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Molecular Phylogenetics and Evolution 34 (2005) 438–447
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Short communication

Mitochondrial DNA phylogeny and rates of larval evolution in *Macrophiothrix* brittlestars

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Received 6 September 2004; revised 23 September 2004
Available online 19 November 2004

Abstract

Phylogenetic analysis has led to significant insights into the evolution of early life-history stages of marine invertebrates. Although echinoderms have been a major focus, developmental and phylogenetic information are relatively poor for ophiuroids, the most species-rich echinoderm class. We used DNA sequences from two mitochondrial genes to develop a phylogenetic hypothesis for 14 brittlestar species in the genus *Macrophiothrix* (Family Ophiotrichidae). Species are similar in adult form and ecology, but have diverse egg sizes and modes of larval development. In particular, two species have rare larval forms with characteristics that are intermediate between more common modes of feeding and non-feeding development. We use the phylogeny to address whether intermediate larval forms are rare because the evolution of a simplified morphology is rapid once food is no longer required for development. In support of this hypothesis, branch lengths for intermediate forms were short relative to those for species with highly derived non-feeding forms. The absolute rarity of such forms makes robust tests of the hypothesis difficult.
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Keywords: Ophiuroid; Brittlestar; Facultative feeding; Pluteus; Mode of development; Life-history transition; Mitochondrial DNA

1. Introduction

The evolutionary analysis of early life cycle diversity in marine invertebrates has advanced significantly over the last decade from the use of phylogenetically based comparative methods (Cunningham, 1999; Hadfield et al., 1995; Hart et al., 1997; Jeffery and Emlet, 2003; Levitan, 2000; Rouse and Fitzhugh, 1994; Villinski et al., 2002). These analyses have confirmed or challenged a number of assumptions about the order, timing, and frequency of change in developmental and life-history characters (reviewed in Hart, 2000).

Certain echinoderm taxa feature prominently in this work (Arndt et al., 1996; Emlet, 1990; Hart et al., 1997; Jeffery and Emlet, 2003; Smith et al., 1995; Wada et al., 1996; Wray, 1996). Gametes and embryos of sea stars (Asteroidea) and sea urchins (Echinoidea) are easy to obtain and culture (Strathmann, 1987), and some larval traits can be inferred indirectly from egg size (Emlet et al., 1987) or adult traits (Emlet, 1989). In contrast, the study of development and larval biology in brittlestars (Ophiuroidea), the most-species rich echinoderm class, has been hindered by the cryptic lifestyles of adults, a lack of reliable methods for inducing spawning and oocyte maturation (Selvakumaraswamy and Byrne, 2000; Strathmann, 1987), and the absence of adult characters that can diagnose larval traits (Emlet, 1989; Hendler, 1978). As in better documented classes, egg size in ophiuroids is correlated with mode of development (Hendler, 1991) and developmental patterns are diverse

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doi:10.1016/j.ympev.2004.09.011

Table 1
Modes of larval development and maximum likelihood branch lengths for *Macrophiothrix* species with obligate non-feeding (group 1) or intermediate (group 2) larval forms and feeding modes

Species	Egg volume (nl)	Larval morphology	Can feed?	Must feed?	Branch length					
					No clock			With clock		
					16S	COI	Both	16S	COI	Both
M. belli	35.1	Non-pluteus	No	No	.129	.239	.208	.123	.286	.214
M. lampra	12.6	(Non-pluteus)	(No)	(No)	.110	na	na	.111	na	na
M. nereidina	9.7	Non-pluteus	No	No	.154	na	na	.123	na	na
M. caenosa	7.4	Pluteus	No	No	.082	.097	.086	.071	.148	.115
M. rhabdota[a]	6.3	Pluteus	Yes	No	.023	na	na	.018	na	na
(to *lorioli*)					.062	.089	.084	.042	.081	.062
M. lorioli	2.4	Pluteus	Yes	Yes		na			na	
M. longipeda	1.9	Pluteus	Yes	Yes		na			na	
M. koehleri	1.6	Pluteus	Yes	Yes		na			na	
M. paucispina	<1.0	(Pluteus)	(Yes)	(Yes)		na			na	

