Table 1). Symbols represent different regions of the protein that are either conserved (filled), partially conserved (stripped) or absent (open). **b,** AlphaFold2 predicted structures from each of the animal PHADs aligned well (RMSD 0.668 to 0.904 Å) to the PHAD crystal structure from *P. funiculosum*'s (pdb 2d81). The catalytic triad (pink), oxyanion hole (purple), the residue that holds the polymer in place (cyan) and substrate binding site (cyan) showed high overlap between the fungal homolog and the animal enzymes. A PHB monomer (brown) is predicted to fit within the catalytic pocket of each of the enzymes (pLDDT = 87 – 97).

Supplementary Figure 8 | The clustering of Rotifera and Burkholderiales PHADs suggests a recent horizontal gene transfer (HGT) event between the two groups. A maximum likelihood phylogenetic tree inferred with IQ TREE using ultrafast bootstrap showed that Rotifera PHADs formed two separate groups. The first group clustered within the animal PHAD clade (orange star). The second group (highlighted in orange) clustered with PHADs from the *Burkholderiales*, which formed a sister group to the fungal PHADs. The pattern observed in the second Rotifera group suggests Rotifers may have obtained one of their PHAD copies during recent HGT event from bacteria.

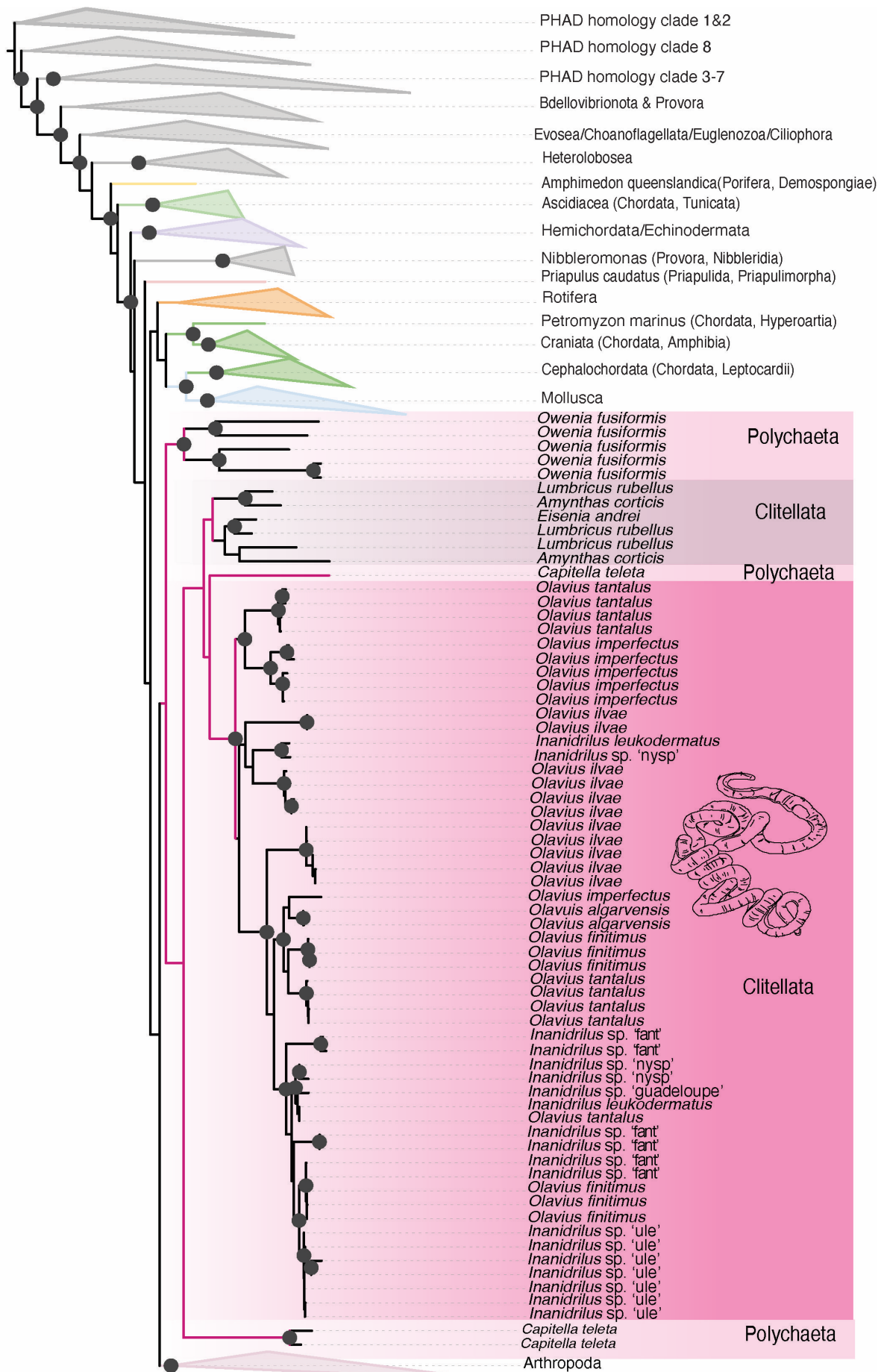


A maximum likelihood phylogenetic tree inferred with IQ TREE using ultrafast bootstrap showed that Mollusca PHADs were split into two classes: Bivalvia and Gastropoda. The splitting of the two PHAD groups is in accordance with the phylum's phylogeny.

