Genetic Identification of Marine Invertebrate Larvae from the Central Oregon Coast

Laurel Hiebert **&** **Greg Gavelis**
lhiebert@uoregon.edu gavelis@uoregon.edu

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Instructor: Svetlana Maslakova
TA: Erin (Ezzy) Cooper

INTRODUCTION

Coos bay is located on the southwestern coast of Oregon. It forms an S-shaped junction between the Pacific Ocean and the Coos River, 16 km long and 3 km wide (Figure 1). Subject both to tidal flux and freshwater outflow, it is an estuary, and serves as a productive habitat for various invertebrates (Roye, 1979).

Many marine invertebrate species inhabiting Coos Bay have planktonic larvae, which are morphologically and ecologically distinct from the adults. In the dynamic estuarine system of Coos Bay, exchange may occur between local and distant larval populations, contributing to a complex planktonic community.

Understanding the composition of this rich larval community provides not only a measure of species diversity but also provides insights into the timing of reproduction and development of contributing species. With this information on larval composition, inferences can be also made about population connectivity, dispersal patterns, and recruitment. Additionally, investigation of larval species composition can reveal cryptic species and invasive species that might otherwise go undetected.

Various methods are available for identification of marine invertebrate larvae. While morphology-based taxonomy is extremely valuable for marine invertebrate larval identification, this method becomes limited in certain circumstances. Complications arise due to phenotypic plasticity within species, and low morphological variability between species, which often make it difficult to identify larvae beyond genus, family, or even phylum. Larvae may also be collected at stages too young to exhibit specific diagnostic characters.

Culturing may also be used for larval identification, but in the case of broad planktonic sampling, this method may be impractical. It is difficult to raise a larva to metamorphosis without knowledge of its specific food preferences or settlement cues, and the process can be further complicated by the organism's sensitivity to the laboratory environment.

DNA barcoding has emerged as an efficient method for biological identification when morphology-based identification becomes problematic (Hebert *et al.*, 2003). For example,

genetic information has been useful to associate different developmental stages and to identify partially preserved specimens. In this study, larval identification was accomplished using DNA barcoding of three genes, cytochrome *c* oxidase subunit I, histone 3, and 16S rRNA. Larval sequence information was compared to previously cataloged adult sequences using the Basic Local Alignment Search Tool (BLAST) and phylogenetic analysis.

MATERIALS AND METHODS

Planktonic invertebrate larvae were collected at three locations near Coos Bay, OR (43° 20′ 25.01″ N, 124° 19′ 44″ W) on October 9 and 30, 2008. Larvae were collected between depths of 1 and 3m from the Charleston Marina (F Dock) using a 243μm mesh size plankton net. A 202μm mesh size plankton net was used to collect larvae from just inside and just outside of the Coos Bay mouth near buoy 1 at depths of 12 and 20m respectively.

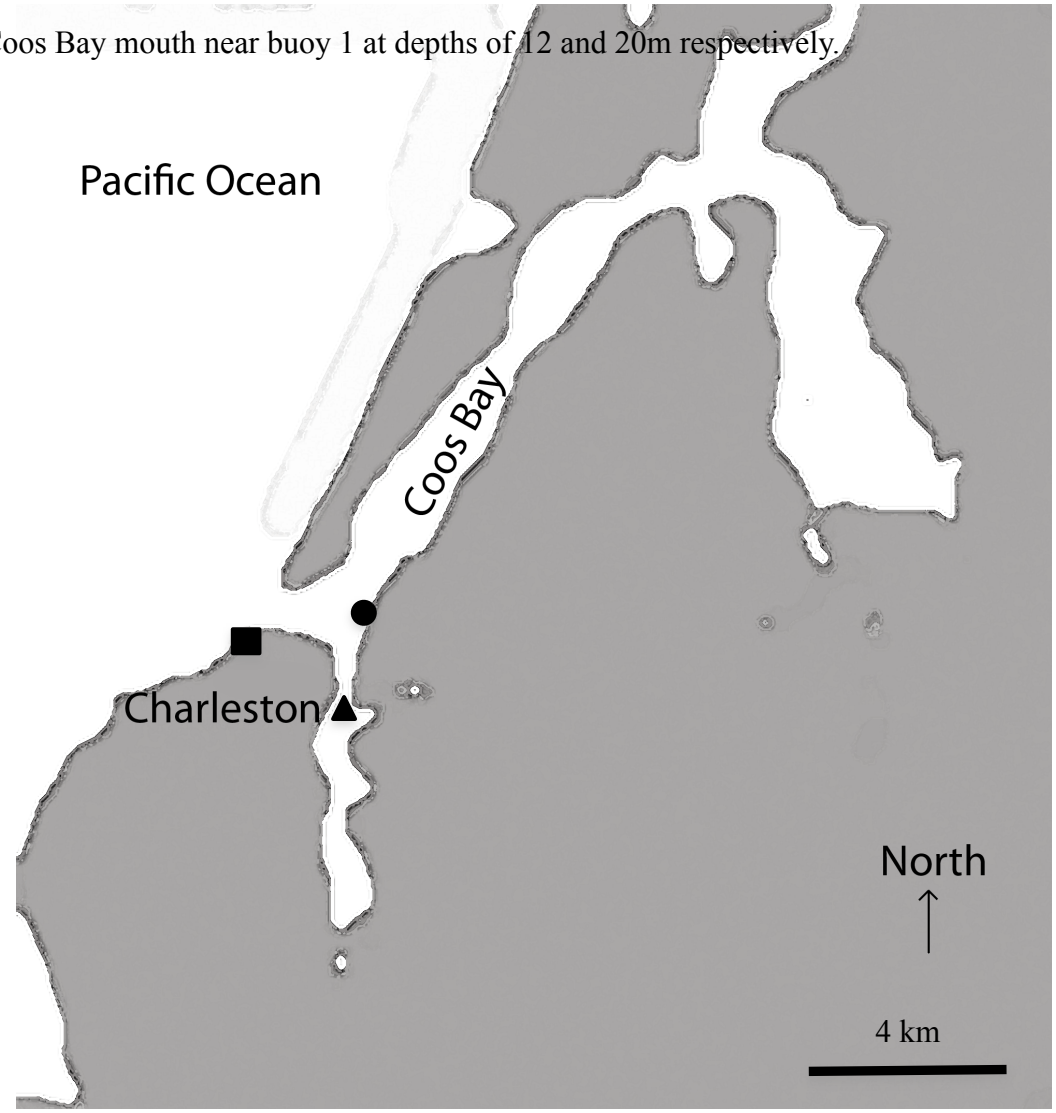

Figure 1: Sampling sites in Coos Bay, OR. Charleston Marina site designated by a triangle. Coos Bay site designated by a circle. Just outside Coos Bay Mouth designated by a square.