Also shown are obligate feeding species (group 3) that are important to the analysis of branch lengths. Larval character states are known from larval culture (R. Podolsky, unpublished data) except for those of *M. lampra* and *M. paucispina*, which are inferred from egg size relative to other species. Of nine additional *Macrophiothrix* species for which we have egg size data, all are likely to be obligately feeding plutei. Because COI data were missing for some species that are relevant to the branch length analysis (*M. lampra*, *M. nereidina*) and COI genetic distances were saturated for the two outgroups, branch lengths in units of expected change per nucleotide site are given for 16S (TVM + G model, 18 taxa), COI (GTR + G + I, 12 ophiotrichid taxa only), and both genes analysed together (GTR + G, 12 ophiotrichids plus *Ophionereis*) under maximum likelihood substitution models estimated independently for each analysis. na, not available or applicable.

[a] The relevant branch length for estimating persistence time of the intermediate larval form of *M. rhabdota* depends on whether the closest relative with feeding larval development is *M. paucispina* (inferred from egg size, first line) or *M. lorioli* (known from larval culture, second line); see Fig. 1.

(sensu McEdward and Janies, 1997). However, no species-level phylogenies have been published for ophiuroids that would aid the analysis of early life cycle evolution (McEdward and Miner, 2001).

The brittlestar genus *Macrophiothrix* (Clark; F. Ophiotrichidae) is common to certain Indo-west Pacific coral reef habitats and especially diverse (21 species) in tropical Australia (Hoggett, 1990). Despite the occurrence of exceptionally high local diversity, adults of different *Macrophiothrix* species are similar in size, form, and ecological habits (Hoggett, 1991). The genus, however, exhibits remarkable variation and specificity in early life cycle characters, involving species-specific sperm chemotaxis (Miller, 1997) and extensive variation in egg size and development mode. At Lizard Island, Australia, 12 *Macrophiothrix* congeners vary more than 60-fold in average egg volume (Podolsky, unpublished data) and produce larval forms that include a typical and morphologically complex feeding pluteus, a morphologically simple non-feeding larval form, and two forms that are functionally intermediate (Table 1). Larvae of *M. rhabdota* are facultative planktotrophs that do not require food to complete development, whereas larvae of *M. caenosa* do not feed but retain the pluteus morphology (R. Podolsky, unpublished data). The rarity of these intermediate larval forms–relative to the large number of evolutionary transitions from feeding to non-feeding larval forms (Duda and Palumbi, 1999; Hart et al., 1997; Jeffery and Emlet, 2003; Lieberman et al., 1993; Wray, 1996)–led Wray (1996) and others to propose that intermediate forms are evolutionarily unstable and short-lived. Phylogenetic information can be used to address this hypothesis (Hart, 1996).

We use information from mitochondrial DNA sequences to resolve relationships among 14 species classified in the genus *Macrophiothrix*, two species in the genus *Ophiothrix* not recently referred to *Macrophiothrix* (Hoggett, 1990), and two outgroups from related families. We use the phylogeny to infer relationships between species with functionally intermediate, putatively transitional larval forms and species with non-feeding larval morphologies, and to test the hypothesis that the former species are more recently derived (Hart, 1996).

2. Materials and methods

We obtained new mtDNA sequences from the large subunit ribosomal RNA (16S) and cytochrome *c* oxidase subunit I (COI) genes for 16 ophiotrichid brittlestar species in the genera *Macrophiothrix* and *Ophiothrix* and for two outgroup taxa (the ophiocomid *Ophiarthrum pictum* and the ophionereid *Ophionereis porrecta*; see Appendix A for details of laboratory methods). We analyzed phylogenetic information from both genes together by maximum likelihood (ML) methods, and used bootstrapping and posterior probabilities as estimates of clade support (see Appendix B for details of analytical methods).

To illustrate the utility of the phylogeny for testing hypotheses about life history evolution, we inferred and compared times of persistence for species with highly

derived, non-feeding larval forms and for species with more intermediate modes of larval development. The intermediate forms develop from relatively large eggs and do not require food, but retain phenotypic traits of suspension feeding larvae that are absent from the more derived forms. If branch lengths leading to intermediate forms are short relative to those leading to highly derived non-feeding forms, this pattern would support the hypothesis that intermediate phenotypes are short-lived steps in a transition from obligate feeding to simplified non-feeding development, rather than stable developmental modes (Hart, 1996). (This hypothesis assumes that rates of molecular evolution are similar across lineages with different derived modes of development.)

We asked whether the ML branch lengths leading to two ophiotrichids with intermediate larval phenotypes (*M. caenosa* and *M. rhabdota*) are shorter than those leading to three species that are known (*M. nereidina*, *M. belli*) or inferred (*M. lampra*) to have obligate non-feeding larvae. Other species (*M. longipeda*, *M. koehleri*, *M. paucispina*, and *M. lorioli*) that are known or inferred to produce obligately feeding plutei (R. Podolsky, unpublished data) were used to establish the earliest possible times of divergence from species with obligate feeding larval forms (Table 1).

3. Results

General sequence characteristics (including missing COI sequence data for some species) are summarized in Appendix A. Within- and among-species genetic distances are summarized in Appendix B.

3.1. Tree topology

We found a single ML tree with a $-\ln(L)$ score of 7788.9 (Fig. 1). Seven clades were moderately or strongly

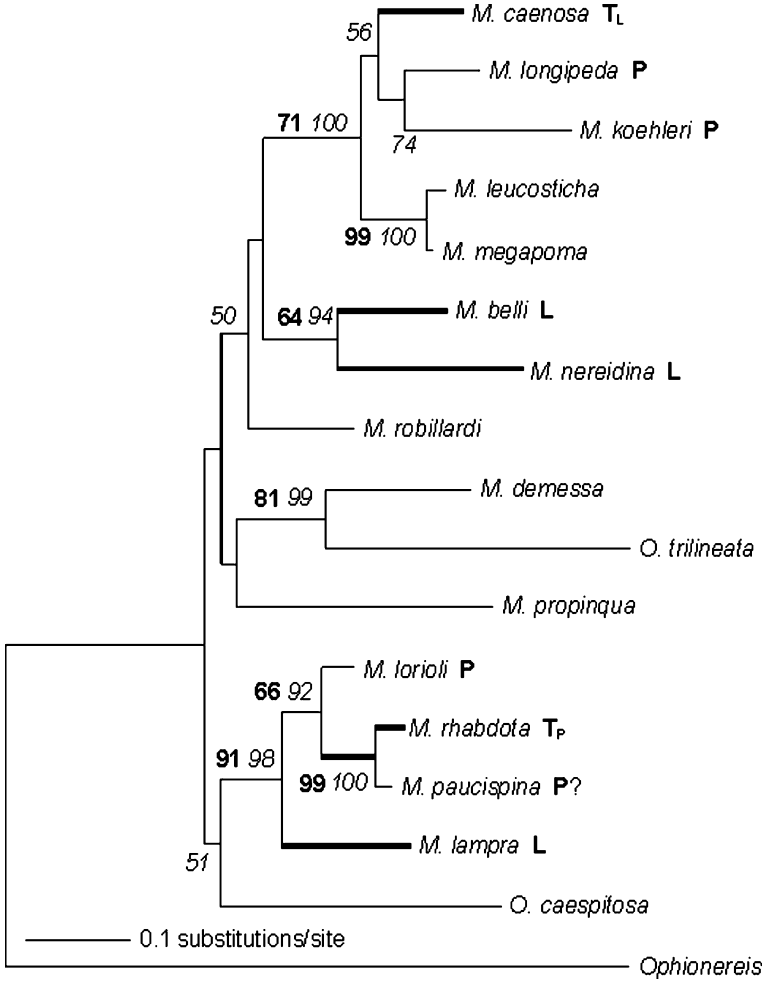

Fig. 1. Phylogram for 16 *Macrophiothrix* and *Ophiothrix* brittlestar species and an outgroup (*Ophionereis*) based on maximum likelihood analysis of combined 16S rDNA and COI sequences (six-rate GTR + G model). Numbers beside branches are bootstrap percentages (bold) and Bayesian posterior probabilities (italics); only values >50% are shown. Heavy lines show branch lengths relevant to the analysis of modes of larval development (shown as abbreviations to the right of some taxon names): feeding planktonic larvae (P), non-feeding lecithotrophic larvae (L), transitional pluteus larvae that cannot feed (T_L), transitional pluteus larvae that can feed (T_P) (see Table 1). The mode of larval development for *M. paucispina* is inferred from egg size in a museum specimen and is considered uncertain but probably a feeding planktonic larva (P?).

supported by high bootstrapping percentages and posterior probabilities: *M. leucosticha* + *M. megapoma*; this species pair in a larger clade with *M. caenosa*, *M. koehleri*, and *M. longipeda*; *M belli* + *M. nereidina*; *M. demessa* + *O. trilineata*; and the clade (*M. lampra*) (*M. lorioli* (*M. rhabdota* + *M. paucispina*). The three most basal nodes, and some other interior branches in the tree, were short and poorly supported, which could reflect low resolving power of these genes for these taxa or rapid divergence of clades. We were unable to identify the sister groups of three species: *M. robillardi*, *M. propiniqua*, *O. caespitosa*. Only one of these (*M. robillardi*) lacks COI data (see Appendix A), which suggests that including these taxa with missing data did not greatly impede our ability to find some well-supported relationships based on 16S only (see also Appendix B).

Ophiothrix and *Macrophiothrix* species did not appear to form reciprocally monophyletic groups: *O. trilineata* was strongly inferred to be sister species to *M. demessa* (Fig. 1). The unresolved relationship between *O. caespitosa* and other ophiotrichids is consistent with some *Ophiothrix* as sister group to a paraphyletic *Macrophiothrix*. However, the low resolution of basal relationships prevents us from drawing a firm conclusion about monophyly for these two genera: a tree in which *O. caespitosa* + *O. trilineata* was the sister group to *Macrophiothrix* species (with other relationships among *Macrophiothrix* as shown in Fig. 1) had a $-\ln(L)$ score of 7799.5 and was not significantly less likely than the ML tree (7788.9) by the Shimodaira–Hasegawa test in PAUP* (Swofford, 2002, $P = 0.177$). We therefore cannot reject the hypothesis that *Macrophiothrix* (sensu Hoggett, 1991) is a monophyletic clade with respect to *Ophiothrix*.

3.2. Branch lengths of derived larval forms

The terminal branches leading to species with intermediate larval forms (*M. rhabdota*, *M. caenosa*) were relatively short (e.g., 0.023, 0.082, for 16S without a clock) compared to terminal branches for species with simplified, non-feeding larval forms. We obtained qualitatively the same result when we allowed sister lineages to differ in rates of molecular evolution and when we enforced a molecular clock (Table 1). Patterns were similar for 16S sequences alone, for COI, and for both genes analyzed together. Both species with intermediate forms were close relatives of species known to develop as feeding larvae (*M. lorioli*; *M. longipeda*, and *M. koehleri*), so the intermediate form is assumed to have arisen along the short terminal branches (Table 1). If *M. paucispina* (whose mode of larval development is uncertain) were also a transitional non-feeding pluteus larva, then the persistence time for that transitional form could be as long as the sum of the terminal and internal branches separating *M. rhabdota* and *M. paucispina* from their common ancestor with *M. lorioli*. Measurements of egg size from a single museum specimen of *M. paucispina* (Australian Museum J22076)–which was dried before ethanol preservation, and could have experienced a reduction in egg dimensions–indicated an egg volume (<1 nl) characteristic of species with planktonic, feeding pluteus larvae (Table 1). This observation suggests that the transitional larval form of *M. rhabdota* evolved after its recent divergence from *M. paucispina*, and supports the inference of the shorter branch lengths in Table 1.

In contrast, the terminal branches leading to sister species (*M. belli*, *M. nereidina*) with obligate non-feeding larval development were longer (e.g., 0.129, 0.154 for 16S without a clock). The terminal branch lengths for these sister species give a minimum estimate of persistence time for species with obligate non-feeding development if this larval form and life history evolved once in their common ancestor. A third, independent terminal branch for a species assumed to have non-feeding larvae (*M. lampra*) gives a maximum estimate of this persistence time that is also longer (0.110) than branches leading to species with intermediate larval forms. Branch lengths for *M. belli* based on COI (which were missing for *M. lampra* and *M. nereidina*) and the combined sequences were also longer than the comparable branch lengths for species with intermediate forms. Of course, any of these derived larval forms could have evolved at any time since the divergence of the species from an ancestor with obligate feeding larvae, and the number of such lineages is small. In addition, some long terminal branches might be shortened by the discovery of sister group relationships with other *Macrophiothrix* species that we were not able to sample.

4. Discussion

4.1. Taxonomic and phylogenetic resolution in Macrophiothrix

Analysis of mtDNA sequences from *Macrophiothrix* and *Ophiothrix* species identified some sister-group relationships that are statistically well-supported, but left other relationships unresolved. Improved taxonomic sampling of more *Macrophiothrix* species using more slowly evolving molecular characters is needed to fully resolve these basal relationships. Nevertheless, our current results provide a useful context for comparative analyses of developmental evolution among ophiotrichids.

Our mtDNA sequence comparisons add to earlier efforts of Hoggett (1991) to resolve generic boundaries involving species in *Macrophiothrix* and related subgenera of *Ophiothrix*. Among the 16 species for which we generated mtDNA sequences, we found some well-supported clades of species that Hoggett had assigned to

Macrophiothrix. However, two other *Macrophiothrix* were not reliably grouped with other members of the genus. First, morphological characters reliably placed *M. propinqua* and *M. robillardi* with other *Macrophiothrix* (Hoggett, 1990), but 16S and COI characters could not reliably identify the sister group of either. Second, analyses of mtDNA tend to group *M. demessa* with *O. trilineata*, though Hoggett (1990) assigned each species with confidence to its respective genus on the basis of adult morphological and allozyme evidence.

We also found examples of strong conflict between our mtDNA phylogeny and cladograms based on morphology or allozymes (Hoggett, 1990). Some species that share numerous adult morphological apomorphies, such as *M. belli*, *M. caenosa*, and *M. paucispina* (Hoggett, 1990; Fig. 2.17), are distant relatives in the mtDNA trees. Other species with highly divergent allozyme alleles, such as *M. leucosticha* and *M. megapoma* (Hoggett, 1990; Fig. 4.3), are strongly supported as sister species in the mtDNA trees. In general, the mtDNA data more finely and robustly resolved species relationships for the clades identified in common by the three analyses.

Our analyses of mtDNA and life-history variation support other taxonomic revisions. For example, Hoggett (1990) reversed a long history of classification (Clark, 1938) by distinguishing *M. caenosa* (sp. nov.) from *M. longipeda.* The main diagnostic character (shape of papillae on the adult dental plate) is difficult to resolve with live specimens. In contrast, the species are easily distinguished by a 3.8-fold difference in egg volume and qualitative differences in mode of development (R. Podolsky, unpublished data). Mitochondrial DNA sequences extend this conclusion by suggesting that these taxa might not even be sister species (Fig. 1). Similar surveys of reproductive traits could be useful for identifying cryptic species diversity in putative morphospecies such as *M. demessa* that have broad geographic ranges (Philippines through Australia and several oceanic islands), long mtDNA terminal branches (Fig. 1), and high within-species mtDNA sequence variation (Appendix B).

4.2. Evolutionary analysis of early life cycle characters

Efforts to understand the origins of developmental diversity have focused on processes that underlie transitions between modes of development (Wray, 1995a). In marine invertebrates, one widespread transition involves the loss of a complex feeding larval morphology in association with the evolution of large, nutrient-rich eggs (Emlet et al., 1987; Strathmann, 1985). Intermediate phenotypes, such as facultative planktotrophy, could represent short-lived steps on the path toward non-feeding development and morphology (Wray and Raff, 1991) or advantageous and evolutionarily stable modes of larval development (Emlet, 1986; McEdward, 1997; Levitan, 2000). Estimates of relative persistence times for these larval forms are a powerful and novel way to evaluate these two hypotheses (Hart, 1996; Wray, 1996).

Intermediate larval forms are taxonomically rare and phylogenetically scattered (Alatalo et al., 1984; Crump, 1989; Emlet, 1986; Hart, 1996; Kempf and Hadfield, 1985; Kempf and Todd, 1989; Perron, 1981). As the first genus documented to include more than one species with an intermediate larval form–in addition to several species with more derived, non-feeding forms–*Macrophiothrix* offered a unique opportunity to apply phylogenetic information to analysis of this transition. Branch lengths from our phylogeny provided estimates of maximum persistence times for intermediate forms. All of these estimates (from two species, and two genes analyzed separately or in combination, with and without rate variation among lineages) were shorter than the relevant branch lengths for species or clades with obligate non-feeding development and simple larval morphology.

These data are consistent with predictions of the rapid transitions hypothesis (Hart, 1996; Wray, 1995b). Notably, comparison between the two transitional larval forms is also consistent with this hypothesis: the species (*M. rhabdota*) with larvae that retain the capacity to feed occurs on a shorter branch than the species (*M. caenosa*) with larvae that retain a feeding morphology but have lost the capacity to feed (e.g., 0.081 versus 0.148 for COI with a molecular clock). This observation provides indirect support–because these two species are part of separate lineages–for the concept of an ordered transformation series (Wray, 1996) in which feeding capacity and feeding morphology are lost in sequential steps (R. Podolsky, unpublished data). Whether persistence times for these intermediate forms are transient enough to explain their absence in most well-sampled taxa that include both feeding and simplified non-feeding forms is uncertain, but we have failed to reject this hypothesis for the most extensive phylogenetic example available. As the first species-level phylogeny to address early life cycle evolution in ophiuroids, the analysis presented here is a starting point for improved understanding of a large, diverse, and ecologically important class for which developmental and life-history data are relatively scarce.

Acknowledgment

We thank J. Allen and J. McAlister for discussions and contributions to larval culture and measurement, J. Mader and K. Kemp for collection of samples from museum collections, J. Addison for assistance with sequencing, P. Berents (Australian Museum) and J. Fromont (Western Australian Museum) for access to and assistance with collections, D. Posada for software assistance, K. Beckenbach and M. J. Smith for sequence data, and B. Schierwater and four anonymous reviewers for

constructive criticism. A. Hoggett and L. Vail provided research space and logistical support at Lizard Island Research Station. Supported by National Science Foundation grant OCE-9811121, the Canada Foundation for Innovation, and the Natural Sciences and Engineering Research Council.

Appendix A. Laboratory methods and sequence characteristics

The table below shows reference information for the mtDNA data. Australian Museum accession numbers for tissue samples are in parentheses after the species name; other samples were collected from fresh specimens at Lizard Island, Australia, and fixed in 70% ethanol. Identifications of live material were based on Hoggett (1990) and verified by A. Hoggett (personnal communication). Two GenBank numbers are given for species in which 16S sequences were obtained from two individuals. We used standard digestion and extraction methods (e.g., Grosberg et al., 1996) to obtain genomic DNA from gonads, gametes, or arms. From serial dilutions of these DNA samples, we amplified part of each of the 16S and COI genes using 'universal' PCR primers (Folmer et al., 1994; Palumbi, 1996). For six species (*M. caenosa*, *M. demessa*, *M. koehleri*, *M. longipeda*, *M. lorioli*, and *M. nereidina*) we obtained 16S sequences from two individuals. Only four species (*M. belli*, *M. demessa*, *M. longipeda*, and *Ophionereis*) could be reliably amplified and sequenced using the universal COI primers; all other COI amplifications required some variable combination of ten other oligonucleotide sequences (available from the authors on request) that we designed from preliminary sequence data and comparison with complete COI sequences for two other brittlestars (Scouras et al., 2004). We were unable to amplify and sequence COI from *M. lampra*, *M. nereidina*, *M. paucispina*, and *M. robillardi* using the universal or modified primers. We sequenced these PCR products directly using the PCR primers, Thermosequenase (USB), and infrared dye-labeled dideoxy terminators (Li-Cor), and we visualized the fragments on a Li-Cor 4200L-2 DNA sequencer. We used the manufacturers' standard cycle sequencing and electrophoresis conditions. Complementary sequences were compared and edited in AlignIR (Li-Cor). Most PCR products were sequenced from both strands through the end of the opposite primer, but in some cases we obtained only partial sequences of the product or sequences from just one strand, so that the lengths of sequences for each gene varied among taxa.

The 24 aligned 16S sequences were 596 bp long; 184 of these sites were parsimony-informative (with gap sites coded as missing). Many alignment gaps corresponded to insertions in the *Ophiarthrum* sequence relative to *Ophionereis* and the ophiotrichids. Some gaps were large (e.g., a 37-base insertion near the 3′ end of the 16S sequence of *M. propinqua*). The 14 aligned COI sequences were 654 bp; 225 of these sites were parsimony-informative. We found one amino acid deletion: a Gly/Gln codon near the 5′ end of the *Ophiarthrum* sequence.

Taxon names, sequence lengths, and GenBank accession numbers

Species	16S	COI
Ophiotrichidae		
Macrophiothrix belli	492 (AY365143)	571 (AY365144)
M. caenosa	492 (AY365145) 491 (AY365147)	654 (AY365146)
M. demessa	483 (AY365148) 508 (AY365150)	654 (AY365149)
M. koehleri	490 (AY365151) 489 (AY365153)	654 (AY365152)
M. lampra (J14001)	494 (AY365154)	
M. leucosticha	511 (AY365155)	565 (AY365156)
M. longipeda	491 (AY365158) 489 (AY365160)	654 (AY365159)
M. lorioli	511 (AY365161) 493 (AY365163)	654 (AY365162)
M. megapoma	494 (AY365165)	654 (AY365166)
M. nereidina	494 (AY365167) 492 (AY365169)	
M. paucispina	498 (AY365170)	
M. propinqua	542 (AY365172)	654 (AY365173)
M. rhabdota	493 (AY365174)	654 (AY365175)
M. robillardi (J19445)	491 (AY365176)	
Ophiothrix caespitosa (J16397)	442 (AY365179)	654 (AY365180)
O. trilineata (J19233)	491 (AY365182)	654 (AY365183)
Ophionereidae		
Ophionereis porrecta	507 (AY365184)	571 (AY365185)
Ophiocomidae		
Ophiarthrum pictum	523 (AY365186)	651 (AY365187)

Appendix B. Phylogenetic methods and genetic distance results

The table below shows pairwise genetic distances based on maximum likelihood (ML) models. 16S nucleotide sequences were aligned in ClustalX using the default

multiple alignment parameters (Jeanmougin et al., 1998). COI sequences were translated using the echinoderm mtDNA translation table in GeneJockey (http://www.biosoft.com), checked to confirm that they contained no frame shifts, nonsense codons, or stop codons, and aligned as amino acid sequences in ClustalX.

We analyzed phylogenetic information from both genes together (total evidence; Kluge, 1989). We favored this approach because we preferred to include some data for taxa where we lacked COI sequences (see Appendix A) rather than to leave these taxa out of some analyses entirely. Other recent studies show that adding taxa with missing sequence characters tends to improve the quality of total evidence analyses (Hughes and Vogler, 2004; Wiens, 1998). As a quantitative check on this approach, we used the incongruence length difference (ILD) test in PAUP* (Swofford, 2002). We inferred a preliminary maximum parsimony (MP) tree in PAUP* with equal weighting for transitions and transversions, then estimated likelihood scores and the Ti/Tv ratio for sequence data mapped onto that MP tree under a two-parameter ML substitution model. We estimated the appropriate transversion weighting separately for three classes of characters: 16S, COI 3rd codon positions, and all other COI nucleotides. We applied these estimated transversion weightings in the ILD test by use of step matrices with accelerated character transformations, heuristic searching, TBR branch swapping, and 1000 iterations of the test. Although we are aware of the uncertainty surrounding use of the ILD test for identifying significant conflict between data partitions (e.g., Darlu and Lecointre, 2002; Dowton and Austin, 2002; