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Who's Who in the Sipuncula: Matching Larvae and Adults Using DNA

Anja Schulze,[1] Jacquelin Hipes,[2] Elizabeth Borda,[3] and Mary E. Rice[4]*

ABSTRACT. Sipunculan pelagosphera larvae are represented by many distinct morphotypes that can be distinguished by size, color, ciliation pattern, texture of the body surface, and head morphology. Some larval types have been reared to adulthood in the lab, but for many of the morphotypes species identification has previously not been possible. We sequenced larvae of 14 different morphotypes for mitochondrial and nuclear markers and performed phylogenetic analyses including larval and adult sequences. The adult sequences covered 16 of the 17 currently recognized sipunculan genera and more than one-third of the 150 known sipunculan species. Analyses were conducted in two phases: the first phase involved the full data set of adult sequences and eight larval morphotypes; in the second phase, individual clades were analyzed separately on the basis of only one of the markers. Of the 14 larval morphotypes included in this study, 11 were identified to species, and 3 were identified only to genus level. We also reconciled the terminology for the larval types used in this study with that of previous studies.

INTRODUCTION

Sipunculan pelagosphera larvae can be common in plankton samples from surface or near-surface tows, especially in warmer waters. Pelagospheras are easily recognizable as such because they tend to be relatively large and often stand out because of their brilliant coloration. They may remain planktonic for months and are regarded as the primary means of dispersal in sipunculans (Scheltema and Hall, 1975).

Larval development is not uniform throughout Sipuncula. Some species are direct developers or have abbreviated larval development (Rice, 1967, 1975a, 1975b, 1976). However, the majority of species for which development has been studied go through two consecutive larval stages: a lecithotrophic trochophore and a planktotrophic pelagosphera. The trochophore is small, relatively short-lived, and not usually found in plankton samples. In this chapter, we consider only the pelagosphera larvae.

The spherical to elongate body of the pelagosphera is separated from the retractable head region by a distinct constriction (Figure 1). Swimming is accomplished by ciliary action of a pronounced metatroch that is located just anterior to the constriction. A prototroch is usually also present but is less conspicuous than the metatroch. The head morphology, with a characteristic lower lip, is distinctive as well. The posterior end often has a telescopic terminal organ, which is used for temporary attachment and possibly other purposes. Following disturbance, the larvae can retract the entire head region, including the metatroch, into the trunk. This behavior temporarily renders them incapable of swimming. Many other unique behaviors have been observed in pelagosphera larvae and are described in more detail by Rice et al. (this volume).

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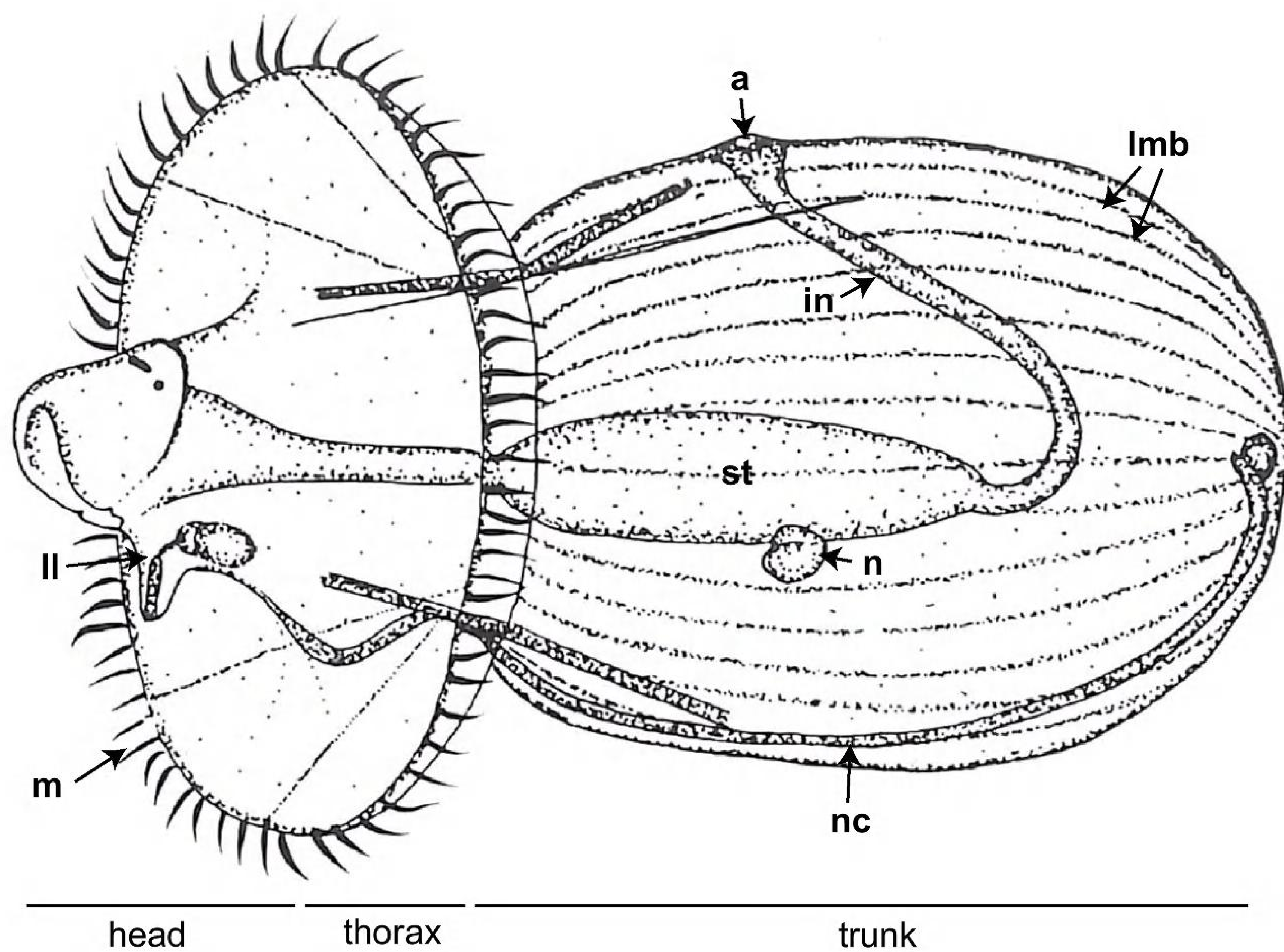

FIGURE 1. Morphology of a sipunculan pelagosphera larva, belonging to *Sipunculus polymyotus* (large transparent or type S; modified from Hall and Scheltema, 1975). Abbreviations: a = anus, in = intestine, ll = lower lip, lmb = longitudinal muscle bands, m = metatroch, n = nephridium, nc = nerve cord, st = stomach.

Many different morphotypes of pelagosphera larvae can be distinguished. Detailed descriptions of their morphology, development, and behavior can be found in Rice et al. (this volume). They differ with regard to body shape and texture, pigmentation, ciliation patterns of the head, and the shape of the lower lip and of the terminal organ, if present. Some of these morphotypes are more common than others and, consequently, have been observed and described in more detail. The larvae do not readily metamorphose in culture and usually die before showing any adult characteristics. However, some attempts at cultivating oceanic sipunculan larvae have been successful (Rice, 1986, 1988, unpublished data). Hall and Scheltema (1975) described 10 larval morphotypes from open-ocean plankton tows taken throughout the Atlantic but could only identify one of them to species on the basis of the number of longitudinal body wall muscles.

We have utilized a DNA barcoding approach to identify individual larvae to species. Species identification of pelagosphera larvae will contribute to a better understanding of zooplankton diversity and provide new insights into population connectivity of geographically widespread species. Furthermore, given the relatively simple and conserved body plan of adult sipunculans, larval morphology reveals an additional suite of characters useful for phylogenetic studies.

Identification of pelagosphera larvae via DNA barcoding is possible because an extensive database of DNA sequences from carefully identified adult sipunculans has been generated in several phylogenetic studies (Maxmen et al., 2003; Staton, 2003; Schulze et al., 2005, 2007; Kawauchi et al., 2012). The reference sequences encompass six gene regions, more than one-third of all sipunculan species, and all but one of the currently recognized sipunculan genera.

MATERIALS AND METHODS

Collections

Larvae were collected from zooplankton tows with nets of 100–200 μm mesh size, towed behind small boats or larger research vessels (Table 1). Zooplankton samples were microscopically sorted, and pelagosphera larvae were separated from other planktonic organisms. Larvae were relaxed in a 1:1 solution of 7.5% magnesium chloride and seawater or by adding drops of

TABLE 1. Larval samples, collection information, and GenBank accession numbers for the samples analyzed in this study. Gene markers used were mitochondrial cytochrome *c* oxidase subunit I gene (COI), nuclear histone H3 gene (H3), and nuclear 18S ribosomal RNA gene (18S rRNA). A dash (—) indicates not applicable.

Code	Larval type	Sampling location	Vessel and station code	Date	18S rRNA	COI	H3
LT1	Large transparent	Carrie Bow Cay, Belize	CB03-13A	25 Apr 2003	EU266987	—	EU266978
ST1	Smooth transparent	Florida Current	R/V *Sunburst* SB567	9 May 2005	—	JX989041	JX989052
SO1	Smooth orange	Florida Current	R/V *Sunburst* SB295-297	11–13 Nov 1993	—	JX989042	JX989053
SO2	Smooth orange	Florida Current	R/V *Sunburst* SB555	26 Jul 2004	—	—	JX989054
YG1	Yellow green	Gulf Stream, North Carolina	R/V *Cape Hatteras* St. 14	23 May 2011	—	—	JX989055
YG2	Yellow green (juvenile)	Florida Current	R/V *Sunburst* SB332	6 Dec 1993	—	—	JX989056
YG3	Yellow green	Carrie Bow Cay, Belize	CB03-13B	25 Apr 2003	EU266991	EU266996	EU266982
TG1	Transverse groove	Florida Current	R/V *Sunburst* SB555	26 Jul 2004	—	JX989043	JX989057
TG2	Transverse groove	Gulf Stream, North Carolina	R/V *Cape Hatteras* St. 8	20 May 2011	—	—	JX989058
TG3	Transverse groove	Florida Current	R/V *Sunburst* SB407	21 Jul 1997	EU266989	EU266994	EU266980
TG4	Transverse groove	Florida Current	R/V *Morning Watch*	20 Sep 1991	—	JX989044	JX989059
KN1	Knobby	Gulf Stream, North Carolina	R/V *Cape Hatteras* St. 8	20 May 2011	—	JX989045	JX989060
KN2	Knobby	Gulf Stream, North Carolina	R/V *Cape Hatteras* St. 14	23 May 2011	—	JX989046	JX989061
KN3	Knobby	Florida Current	R/V *Sunburst* SB567	9 May 2005	—	—	JX989062
WB1	White blackhead	Carrie Bow Cay, Belize	CB03-13F	25 Apr 2003	—	—	JX989063
WB2	White blackhead	Bahamas	R/V *Edwin Link*	16 Apr 1994	EU266990	EU266995	EU266981
SV1	Spotted velvet	Florida Current	R/V *Sunburst* SB295	7 Jan 1993	—	JX989047	JX989064
SV2	Spotted velvet	Florida Current	R/V *Sunburst* SB253	31 Dec 1991	EU266988	EU266993	EU266979
WW1	White white	Carrie Bow Cay, Belize	CB03-13D	25 Apr 2003	EU266992	EU266999	EU266985
YP1	Yellow papillated	Bahamas	R/V *SeaDiver*	23 Oct 1994	—	JX989048	JX989065
YP2	Yellow papillated	Bahamas	R/V *Edwin Link*	20 Aug 1991	—	EU266997	EU266983
YP3	Yellow papillated	Florida Current	R/V *Sunburst* SB253	31 Dec 1991	—	—	JX989066
WO1	White orange metatroch	Florida Current	R/V *Sunburst* SB567	9 May 2005	—	JX989049	JX989067
WP1	White papillated	Florida Current	R/V *Morning Watch*	19 Sep 1991	—	JX989050	JX989068
PP1	Pinkish papillated	Carrie Bow Cay, Belize	CB03-13C	25 Apr 2003	—	—	JX989069
PW1	Pink white papillated	Florida Current	R/V *Sunburst* SB567	9 May 2005	—	JX989051	JX989070

menthol dissolved in ethanol in a small petri dish with seawater and chilling it. Larvae usually showed reduced movement and ciliary beating after 10–20 min and no longer retracted their heads. However, the relaxation techniques were not always successful. Whenever possible, the larvae were photographed by light microscopy and scanning electron microscopy. Photographs of all but four larval types included in this analysis are shown in Rice et al. (this volume: figs. 4–32). They show representatives of the morphotypes but not necessarily the specimen that was used to generate DNA sequences. Larvae were fixed in 95% ethanol and stored at −80°C or directly frozen at −80°C with a minimal amount of seawater. The larval types considered in this paper, their collection information, and abbreviations used in the figures are listed in Table 1.

Sequence Generation

DNA extraction from individual larvae was accomplished using the DNeasy Blood and Tissue kit (Qiagen), following the instructions of the manufacturer. The desired gene regions were amplified from the genomic DNA using polymerase chain reaction (PCR) following protocols described in Schulze et al. (2007; Table 2). Amplified gene regions include the mitochondrial cytochrome *c* oxidase subunit I gene (COI; 649 bp), the nuclear histone H3 gene (327 bp), and a portion of the nuclear 18S ribosomal RNA gene (744 bp). In the case of COI, several combinations of forward and reverse primers were used, as some of them yielded results for only a limited number of samples. PCR products were cleaned using ExoSap-IT (Affymetrix). Cycle sequencing with BigDye Terminator version 3.1 (Applied Biosystems) was conducted using the same primers as for the PCRs. Sequence reactions were cleaned using the BigDye Exterminator (Applied Biosystems) chemistry, and sequences were analyzed on an ABI 3130 Genetic Analyzer.

Electropherograms were visualized in Sequencher 4.8, and forward and reverse fragments were assembled. In the case of 18S rRNA, the two fragments were joined into a single sequence. External primer sequences were cropped and discarded. Sequences were aligned in BioEdit Sequence Alignment Editor (Hall, 1999) using the ClustalW algorithm. There were no alignment ambiguities for COI and H3. The 18S rRNA sequences were manually aligned using the alignment in Schulze et al. (2007) as a reference. This alignment is based on a direct optimization analysis and has annotations for secondary structure. The annotations are based on a secondary structure model of 18S rRNA for *Katharina tunicata,* available from the European Ribosomal RNA Database (Van de Peer et al., 2000). The final data set contained the alignment of the complete 18S rRNA sequence (2,053 bp) even though the larval sequences were shorter. All larval sequences were deposited in GenBank under accession numbers EU266987 through EU267000 and JX989041 through JX989070.

Analysis

All sequences were submitted to a BLAST (Basic Local Alignment Search Tool) search in GenBank to confirm that they were, indeed, sipunculan sequences and to identify the closest matches within the Sipuncula. This enabled us to assign all the larval sequences to the major clades of the Sipuncula. We felt comfortable with the quality of the GenBank sequences because the majority originated from the Giribet lab at Harvard University, where various experienced sipunculan taxonomists identified the species and performed the sequencing work.

Phylogenetic analyses were performed in two phases. In a first step, eight larval morphotypes for which COI, 18S, and H3 sequences were available were analyzed together with sequences from the data set previously generated by Schulze et al. (2007). The initial analysis was performed using Bayesian statistics, following protocols from Schulze et al. (2007). The present analyses include fewer terminals while maintaining the same number

TABLE 2. Primer sequences used for PCR and cycle sequencing of the three markers used in this study. Abbreviations: F, forward; R, reverse.

Marker	Primer a	Primer sequence	Reference
COI	F: LCO-1490	5′-GGTCAACAAATCATAAAGATATTGG-3′	Folmer et al. (1994)
	R: HCO-2198	5′-TAAACTTCAGGGTGACCAAAAAATCA-3′	Folmer et al. (1994)
	F: COI-7	5′-ACNAAYCAYAARGAYATYGGNAC-3′	Kojima et al. (1997)
	R: COI-D	5′-TCNGGRTGNCCRAANARYCARAA-3′	Kojima et al. (1997)
H3	F: H3aF	5′-ATGGCTCGTACCAAGCAGAC[ACG]GC-3′	Colgan et al. (1998)
	R: H3aR	5′-ATATCC TT[AG]GGCAT[AG]AT[AG]GTGAC-3′	Colgan et al. (1998)
18S rRNA	F: 3F	5′-GTTCGATTCCGGAGAGGGA-3′	Giribet et al. (1996)
	R: 18Sbi	5′-GAGTCTCGTTCGTTATCGGA-3′	Giribet et al. (1999)
	F: 18Sa2.0	5′-ATGGTTGCAAAGCTGAAAC-3′	Giribet et al. (1999)
	R: 9R	5′-GATCCTTCCGCAGGTTCACCTAC-3′	Giribet et al. (1996)

of species. Multiple individuals per species were included in cases where cryptic speciation was suspected.

The three markers were analyzed simultaneously under mixed models. The choice of models was estimated using MrModeltest 2.2 (Nylander, 2004). The COI and H3 sequences were analyzed under a general time reversible model. The loop regions of the 18S rRNA were analyzed under a symmetrical model that assumes equal base frequencies (Zarkihk, 1994), whereas the stem regions were analyzed under a doublet model (Schöniger and von Haeseler, 1994). Two runs with four chains each were performed for 1,500,000 generations, and the initial 500,000 generations were discarded as burn-in.

During the second phase, phylogenies for five clades recovered in the first step were reconstructed separately on the basis of only H3 because a complete data set was available for this marker. Analyses included additional larval morphotypes as well as additional adult sequences available from GenBank (primarily from Schulze et al., 2007, and Kawauchi et al., 2012). Histone H3 is conserved at the amino acid level but shows sufficient variation in the nucleotide sequences to resolve families and genera in sipunculans (Maxmen et al., 2003; Schulze et al., 2007; Kawauchi et al., 2012). This phase of the analysis included a total of 14 larval morphotypes, some of them represented by several individuals. Outgroups were chosen to represent the sister groups to the clades under analysis. All individual clades were analyzed under a general time reversible model with Bayesian inference in MrBayes 3.2, using two runs of four Monte Carlo Markov chains with 1 million generations each, sampling every 100th tree. The first 500,000 generations were discarded as burn-in. All trees are presented as 50% majority consensus trees generated from the tree distribution after discarding the burn-in.

Average genetic distances within species or clades, as indicated in Figure 2, were calculated for COI and H3 in MEGA 5 (Tamura et al., 2011) under a Kimura two-parameter (K2P) model (Table 3).

Nomenclature

Both Hall and Scheltema (1975) and Rice et al. (this volume) have created their own nomenclatures for the larval morphotypes. In this chapter, we adopt Rice et al.'s nomenclature of descriptive names (e.g., smooth orange and smooth small transparent) but reconcile the names from previous publications and assign them to taxonomic species. We based the matches on descriptions, as well as light and scanning electron microscopic images.

RESULTS

The first phase of analysis revealed that the eight larval morphotypes fall into five distinct clades within the sipunculan phylogeny (Figure 2). The trees from the analyses of individual clades are shown in Figure 3. Four larval morphotypes (large transparent, smooth transparent, smooth yellow-green, and smooth orange) fall into the most basal clade in the sipunculan phylogeny, consisting of *Sipunculus* and *Xenosiphon* (Figure 3A). The transverse groove and knobby larvae groups are associated with *Siphonosoma cumanense* and *Siphonosoma vastum*, respectively (Figure 3B). The white blackhead and spotted velvet larvae both group with *Apionsoma misakianum* (Figure 3C). The only larval type associated with members of the genus *Phascolosoma* is white white, which falls into a clade of *Phascolosoma nigrescens* (Figure 3D). Three larval types (yellow pap, white orange metatroch, and white papillated) are associated with *Aspidosiphon laevis* (Figure 3E). Pinkish papillated is most closely related to *Aspidosiphon albus*. Pink white papillated falls into a clade containing several *Aspidosiphon parvulus* but also *Aspidosiphon gosnoldi* and *Aspidosiphon gracilis*.

We were able to match seven of the larval types described in Hall and Scheltema (1975) to larval forms described in Rice et al. (this volume) and thus assign them to species or genera (Table 4).

DISCUSSION

Phylogenetic Analyses

The initial phylogenetic analysis of three molecular markers resulted in a tree (Figure 2) similar to that of Schulze et al. (2007) with many commonalities to that of Kawauchi et al. (2012). As our goal was not to reanalyze sipunculan phylogeny but to assign larval morphotypes to sipunculan species or clades, we will focus our discussion on those clades that contain larval sequences. Only one major clade in the sipunculan phylogeny does not include any sequences from larval morphotypes. This clade is represented by multiple genera, primarily *Golfingia, Nephasoma, Phascolion,* and *Themiste*, and corresponds to clade III in Schulze et al. (2007). It is uncertain whether typical teleplanic larvae do not exist in this clade or whether they have simply not been sampled. Several members of this clade are known to have abbreviated development, such as *Phascolion cryptum* (Rice et al., 1983), *Phascolion strombus* (Åkesson, 1958), *Phascolion psammophilum* (Rice, 1993), *Themiste lageniformis* (Pilger, 1987), *Themiste pyroides* (Rice, 1967), and *Thysanocardia nigra* (Rice, 1967). *Nephasoma pellucidum*, another member of the clade, does produce planktotrophic pelagosphera larvae, but they are small and comparatively short-lived (Schulze and Rice, 2009) and have not been reported from plankton samples.

Larval Identifications

The large transparent pelagosphera is usually not abundant in plankton samples, but it is very conspicuous if present. Our observations of this larval type match very closely the description of larval type S by Hall and Scheltema (1975) in terms of size, pigmentation, and internal anatomy visible through the transparent body wall (Table 4, Figure 1). Primarily on the basis of

FIGURE 2. The 50% majority consensus tree from initial analysis based on three markers (CO1, H3, and 18S rRNA) from the data set generated by Schulze et al (2007) and including eight larval morphotypes. Branch support is given as Bayesian posterior probabilities in percentages. Asterisks indicate 100% posterior probability. Only values over 70% are shown. Larval samples are in bold; for abbreviations, refer to Table 1. The six-digit numbers after species names refer to the Harvard University Museum of Comparative Zoology DNA accession numbers. Colors indicate those clades that were analyzed in more detail with H3 alone (see Figure 3).

TABLE 3. Average genetic distances (Kimura two-parameter model) for H3 and COI within species or clades as defined in the phylogenetic analyses, including the larval sequences, if available. A dash (—) indicates not applicable.

Species and clade	Group in Figure 3	Average distance	
		H3	COI
Sipunculus phalloides/Sipunculus polymyotus	1	0	0.050
Sipunculus nudus	2	0.075	0.257
Xenosiphon branchiatus	3	0.049	0.193
Siphonosoma vastum	4	0.005	0.003
Siphonosoma cumanense	5	0.019	0.220
Apionsoma misakianum, spotted velvet clade	6	0.014	0.162
Apionosoma misakianum, white blackhead clade	7	0.001	0.007
Apionsoma misakianum	8	0.025	0.190
Phascolosoma nigrescens	9	0.049	0.240
Aspidosiphon laevis	10	0.032	0.216
Aspidosiphon albus	11	0.006	—
Aspidosiphon spp.	12	0.013	—

the large number of bands in the longitudinal body wall musculature, these authors concluded that the larva belonged to *Sipunculus polymyotus*. This larval type was first described by Fisher (1947). Our analyses did not resolve whether the large transparent larva represented *S. polymyotus* or *S. phalloides* (Figures 2, 3A). The H3 and 18S sequences are identical for *S. polymyotus* and *S. phalloides*. For COI, the two species are 5% different (K2P), but no COI sequence is available for the larva. The two species are morphologically similar, with the number of longitudinal muscle bands being the main distinguishing characteristic (35–41 in *S. phalloides* and 42–55 in *S. polymyotus*). On the basis of this feature, we agree with Hall and Scheltema (1975) that the large transparent pelagosphera corresponds to *Sipunculus polymyotus*. The large transparent pelagosphera utilized for this study was collected at Carrie Bow Cay, Belize. This occurrence is within the reported distribution range of *S. polymyotus,* which occurs in the western Atlantic, the Caribbean, and the Gulf of Mexico, as well as in the eastern Pacific (Cutler, 1994; Kawauchi and Giribet, 2014).

The smooth small transparent larva falls into a diverse clade of specimens labeled *Sipunculus nudus* (Figure 3A), but the basal branches in this clade are not fully resolved. Kawauchi and Giribet (2014) have shown that *S. nudus* represents a species complex with a worldwide distribution but have not formally described any new species. At this point, the best species assignment for the smooth small transparent larva is *S. nudus*, keeping in mind that future work may result in the erection of multiple new species within the *S. nudus* complex.

The genetic diversity within the clade that contains *Xenosiphon branchiatus,* the smooth yellow-green and the smooth orange larvae, is also fairly high ($K2P_{COI}$ = 19.3%; Figure 3A). Cutler (1994) lists two species of *Xenosiphon*, *X. branchiatus* and *X. absconditus*, but expresses doubts about the validity of *X. absconditus,* which was described on the basis of museum material from uncertain localities. Although adult *Xenosiphon* spp. are large, they are never encountered in high densities, probably because they burrow very deeply into sediment. It would therefore be difficult to conduct a thorough analysis of the genetic structure of this species throughout its distribution range on the basis of adults alone. However, the presence of two distinct larval forms provides further support that the clade includes at least two species. Interestingly, one of the three smooth yellow-green larvae (YG3) is divergent ($K2P_{H3}$ = 6.8%) from the other two (YG1 and YG2) and appears to be more closely related to the smooth orange larvae and the single adult of *X. branchiatus*. The apparent paraphyly of the smooth yellow-green larval type could indicate that it is the ancestral larval form within the clade, but a larger sample size of individuals and molecular markers are desirable to appropriately address this question. The smooth orange larva corresponds to Hall and Scheltema's (1975) larval type B, smooth, on the basis of the smooth cuticle and characteristic pigmentation, although Jägersten's original description of the "smooth" larva encompasses other smooth-skinned pelagosphera larvae as well (Jägersten, 1963).

The analysis of the *Siphonosoma* clade (Figure 3B) confirmed that the transverse groove larva belongs to *Siphonosoma cumanense*. This affiliation was previously shown in rearing experiments (Rice and Reichardt, 1984; Rice, 1988). The circular annulations in the trunk region of this larva and the greenish intestine are very characteristic and have also been described for the type E larva in Hall and Scheltema (1975), which we have matched to the transverse groove. Average genetic distances

Siphonosoma cumanense JN865159
Sipunculus norvegicus DQ300090
Sipunculus phalloides DQ300094
Sipunculus polymyotus DQ300095
*
LT1
1
Sipunculus sp. AF519295
Sipunculus nudus JN865127
99
Sipunculus nudus DQ300093
84
Sipunculus nudus DQ300091
85
82
Sipunculus nudus JN865167
Sipunculus nudus JN865128
ST1
2
Sipunculus nudus JN865132
*
Sipunculus nudus DQ300092
81
91
Sipunculus nudus JN865148
Xenosiphon branchiatus DQ300101
75
SO1
99
SO2
99
YG3
3
99
YG1
*
YG2
A
0.04

Sipunculus nudus JN865167
Siphonosoma vastum AY326297
Siphonosoma vastum AY326298
KN2
*
KN1
KN3
4
Siphonosoma cumanense JN865159
Siphonosoma cumanense EU266980
Siphonosoma cumanense AY326296
TG4
*
TG2
TG3
TG1
5
95
Siphonosoma cumanense DQ300088
Siphonosoma cumanense DQ300089
Siphonosoma cumanense AF519296
B
0.02

Apionsoma misakianum JN865155
Apionsoma misakianum DQ300052
SV2
97
SV1
6
Apionsoma misakianum EU266986
8
WB2
*
WB1
88
Apionsoma misakianum JX989071
Apionsoma misakianum JX989072
7
Apionsoma pectinatum AY326300
C
0.009

Apionsoma misakianum DQ300052
Phascolosoma capitatum DQ300079
*
Phascolosoma turnerae DQ300087
Phascolosoma agassizii DQ300078
Phascolosoma scolops JN865162
Phascolosoma stephensoni JN865133
86
98
Phascolosoma stephensoni JN865125
97
Phascolosoma stephensoni AF519307
Phascolosoma stephensoni JN865138
*
Phascolosoma scolops AF519310
Phascolosoma scolops AF519309
95
Phascolosoma stephensoni DQ300086
Phascolosoma stephensoni AF519308
Phascolosoma nigrescens JN865165
99
Phascolosoma nigrescens JN865131
Phascolosoma nigrescens JN865137
WW1
9
75
98
Phascolosoma nigrescens AY326299
Phascolosoma nigrescens DQ300080
94
Phascolosoma nigrescens DQ300081
Phascolosoma agassizii JN865130
84
Phascolosoma scolops JN865144
Phascolosoma albolineatum JN865166
*
Phascolosoma perlucens JN865129
73
Phascolosoma perlucens JN865141
*
Phascolosoma perlucens JN865160
Phascolosoma perlucens JN865135
77
98
Phascolosoma perlucens DQ300082
Phascolosoma perlucens DQ300083
99
Phascolosoma perlucens AF519306
Phascolosoma scolops DQ300085
Phascolosoma noduliferum AF519305
Phascolosoma granulatum AF519309
D
0.06

Antillesoma antillarum JN865136
Aspidosiphon elegans JN865147
95
Aspidosiphon steenstrupii JN865143
Aspidosiphon misakiensis AF519312
*
*
Aspidosiphon steenstrupii AF519315
Aspidosiphon steenstrupii JN865161
97
Aspidosiphon steenstrupii JN865126
81
Aspidosiphon steenstrupii DQ300064
Aspidosiphon laevis AF519313
Aspidosiphon laevis JN865140
Aspidosiphon laevis DQ300059
*
YP2
10
YP3
78
YP1
WO1
Aspidosiphon laevis JN865164
88
WP1
Aspidosiphon albus DQ300053
94
Aspidosiphon albus DQ300054
11
92
PP1
Aspidosiphon elegans DQ300056
Aspidosiphon fischeri JN865139
92
Aspidosiphon fischeri AY326301
Aspidosiphon gosnoldi DQ300057
*
Aspidosiphon gracilis DQ300058
Aspidosiphon parvulus DQ300062
12
88
Aspidosiphon parvulus DQ300063
Aspidosiphon parvulus AF519314
Aspidosiphon parvulus DQ300061
PW1
80
Aspidosiphon parvulus JN865145
Aspidosiphon spinalis JN865158
85
Aspidosiphon spinalis JN864152
Aspidosiphon muelleri DQ300060
E
0.2

TABLE 4. Species identifications of pelagosphera larval morphotypes, using Rice et al.'s (2018) and Hall and Scheltema's (1975) terminology. A dash (—) indicates not applicable.

Larval type (Rice et al.)	Figure nos. in Rice et al.	Larval type (Hall and Scheltema)	Species
Large transparent	4, 5	Type S	*Sipunculus polymyotus*
Smooth small transparent	6, 7	—	*Sipunculus nudus*
Smooth orange	8–11	Type B, smooth	*Xenosiphon branchiatus*
Smooth yellow-green	12–15	—	*Xenosiphon branchiatus*
Transverse groove	16–19	Type E	*Siphonosoma cumanense*
Knobby	20, 21	Type F	*Siphonosoma vastum*
Spotted velvet	22–24	Type J	*Apionsoma misakianum*
White blackhead	25, 26	Type C, *Baccaria oliva*	*Apionsoma misakianum*
White white	27–29	—	*Phascolosoma nigrescens*
Yellow pap	30–32	Type A, *Baccaria citrinella*	*Aspidosiphon laevis*
White orange metatroch	—	—	*Aspidosiphon* cf. *laevis*
White pap	—	—	*Aspidosiphon* sp.
Pinkish papillated	—	—	*Aspidosiphon albus*
Pink white papillated	—	—	*Aspidosiphon* sp.

within this clade are moderate for H3 (K2P = 1.9%) but high for COI (K2P = 22%). This species might be another candidate for future studies of genetic population structure, as it has a wide geographic distribution in the Atlantic, Pacific, and Indian Oceans. The knobby larva unambiguously groups with the second species in the genus, *Siphonosoma vastum*. On the basis of our limited sample size, genetic diversity within this clade is low for both markers used ($K2P_{COI}$ = 0.3%; $K2P_{H3}$ = 0.5%). The distinct projections (knobs) on the body surface of this larva indicate that it matches the type F larva in Hall and Scheltema (1975). These authors mention similarities in the head structures between type E and type F larvae, providing further support that they both belong to the same genus. Cutler (1994) reported *S. vastum* only from the Pacific, primarily from the western portion, but Cutler and Schulze (2002) provided a first report from the Caribbean island of Barbados. Its presence in Caribbean waters might have previously been overlooked. A resident population of *S. vastum* in the Caribbean would explain the presence of its larva in the Florida Current and Gulf Stream.

High genetic diversity ($K2P_{COI}$ = 19%) also exists in *Apionsoma misakianum* (Figure 3C). The white blackhead and spotted velvet larvae fall into two clearly separated clades, suggesting that *A. misakianum* is not a single, cohesive species. Whereas the white blackhead larva has been successfully reared through metamorphosis to adulthood in the lab (Rice, 1986), the species designation for the spotted velvet larva has not been confirmed. Hall and Scheltema (1975) maintained cultures of their type C, *Baccaria oliva,* and type J larvae through metamorphosis but were not able to identify them to species at the juvenile stage. However, their morphological descriptions of the larvae and juveniles, especially of the pigmentation and the papillae, including SEM images, leave no doubt that their type C larva is the same as the white blackhead and that type J corresponds to the spotted velvet larva. The names "*Baccaria oliva*" and "*Baccaria citrinella*" (see below) go back to Häcker (1898), who recognized that they were sipunculan larvae but did not know their affinities.

Staton and Rice (1999) have suggested the presence of two cryptic species in *Apionsoma misakianum* on the basis of allozyme analysis. They found that the population at Sebastian Pinnacles, off the Atlantic coast of south central Florida, has an allozyme signature distinct from the more southern populations in the Florida Keys and the Bahamas. The southern populations appear to

FIGURE 3. (*Opposite page*) Detailed analysis of individual clades (only H3). (A) Sipunculidae, (B) Siphonosomatidae, (C) *Apionsoma misakianum,* (D) *Phascolosoma*, and (E) *Aspidosiphon*. Colors correspond to the colored clades in Figure 2. Branch support is given as Bayesian posterior probabilities in percentages. Asterisks indicate 100% posterior probability. Only values over 70% are shown. Larval samples are in bold; for abbreviations, refer to Table 1. Adult sequences are listed with their GenBank accession numbers. Vertical bars to the right of the trees delimit groups for which average genetic distances were calculated (see Table 3).

produce the white blackhead larvae, which might drift northward in the Florida Current and Gulf Stream but do not contribute to the recruitment at Sebastian Pinnacles. Our study indicates that the Sebastian Pinnacles population produces the spotted velvet larva. Although the two larval types have many commonalities—their papillae are indistinguishable—their pigmentation patterns are very distinct. They also differ slightly in developmental timing (Rice, 1981; Rice et al., this volume). In our analyses, the white blackhead clade includes one adult from Belize and another one that was reared in the lab from a white blackhead larva from the Florida Current. The adults that group with the spotted velvet larvae are from Sebastian Pinnacles (EU266986), from the Red Sea (JN865155), and from New Caledonia (DQ300052). The spotted velvet clade has high branch support, but more extensive studies are required to examine the genetic structure within this clade throughout its vast distribution range and to study the larval forms from different populations.

The white white (Belize) larva falls into a clade of *Phascolosoma nigrescens* (Figure 3D). As is obvious from the tree, many of the species designations within the genus *Phascolosoma* become questionable when analyzed with molecular tools (see also Kawauchi and Giribet, 2010). However, the only species with multiple representatives that appears to be monophyletic in our analysis is *Phascolosoma nigrescens*, although genetic diversity within this species ($K2P_{COI}$ = 24%) is nearly as high as in *Sipunculus nudus* (Table 3). Again, future studies might reveal that *P. nigrescens* represents a species complex, but at the present time, the white white larva can confidently be assigned to this clade.

Like in *Phascolosoma*, the molecular analyses of the genus *Aspidosiphon* also reveal many uncertainties in species delimitations (Figure 3E; see also Schulze et al., 2007; Kawauchi et al., 2012). Three larval types (yellow pap, white orange metatroch, and white papillated) are most closely related to *Aspidosiphon laevis* (Figure 3E). Among those, yellow pap is connected to adults of *A. laevis* from Belize and Bermuda by very short branch lengths, and its species designation is the most obvious. Yellow pap probably corresponds to type A, *Baccaria citrinella*, in Hall and Scheltema (1975). Although they do not include a detailed description of this larval type, scanning electron micrographs of the body wall papillae closely match ours (Rice et al., this volume). Furthermore, in the key to the larval types, Hall and Scheltema (1975) describe the color as "light pink-yellow to orange brown." White orange metatroch falls between the clade that contains yellow pap and a divergent sequence of *A. laevis* from New Caledonia, but branch support for this placement is low. White orange metatroch is quite distinct morphologically from yellow pap. As the name implies, this larva is white with a distinct orange ring in the metatrochal region, and its body surface is densely papillated. At the present time, we regard its designation as *A. laevis* as preliminary. The remaining papillated larvae associated with *Aspidosiphon* species are difficult to distinguish from each other. The distinctions are primarily based on subtle color differences detected under light microscopy. Additional differences may exist in the shape of the papillae under scanning electron microscopy, but once a specimen is prepared for SEM, it is no longer available for DNA analysis, preventing independent verification of its taxonomic identity. White papillated forms the most basal branch in the *Aspidosiphon laevis* clade and most likely represents a sister species not represented in our data set. Pink white papillated falls into a clade consisting of *Aspidosiphon parvulus*, *A. gracilis*, *A. gosnoldi*, *A. fischeri*, and *A. elegans*, none of which are monophyletic. The closest matches in GenBank for this larva species are *A. parvulus* for H3 and *A. gosnoldi* for COI. Until we have clearer delimitations for the *Aspidosiphon* species in question, we cannot confidently assign pink white papillated to any species, but it clearly belongs to the genus *Aspidosiphon*.

CONCLUSIONS AND FUTURE DIRECTIONS

Some morphotypes of pelagosphera larvae are more distinctive than others. Easily recognizable forms include all the larvae that fall into the *Sipunculus-Xenosiphon* clade, the two *Siphonosoma* larvae, and those belonging to *Apionsoma misakianum*. The relatively small, papillated larvae of *Aspidosiphon* and *Phascolosoma* tend to be more difficult to identify on the level of light microscopy, especially if they lack distinctive pigmentation. The papillae are usually distinctive when examined with SEM, but the conundrum is that once a larva is used for SEM, it cannot be used for DNA extraction any longer and vice versa. The best solution is to collect as many individuals as possible and process several for each of the two methods to confirm that the results are consistent. Unfortunately, although long-term records of pelagosphera larvae in plankton tows from the Florida Current exist, which larval types are caught on a particular day and how abundant they will be are still unpredictable. Therefore, this type of research depends to a large degree on fortuitous findings.

In this study, our larval sampling was restricted to the Caribbean and northwest Atlantic, but from the work of Hall and Scheltema (1975) and Scheltema and Hall (1975) we know that some of the larval types occur across the north Atlantic. It would be interesting to examine the genetic signatures of the larvae from different parts of the Atlantic, which could provide valuable insight into dispersal ranges and population connectivity. Additional sampling in other oceans and climatic zones might reveal new larval types that have not been considered in this study. Recent data indicate that populations of several North Pacific shallow-water sipunculan species are genetically very distinct between the eastern and western boundaries of their distribution, suggesting that larval dispersal across the Pacific basin is limited or nonexistent (Schulze et al., 2012; Johnson and Schulze, 2016; Johnson et al., 2016).

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