Table 1: Information about larvae collected for study

Larva	Location and Tide	Date Collected
Pilidium #1	*Ocean* (1.75m, outgoing)	Oct. 9, 2008
Pilidium #2	*Bay* (1.75m, outgoing) *Ocean* (1.75m, outgoing) *Dock* (1.2m, incoming)	Oct. 9 & 30, 2008
Pilidium #3	*Ocean* (1.75m, outgoing)	Oct. 9 & 30, 2008
Pilidium #4	*Ocean* (1.75m, outgoing) *Dock* (1.2m, incoming)	Oct. 9 & 30, 2008
Pilidium #5	*Ocean* (1.75m, outgoing)	Oct. 10, 2008
Pilidium # 6	*Ocean* (1.75m, outgoing)	Oct. 9, 2008
Palaeonemertean Larva	*Ocean* (1.75m, outgoing)	Oct. 9, 2008
Hoplonemertean Larva	*Bay* (1.75m, outgoing) *Ocean* (2.4m, outgoing)	Oct. 9 & 30, 2008
Flatworm	*Dock* (1.2m, incoming)	Oct. 30, 2008
Pelagosphaera	*Dock* (1.2m, incoming)	Oct. 30, 2008
Trochophore	*Bay* (1.75m, outgoing)	Oct. 9, 2008
Magelona Setiger	*Ocean* (1.75m, outgoing)	Oct. 9, 2008
Spionid	*Bay* (1.75m, outgoing)	Oct. 9, 2008
Veliger	*Ocean* (1.75m, outgoing)	Oct. 9, 2008
Echinoid or Ophiuroid Prism	*Ocean* (1.75m, outgoing)	Oct. 9, 2008
Dendraster Pluteus	*Ocean* (1.75m, outgoing)	Oct. 9, 2008
Brachiolaria	*Dock* (1.2m, incoming)	Oct. 30, 2008

'Dock' refers to collections from the F dock in the Charleston marina. 'Ocean' refers to collections from just outside Coos Bay. 'Bay' refers to collections from just inside Coos Bay Mouth.

Genomic DNA was extracted using InstaGene™ Matrix (Biorad) Kit, using the protocol for bacterial colonies. Live larvae were rinsed in 1 ml DNAse-free water before incubation IntsaGene Matrix. DNA was stored at -20°C.

From the extracted DNA, three genes were PCR-amplified using universal metazoan primers: the nuclear gene encoding histone H3 and the mitochondrial genes encoding

cytochrome *c* oxidase subunit 1 (COI) and ribosomal 16S rRNA. Histone H3 DNA was amplified using H3NF [ATGGCTCGTACCAAGCAGAC] and H3R [ATATCCTTRGGCATRATRGTGAC] (Clogen *et al*., 2000), COI using LCO1490 [GGTCAACAAATCATAAAGATATTGG] and HCO2198 [TAAACTTCAGGGTGACCAAA AAATCA] (Folmer *et al.*, 1994), and 16S16ARL [CGCCTGTTTATCAAAAACAT] and 16BRH [CCGGTCTGAACTCAGATCACGT]16S ARL and 16S BRH (Palumbi *et al*., 1991). The PCR reaction was carried out with 1µl supernatant from InstaGene DNA extraction, 200µM dNTP mix, 1U/Rx GoTaq™ Polymerase (Promega), and 500 nM of the respective primer pairs. Cycling parameters for the initial PCR reaction were as follows: initial denaturation (95°C, 2 min), 35 cycles of: cycle denaturation (95°C, 40 sec), primer annealing (52°C, 40 sec), primer extension (72°C, 1 min), and final extension (72°C, 2 min).

In order to determine whether the desired genes were amplified, gel electrophoresis was carried out using 1% agarose and 0.5X Tris-borate EDTA buffer with 0.1 µg/ml of EtBr. Size comparisons were made with a 1Kb ladder (Promega). The COI gene is 658 bp, H3 sequence is 304 bp and the 16S measures approximately 460 bp in length. Gel images were viewed and documented using an Alpha Innotech Red™ Gel Imager.

Gel images revealed bright bands of the expected length were in some cases accompanied by dim bands of varying lengths. Conditions of the PCR reaction were modified to select for amplification of the desired product in such cases. The annealing temperature was raised to 55°C to increase the stringency of the reaction. If the modification failed to eliminate secondary bands, the samples were reamplified with a 56°C annealing temperature.

In circumstances where the desired band was dimmer or of equal brightness to secondary bands, gel extraction was performed to excise the band of the correct length. Remaining PCR product (approximately 17µl) was run on a 1% agarose gel in 0.5X Tris-borate EDTA buffer with 0.1 µg/ml of EtBr for one hour at 150 volts. Gel slices and PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). DNA was eluted in 15 µl of nuclease-free water, and stored at 4°C.

To quantify the DNA product, 1 µl of each purified sample was loaded onto a 1.5% agarose gel. Band size was quantified by comparison to a Low Mass Ladder (New England

BioLabs). For sequencing, the DNA was diluted to 1ng/100bp/rxn. Labeling was done using BigDye Terminator v 3.1 Cycle Sequencing kit chemistry (Applied Biosystems). Sequencing was done on an automatic sequencer 3130XL Genetic Analyzer (Applied Biosystems).