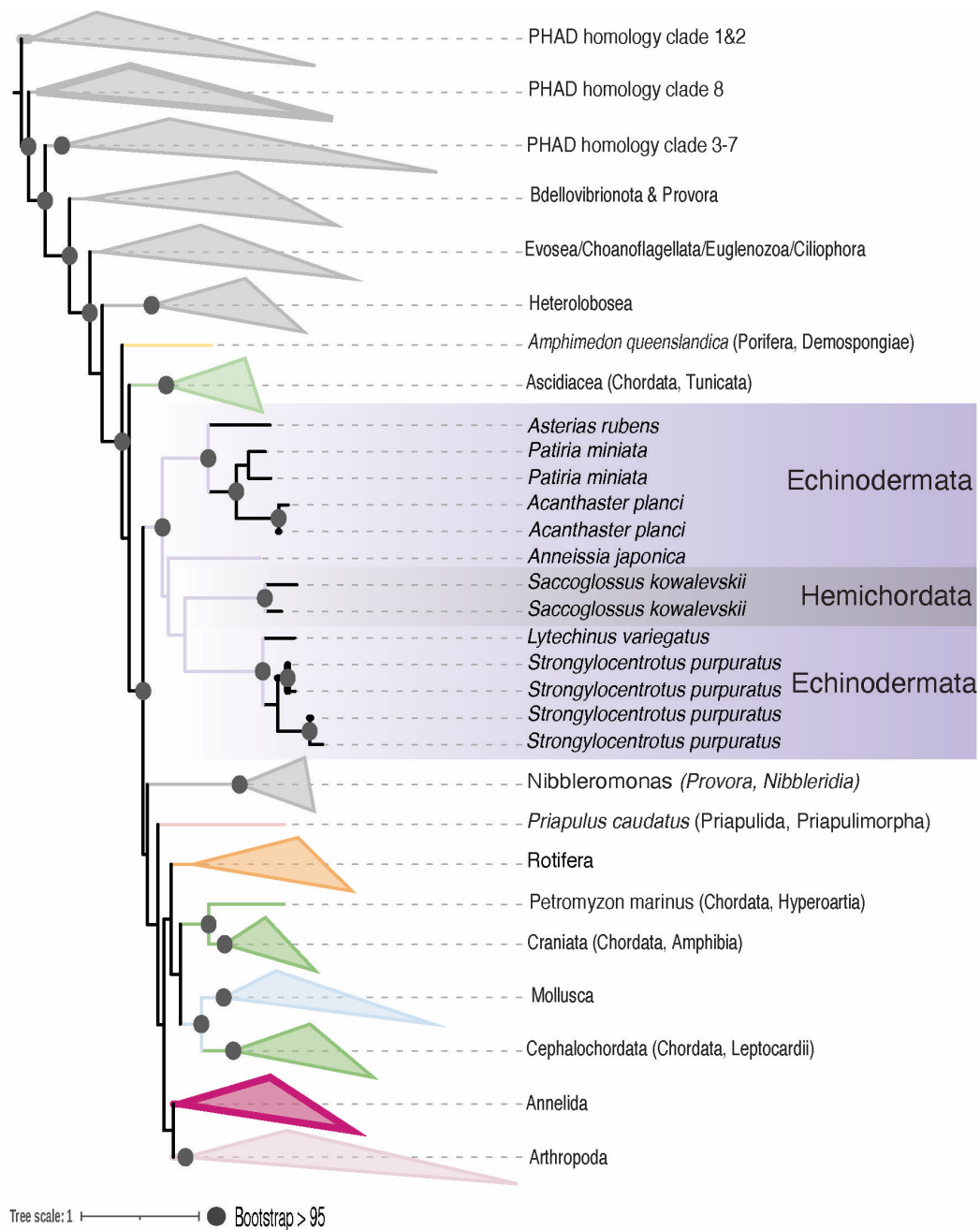


Supplementary Figure 10 | Chordata PHADs split into their proposed subclades Craniata, Cephalochordata and Tunicate. A maximum likelihood phylogenetic tree inferred with IQ TREE using ultrafast bootstrap showed the PHADs from the subphyla Tunicata formed a disparate branch from the Craniata and Cephalochordata.

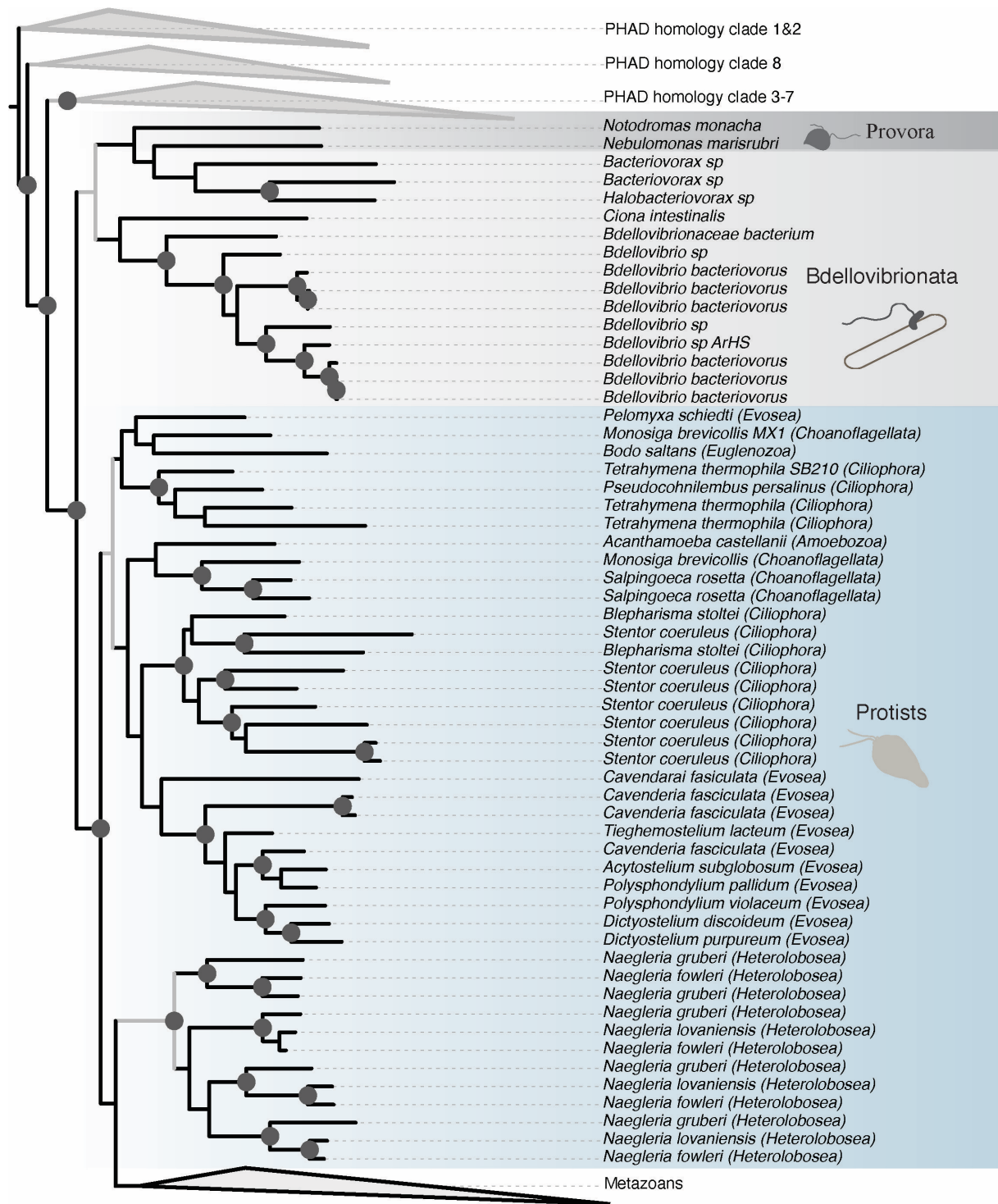
Supplementary Figure 11 | Arthropoda PHADs did not follow the phylogeny of this phylum, likely because of multiple PHAD homologs in some collembola. A maximum likelihood phylogenetic tree inferred with IQ TREE using ultrafast bootstrap showed that while arthropod PHADs formed a monophyletic clade, the PHADs from Collembola formed three disparate branches within the group.



Supplementary Figure 12 | Annelida PHADs did not follow the phylogeny of this phylum, likely because of multiple PHAD homologs in some clitellates. A maximum likelihood phylogenetic tree inferred with IQ TREE using ultrafast bootstrap showed four subclades of Annelida PHADs.



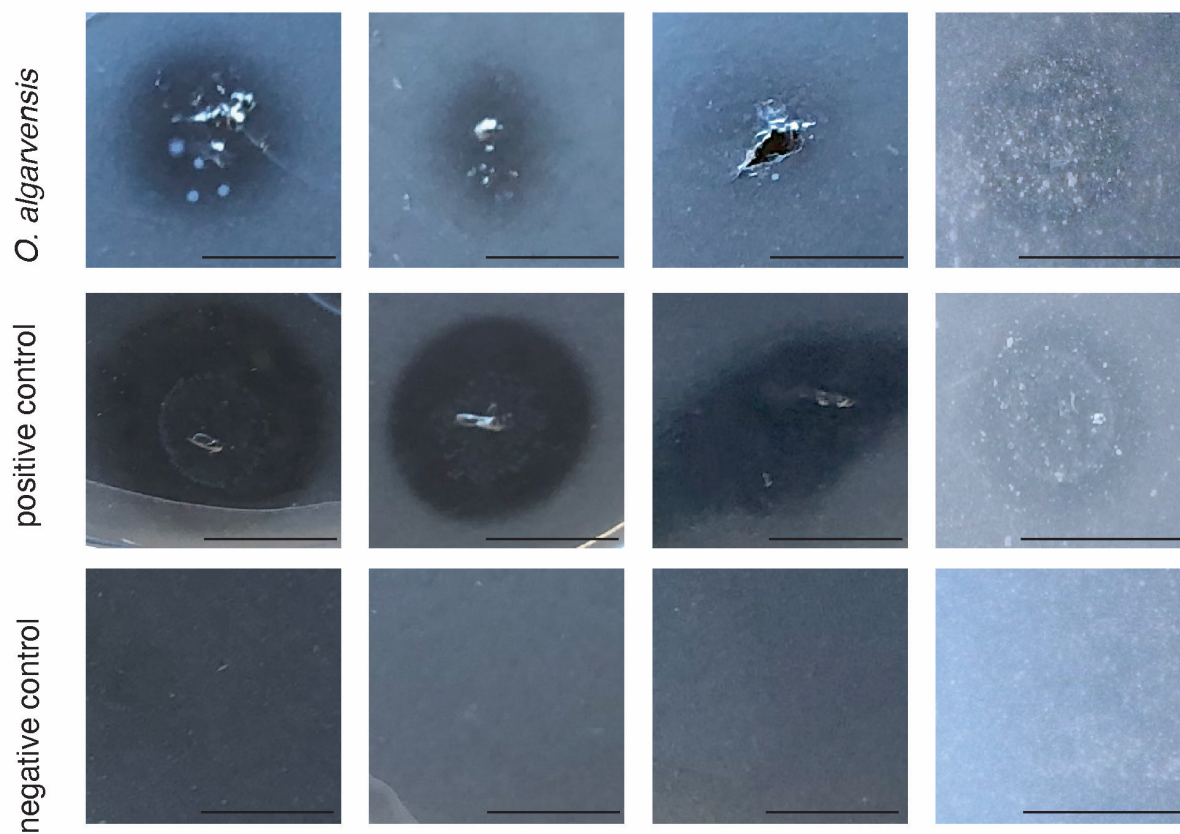
Supplementary Figure 13 | Echinodermata and Hemichordata PHADs formed a monophyletic group. A maximum likelihood phylogenetic tree inferred with IQ TREE using ultrafast bootstrap showed that Echinodermata and Hemichordata PHADs grouped in one clade.



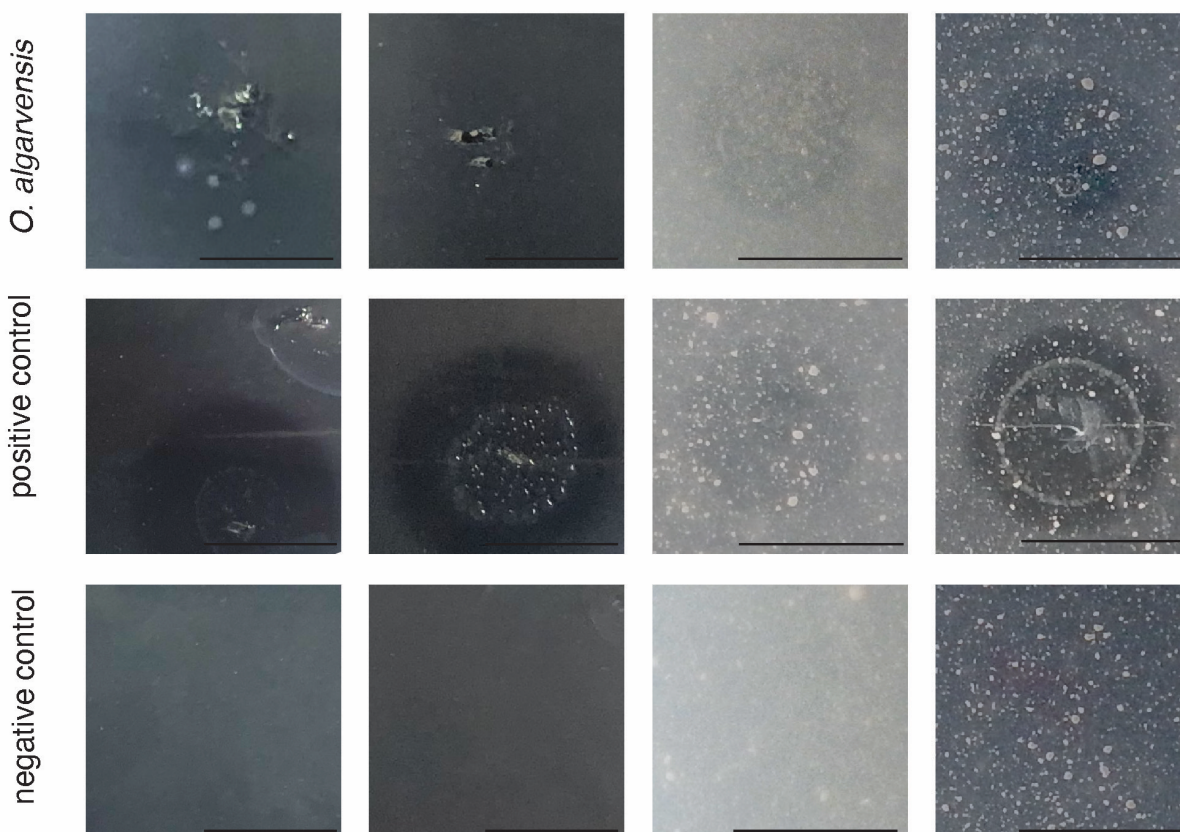
Tree scale: 1 ——— ● Bootstrap > 95

Supplementary Figure 14 | The *Bdellovibrio* PHADs formed an early branching clade to the animal and protist PHADs. A maximum likelihood phylogenetic tree inferred with IQ TREE using ultrafast bootstrap showed that animal and protist PHADs formed a sister clade to the *Bdellovibrio* sp. PHADs.

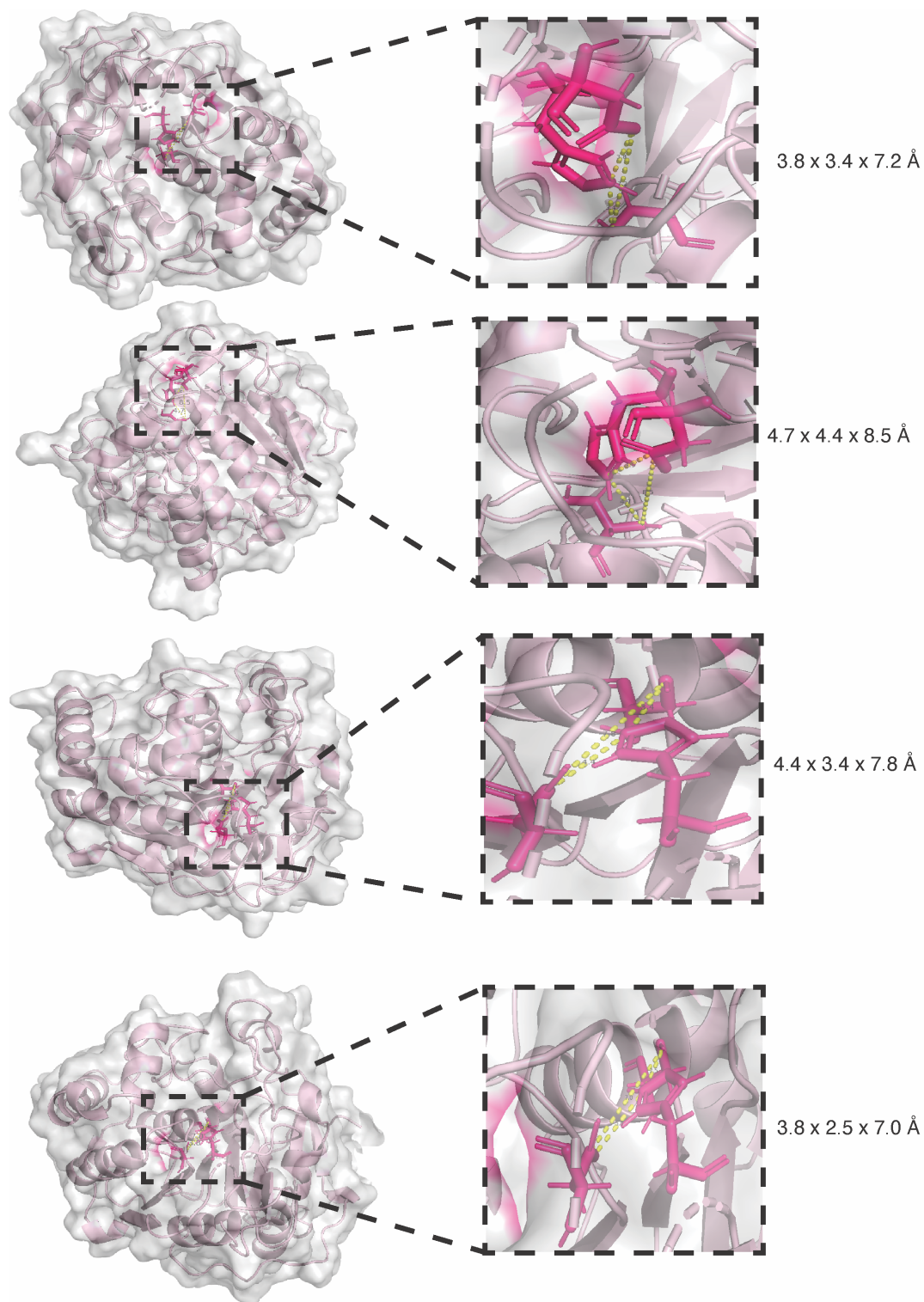
a. PHB



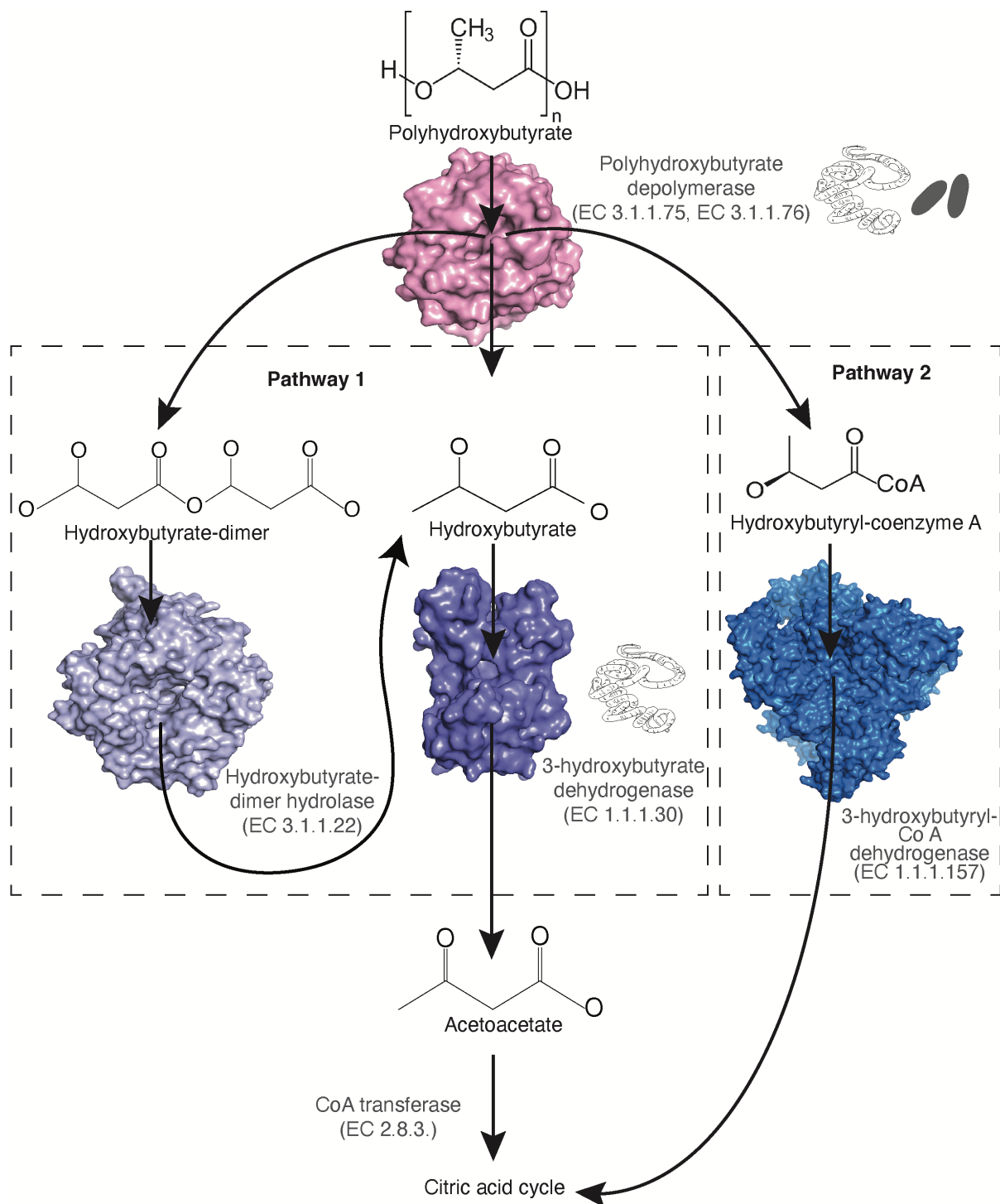
b. PHB/PHV



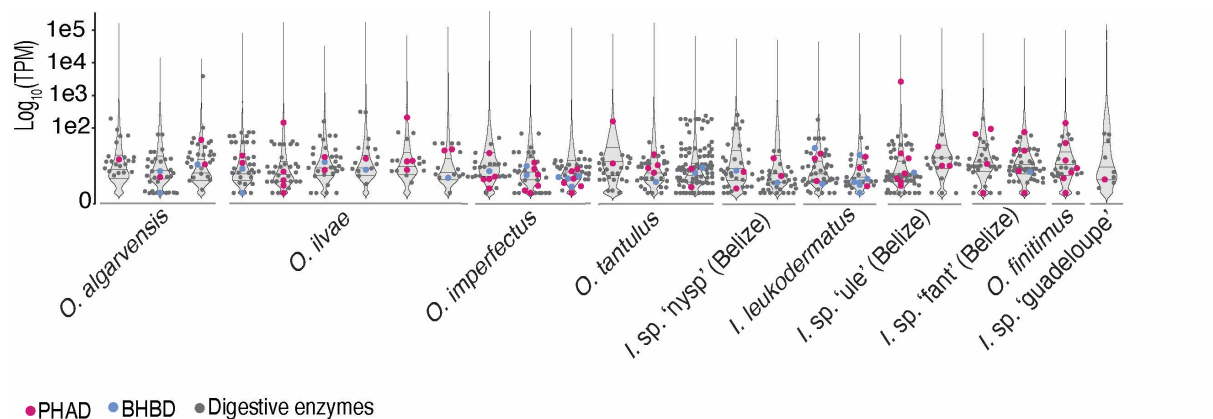
Supplementary Figure 15 | *O. algarvensis* PHAD was active on PHB and PHB/PHV substrates. Replication of the enzyme spot assays (n=5) on **a**, PHB and **b**, PHB/PHV assay plates. The heterologously expressed PHAD from *O. algarvensis* degraded PHA after 24 hours. The clearance zone of the animal PHAD was not as strong as the positive control (a heterologously expressed PHAD from the bacterium *Paucimonas lemoignei*), possibly due to differences in the activity of the *P. lemoignei* and *O. algarvensis* PHADs. Heat inactivation of both the bacterial and animal PHADs eliminated their activity (negative controls). Additionally, PHB/PHV assay plates show an uneven distribution of the polymer due to lower ability to get into solution.



Supplementary Figure 16 | The size of the catalytic site across *D. magna*'s PHADs is variable. AlphaFold2 models from each of *D. magna*'s four PHAD copies had a different size (2.5 x 7.0 Å to 4.7 x 4.4 x 8.5 Å) of their catalytic triad as measured in PyMOL. The variation in size is hypothesized to bind different types of PHA substrates.



Supplementary Figure 17 | Overview of the enzymes and products involved in PHA degradation. PHADs (pdb 280) degrade PHB into monomers, dimers or a mix of oligomers. In **Pathway 1**, hydroxybutyrate-dimer hydrolase (EC 3.1.1.22) degrades the dimers into their monomers. The enzyme BHBD (EC 1.1.1.30; pdb 3w8e) is then required to transform the monomers into acetoacetate, which is then oxidized to acetyl coenzyme A by CoA transferase (2.8.3) and fed into the citric acid cycle. Alternatively, in **Pathway 2**, PHAD activity results in the production of CoA-bound monomers that can be degraded directly to acetyl-CoA by 3-hydroxybutyryl-coenzyme A dehydrogenase (EC 1.1.1.157; pdb 6acq; Pathway 2). In our study, all gutless oligochaete species expressed a PHAD, and eight of the ten species expressed a BHBD. Their primary symbionts lacked a BHBD, a hydroxybutyrate dimer hydrolase and 3-hydroxybutyryl-coenzyme A dehydrogenase needed to breakdown the products of PHAD activity.



Supplementary Figure 18 | Gutless oligochaetes expressed a PHAD and BHBD in their transcriptome. Violin plots, which represented assembled host-enriched transcriptomes of single individuals from ten different gutless oligochaete species, showed the range in normalized transcription ($\log_{10}(\text{TPM})$) per individual worm. We identified at least one copy of the host-specific PHAD (pink) and BHBD (blue) transcript in at least one individual per species. Both proteins were expressed in range with other digestive enzymes (gray). To estimate transcript expression, we assembled one reference assembly per gutless oligochaete species and mapped the raw reads of each library representing one individual to obtain normalized transcript abundances.

Supplementary Tables

(File: Zeidler_et_al_SupplementaryTables_140624.xlsx)

Supplementary Table 1 - Signal peptide prediction of animal PHADs

Supplementary Table 2 – Metadata of the gutless oligochaete metatranscriptomic libraries

Supplementary Table 3 - Identified animal PHADs and analysis of the feeding behavior of animals with a PHAD

Supplementary Table 4 - Analysis of symbiont PHA degradation genes in metagenomes across 12 gutless oligochaete hosts

Supplementary Table 5 - Identified protist PHADs

Supplementary Table 6 - Signal peptide prediction of protist PHADs

Supplementary Table 7 - Sequences of the HCR-FISH probe specific to the symbiont and host PHAD

References

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