Sequences were analyzed and trimmed using Codon Code Aligner (Codon Code Corporation, MA, USA). BLAST searching was used to identify larval sequences (Altschul, 1990). Percent coverage and percent similarity were used to evaluate the plausibility of the closest match for species or genus identity. All nemertean larval sequence data and additional nemertean sequences from NCBI were used to build phylogentic trees (Table 2). Sequences from three local species of unidentified adult nemerteans named "pink *Micrura,*" "white palaeonemertean" and "brown palaeonemertean" were included in the analysis. Nemertean sequence alignment was done with CLUSTALX (Jeanmougin *et al.,* 1998) using 10 – 5 gap-gap length alignment parameters (Thollesson & Norenburg, 2003). Phylogenetic trees were constructed with PAUP* (Swofford, 2000) using maximum parsimony as the optimality criterion with default settings. Trees were viewed in TreeView (Page, 1996).

Table 2: Accession numbers for nemertean sequences and outgroup sequences used for phylogenetic analysis.

Taxa and Gene	Accession Numbers
Nemertean COI	EF157586-89, EU255601-30, DQ11370-97, AY791971-97, AJ436896-949
Nemertean 16S	EF157574-85, AY955227-32, DQ911371-96, AY340467, DQ280022, DQ011575, DQ022549-51, AJ436786-839, AF065097-102, U55845, AF103754-67
Nemertean H3	DQ279996, AJ436952-89, AF19235-94
Outgroup COI	AF077759, U68773
Outgroup 16S	AF192295, AF023541, EF521183, AF331161
Outgroup H3	AF185264

RESULTS, DISCUSSION, AND CONCLUSIONS

For each of the 17 larvae collected, amplification and sequencing was attempted for genes COI, 16S and H3, which yielded data at 53%, 82%, and 35% success rates, respectively. Overall, the sequencing success was 53%. All larvae were represented by at least one sequence.

Non-nemertean Larval Identification

For most of the non-nemertean sequences, the BLAST search closest match fell within a reasonable higher taxon (Table 3). For example, the closest match for the spionid larva fell within the expected order (Spionidae), and the flatworm larva fell within the polyclads, with a few exceptions; the pelagosphaera larva matched most closely with a catfish. Yet, the Gene Bank inventory and sequence resolution could not reliably identity larvae to the proper genus and species, in many cases, for instance, the flatworm larva closest match was a Japanese flatworm species. Only two of the nine non-nemertean larvae had expected closest that fell within the threshold of intraspecific variation. Both of these were echinoderms, whose maximum intraspecific variation for 16S and COI are 1% and 0.62% correspondingly (Mastubara *et al.,* 2004; Ward *et al.,* 2008). High maximum identity (above 99.6%) and coverage values for echinoid or ophiroid prism and the brachiolaria support their placement within *Brisaster latifrons* and *Pisaster ochraceus*, respectively.

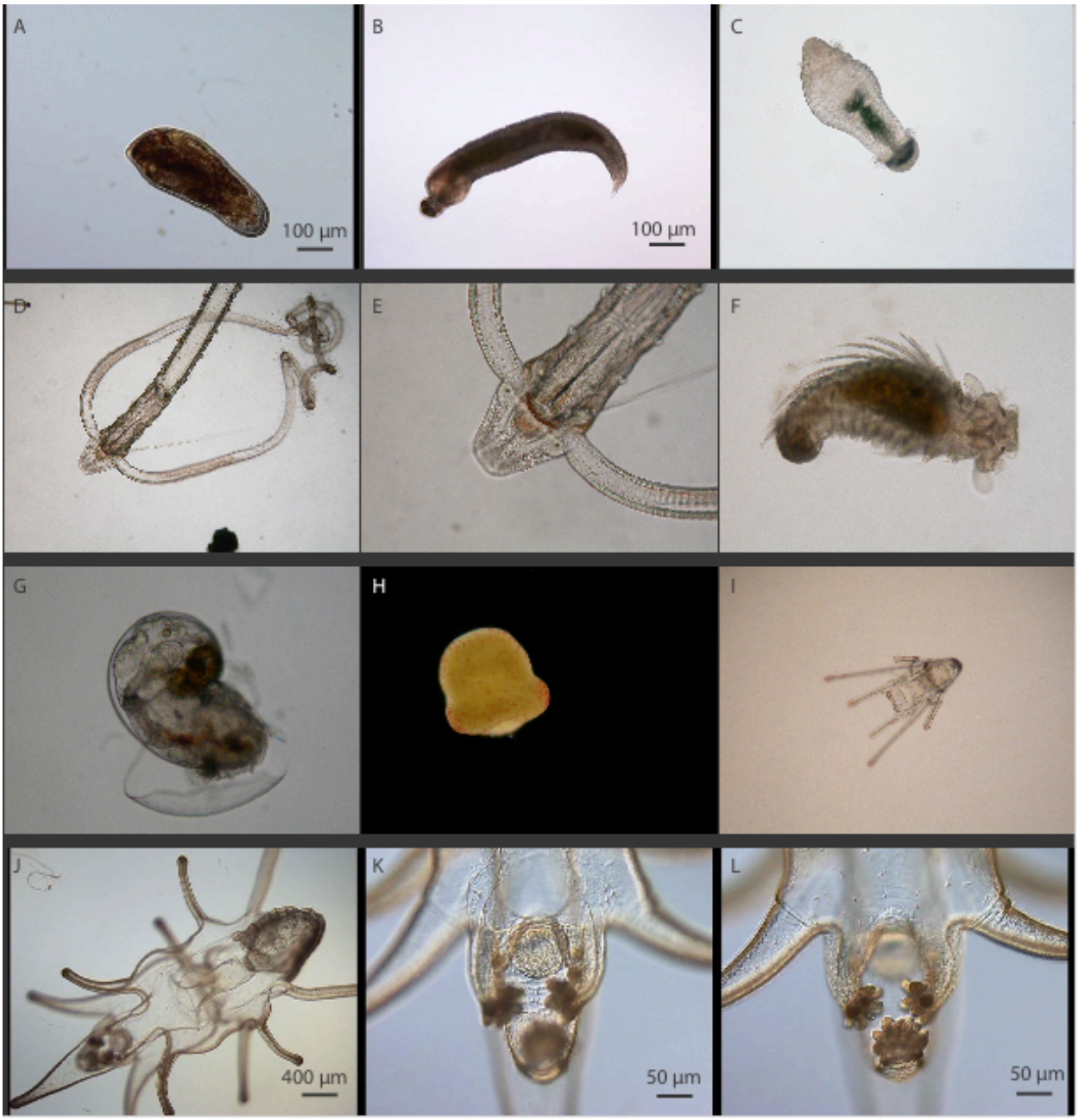

Figure 2: Non-Nemertean Larvae from Coos Bay. A. Flatworm B. Pelagosphaera C. Trochophore. D. & E. *Magelona* setiger. F. Spionid. G. Veliger. H. Echinoid or ophiuroid prism. I. *Dendraster* pluteus. J-L Brachiolaria. K-L Brachiolar arms and adhesive disk.

Figure 3: Nemertean Larvae from Coos Bay. A. & B. *Pilidium Pyramidum* morphotype: Pilidium #1 and Pilidium #5. C. - E. *Pilidium Microdoma* morphotype: Pilidium #2, Pilidium #6, and metamorphosed juvenile. F. & G. *Pilidium Recurvum* morphotype: Pilidium #3. H. & I. *Pilidium Megadoma* morphotype: Pilidium #4 and close-up of juvenile. J. Hoplonemertean larva. K. & L. Palaeonemertean larva and close-up of head.

Nemertean Phylogenetic Analysis

There were eighteen most parsimonious trees generated from COI sequence data. The best tree score was 5,266. From 16S, seven trees were generated, and only one for H3, with scores of 5,207 and 1,028, correspondingly. As expected, pilidiophorans branch high within the COI tree (Figure 4 and 5). Unexpectedly, not all palaeonemerteans are confined to the base. Rather, many are grouped as sister to the pilidiophorans. In the 16S tree, palaeonemerteans were sister to hoplonomerteans, and together they were sister to the pilidiophorans (Figure 6 and 7). In the H3 tree, quite surprisingly, pilidophorans form a basal group, palaeonemerteans branch higher, both exhibiting polyphyly. Hoplonemerteans in the H3 tree form a derived monophyletic clade (Figure 8).

Pilidium #1 & 5: Pilidium Pyramidum

We considered Pilidium #1 and #5 to be a single morphotype (Figure 3A and 3B), which we call *Pilidium Pyramidum*. Both were characterized by a tall pyramidal episphere with dark pigment spots along the lobe margins. The juvenile was positioned perpendicular to the apical axis and displayed a caudal cirrus and cephalic slits. For Pilidium #1, the COI gene BLAST search was restricted to heteronemerteans since initial BLAST search generated a hoplonemertean closest match. Within heteronemerteans, *Lineus geniculatus* was the closest match for the COI gene. The H3 sequence of Pilidium #1 found a closest match in *Cerebratulus marginatus*, however the 10% divergence between the two does not fall within the range of intraspecific variation. As such, we are not confident of the placement of this larva within *C. marginatus,* but possibly within another species of this genus. The 16S sequence for Pilidium #5 places it with *Cerebratulus sp.* from the Swedish coast (Saundberg & Saur, 1998). The presence of longitudinal cephalic slits and a caudal cirrus suggest placement within Lineidae, likely *Cerebratulus* or *Micrura.* Pilidium #1 and 5 are similar to *Cerebratulus* A and B as described by Lacalli (2005). A closest match within *Cerebratulus* suggests that we search for a match among local *Cerebratulus* species, such as *C. albifrons, C. californiensis, C. herculeus, C. longiceps, C. montgomeryi* and *C. occidentalis.*

Pilidium #2 & #6: Pilidium Microdoma

Sequences of Pilidium #2 & #6 differed by six and zero base pairs for COI and 16S respectively, well within the range of intraspecific variation. The pilidium, which we refer to as *Pilidium Microdoma,* had a small rounded episphere that housed a large juvenile with two red eyespots and cephalic slits, suggesting that it is within the family Lineidae. We encountered this morphotype six times within the bay and the ocean. Lacalli (2005) also found a very similar morphotype that was the most common pilidial type in the plankton near Bamfield, British Colombia. Phylogenetic analysis placed it as sister to *Lineus bicolor.* However, divergence for 16S and COI (84-94%) is well outside the range of interspecific variation and perhaps outside the range of intergeneric variation. Additionaly, *Lineus bicolor* is not a strong candidate as it is from the east coast. With confidence, *Pilidium Microdoma* belongs to family Lineidae. We suggest that it is related to those lineids lacking a caudal cirrus. Since the juvenile had eyespots, we should search for candidates among local species of lineids possessing eyespots and lacking caudal cirri. *The Light and Smith Manual* (2007) lists seven species for this area, of these, *Lineus flavescens, L. pictifrons, L. rubescens,* and *L. bilineatus* are potential candidates as they possess eyespots. In the future, we will attempt to collect these adult candidate species and obtain sequence data in order to resolve the identity of these pilidia.

Pilidium #3: Pilidiu Recurvum

The morphology of Pilidium #3 resembles *Pilidium Recurvum* from Vung-ro Bay, Vietnam (Dawydoff, 1940). It also resembles *Pilidium Recuvatum*, from Sweden, described by Cantell (1966), but lacks a horizontal ciliated band. We obtained sequences from all three genes, which agree on the placement of this larva with *Riserius pugetensis*, an aberrant interstitial mesopsammic heteronemertean described by Norenburg (1993) from Puget Sound. Sequence divergence is sufficient to establish that it is a different species and possibly a different genus. Norenburg observed that ripe females of *R. pugetensis* contained up to 300 eggs in the ovaries. All described pilidiophorans with planktonic larvae have thousands of eggs. It is unlikely that

this species has a planktonic larva given its small size (less than 15 mm in length) and low reproductive output. Cantell, in 1966, suggests that *Pilidium Recurvatum* belongs to the family Baseodiscidae since the juvenile lacks a caudal cirrus and cerebral grooves. Analysis of the 16S sequences, including four sequences of baseodiscids does not group *Riserius* or Pilidium #3 in the Baseodiscidae. No baseodiscids have been described north of Monterey Bay. It is unlikely that *Pilidium Recurvum* belongs to *Baseodiscus* and is more likely that it is a macroscopic undescribed non-lineid pilidiophoran closely related to *R. pugetensis,* capable of producing large numbers of eggs. According to *The Lights Manual,* the only non-lineid pilidiophorans species from the west coast of North America are *Baseodiscus punnetti* and *Zygepolia rubens*. According to Coe (1940) *Baseodiscus delineatus* and *B. princeps* can also be found.

Pilidium #4: Pilidium Megadoma

Pilidium #4, which we call *Pilidium Megadoma,* has chromatophores along the lobe margin, which appear yellow-green in reflected light and pink in transmitted light (Figure 3, __). Its spacious episphere houses a juvenile (with caudal cirrus), which is inclined diagonally toward the apical organ. Sequence analysis of the COI gene places Pilidium #4 in a clade with *Cerebratulus marginatus* and *Lineus geniculatus,* whereas 16S groups it among *C. marginatus* and H3 with *Parborlasia corrugatus* and *Micrura alaskensis*. We can say with confidence that it is a pilidiophoran, however, no specific heteronemertean placement is supported. The presence of a caudal cirrus suggests that it belongs to *Cerebratulus, Lineus, Micrura,* or *Zygelopia. Zygelopia* is an especially plausible candidate as the larva has a caudal cirrus and no observable cephalic slits. However, it may be too early in development for detection of these organs. Molecular data likely did not provide definitive identification due to lack of corresponding adult sequences in GeneBank. This was a recurring obstacle throughout our analysis.

Palaeonemertean larva

The palaeonemertean larva displays a single midventral eye anterior to the mouth, a characteristic feature of the *Carinomidae* (Maslakova, 2004; Maslakova, personal communication). This affinity is supported by 16S sequence data, which place the

palaeonemertean as sister to *Carinoma tremaphoros*. Along with *C. mutabilis*, *C. tremaphoros* is one of two *Carinoma* known to North America, which reside on the west and east coasts, respectively. Interestingly, the palaeonemertean larva groups more closely with the Atlantic species, diverging 11.8% from *C. mutabilis* and only 8% from *C. tremaphoros*. These divergence values are comparable to the divergence between the two species of *Carinoma* (13.3%) and appears to place the palaeonemertean larva as a separate species. If this measure is correct, there exists a third, undescribed *Carinoma* species on the west coast of North America. Further sequence data should be taken from presumed *Carinoma mutabilis* adults to sample for potential cryptic biodiversity.

Hoplonemertean larva

Both the 16S and COI sequence data group this hoplonemertean larva with *Carcinonemertes* sp414, with high divergence (14%), which cannot suggest its placement within the genus with confidence. *Carcinonemertes* sp414 was obtained from the south coast from Brazil. The hoplonemertean larva may be a related *Carcinonemertes* from the west coast where only two species, *C. epialti* and *errans,* are known (Stricker & Reed, 1981; Carlton, 2007). These species have only one pair of eyes whereas this larva is characterized by two. The first pair is directly in front of the brain, five times closer together than the pair towards the anterior. It possesses large double eyes, the anterior ocelli measuring 25 µm in diameter. The stylet basis measures 37.5 um, while the stylet measures 12.5 µm, generating a basis-to-stylet ratio of 3. No accessory stylet sacs or cerebral organs were observed. The contracted larva measures 450 by 600 µm. The short bipartite proboscis suggests that it could be within Carcinonemertidae or Emplectonemidae, yet sequence data for *C. epialti* and *errans* are lacking to clarify this issue.

Table 3: Divergence Scores for Non-nemertean Larvae.

Larva	Gene	Closest Match (Probable affiliations in bold)	Query Coverage	Maximum Identity
Flatworm Larva	16S	*Pseudostylochus intermedius* (Polyclad flatworm)	22%	83% (71/85)
Pelagosphaera	COI	*Silurus asotus* (Catfish)	88%	95% (523/548)

Magelona Setiger	16S	*Pygulopsis avernalis* (Gastropod)	100%	79% (393/496)
Trochophore	16S	*Phascolosoma esculenta* (Sipunculid)	42%	81% (161/198)
Spionid	16S	*Polydora giardi* (Spionid)	68%	82% (263/319)
Veliger	COI	*Oliva mustelina* (Gastropod)	96%	82% (423/514)
	H3	*Raphitoma sp.* (Gastropod)	100%	94% (291/308)
Echinoid or Ophiroid Prism	16S	***Brisaster latifrons*** (Heart Urchin)	100%	99.8% (544/545)
Dendraster Pluteus	16S	*Echinolampas crassa* (Sand Dollar)	100%	88% (470/532)
	COI	*Paracalanus parvus* (Copepod)	100%	87% (559/636)
Brachiolaria	16S	***Pisaster ochraceus*** (Asteroid)	100%	99.6% (540/542)

Table 4: Divergence Scores for Nemertean Larvae.

Larva	Gene	Closest Match (Probable affiliations in bold)	Query Coverage	Maximum Identity
Pilidium #1	COI	*Lineus geniculatus*	97%	79% (496/625)
	H3	*Cerebratulus marginatus*	98%	90% (276/304)
Pilidium #2A	16S	***Lineus*** *bicolor*	99%	89% (411/457)
	COI	*Lineus bicolor*	98%	84% (532/626)
	H3	*Lineus bicolor*	100%	94% (291/319)
Pilidium #2B	16S	***Lineus*** *bicolor*	99%	90% (417/463)
	COI	*Lineus bicolor*	98%	84% (532/626)
Pilidium #3: Recurvum	16S	*Riserius pugetensis*	100%	90% (446/492)
	COI	*Riserius pugetensis*	92%	84% (503/593)
	H3	*Riserius pugetensis*	98%	94% (286/304)
Pilidium #4	16S	*Cerebratulus marginatus*	100%	92% (447/484)
	COI	*Lineus longissimus*	99%	83% (528/630)
	H3	*Cerebratulus marginatus*	99%	92% (284/306)
Pilidium #5	16S	***Cerebratulus*** *sp.*	95%	94% (425/452)
Palaeonemertean Larva	16S	***Carinoma*** *tremaphoros*	100%	92% (437/474)
Hoplonemertean Larva	16S	*Carcinonemertes sp. 414*	98%	86% (375/432)
	COI	*Carcinonemertes sp. 414*	100%	86% (549/638)

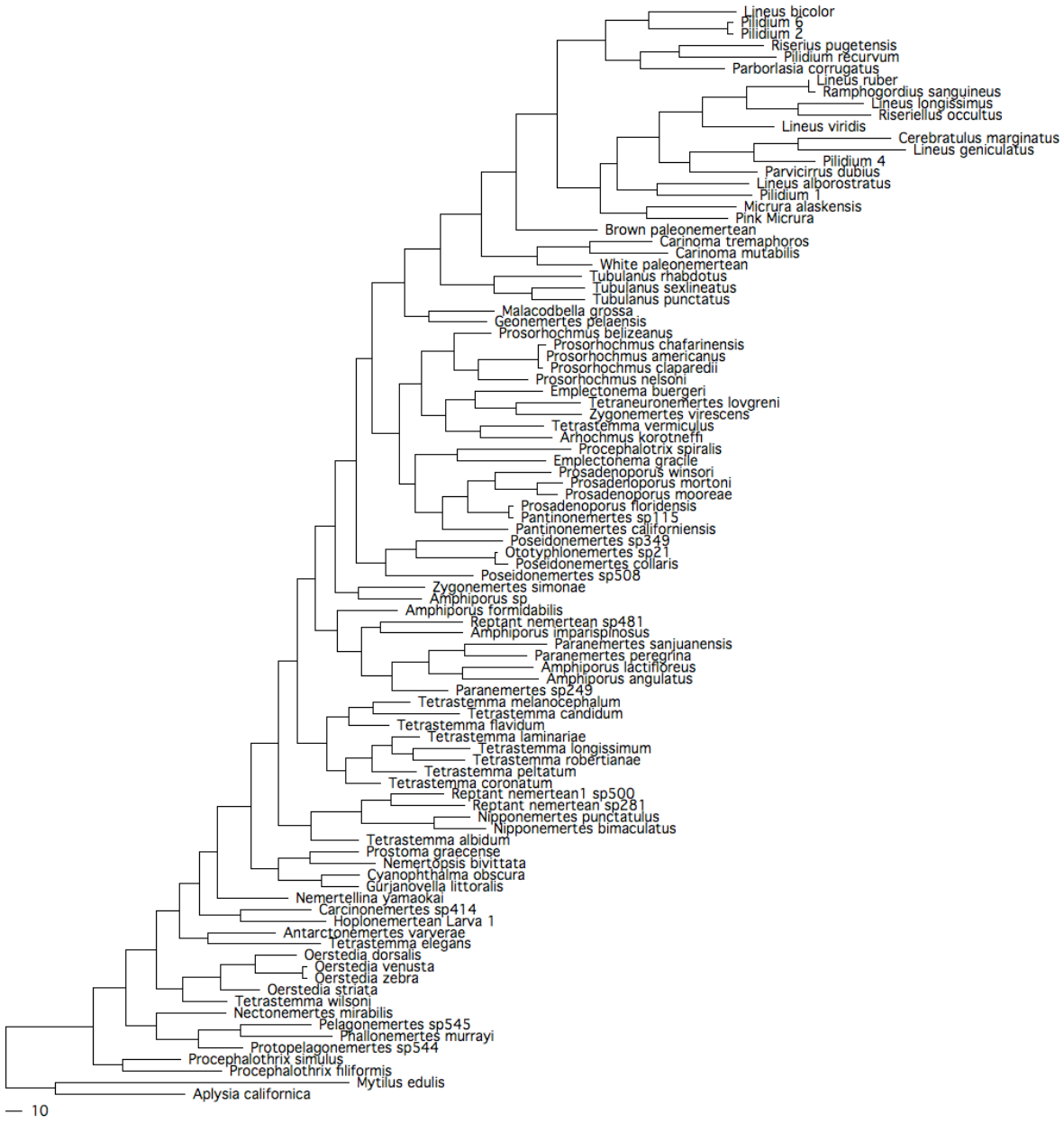

Figure 4: One of 18 most parsimonious trees constructed using COI sequence data of nemerteans and nemertean larvae from Coos Bay.

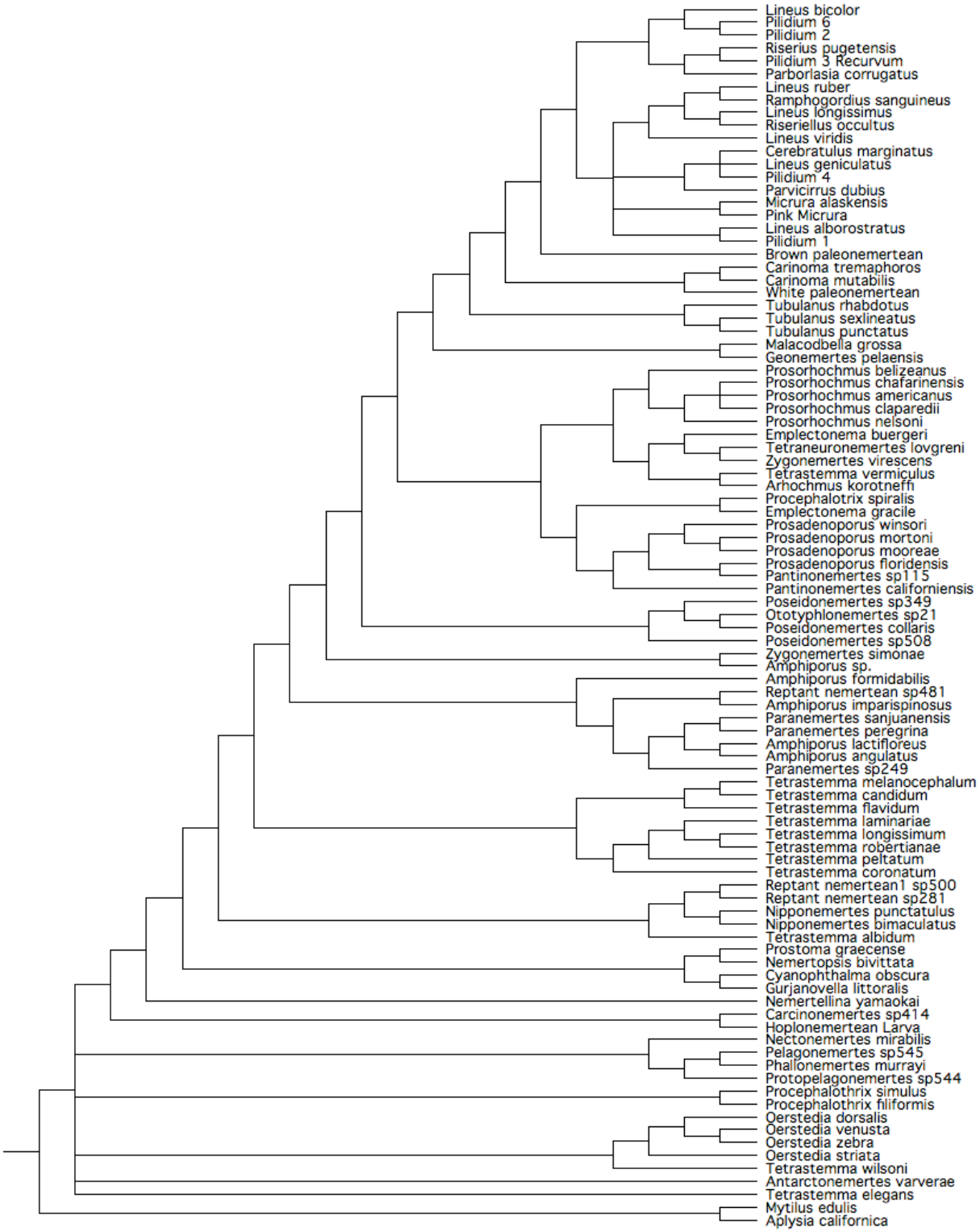

Figure 5: Consensus of 18 most parsimonious trees constructed using COI sequence data of nemerteans and nemertean larvae from Coos Bay.

Figure 6: One of seven most parsimonious tree constructed using 16S sequence data of nemerteans and nemertean larvae from Coos Bay.

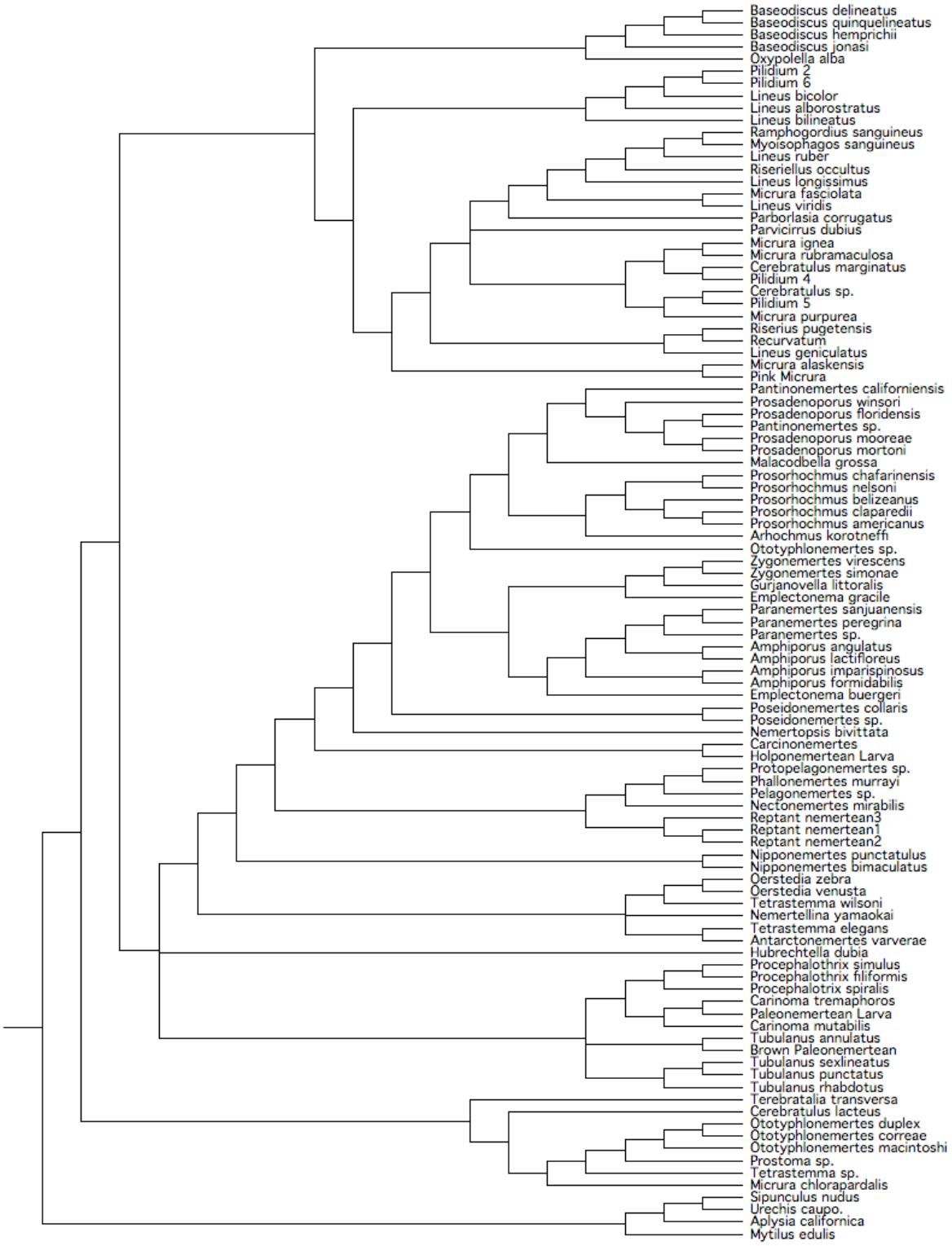

Figure 7: Consensus of seven most parsimonious trees constructed using 16S sequence data of nemerteans and nemertean larvae from Coos Bay.

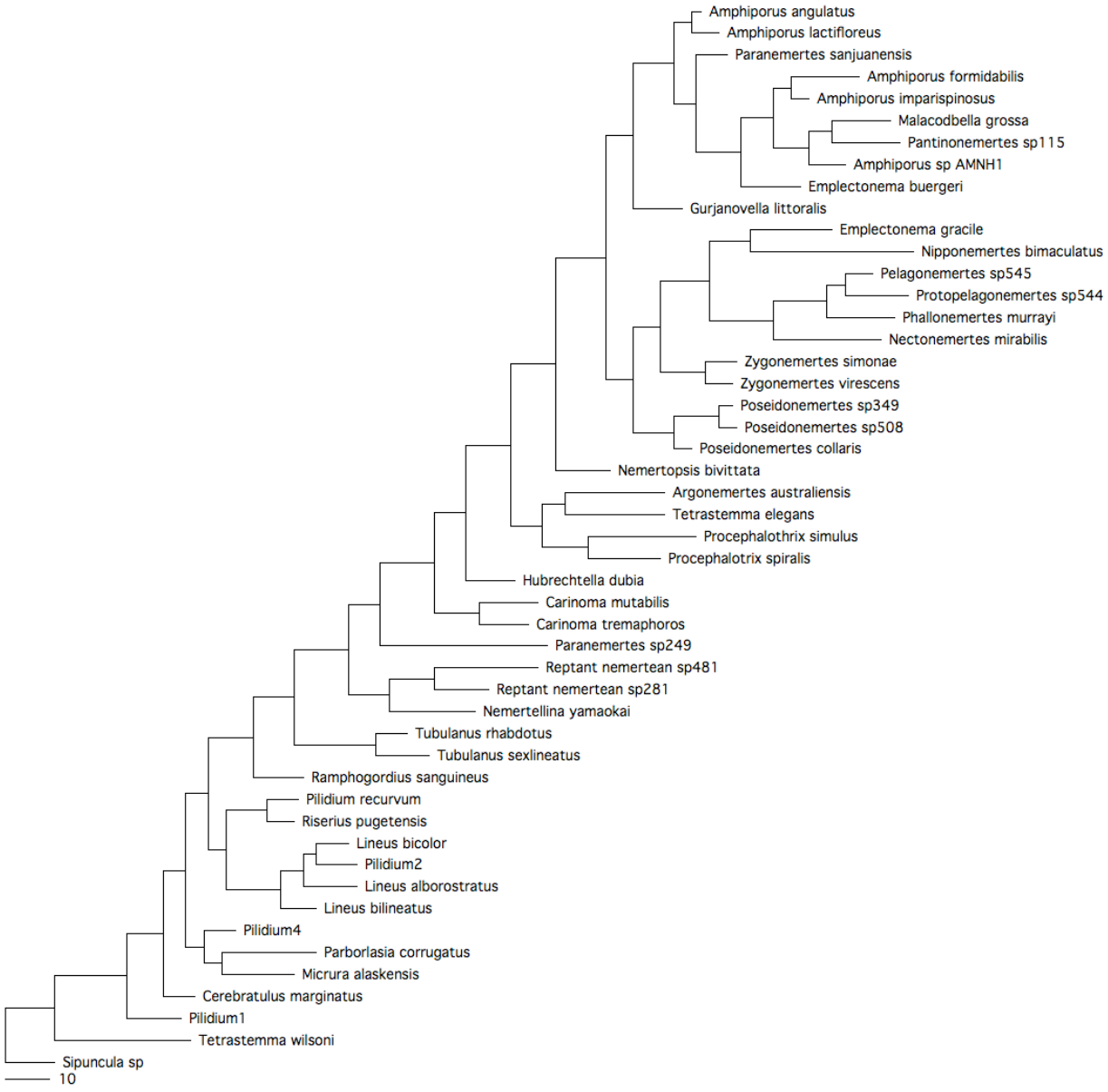

Figure 8: The most parsimonious tree constructed using H3 sequence data of nemerteans and nemertean larvae.

CONCLUSION

As has been demonstrated, the success of sequencing varied for each of the three genes COI, 16S, and H3. Likewise, placement of the larvae within trees and BLAST closest matches varied depending on the genes used to construct them. It was through joint consideration of

multiple genes as well as morphology that some specimens could be classified. A future study could bolster identification through combined phylogenetic analysis of multiple genes, possibly incorporating 28S information as a well, as performed by Thollesson and Norenburg (2003). Yet the immediate task at hand should be the collection and inventory of sequences from local adult nemerteans, with special attention to the candidates discussed previously. For larval identification to be made with confidence, there must be a more comprehensive index of adult sequences with which to compare. In the end, the dearth of sequences in GeneBank was the greatest obstacle to larval identification.

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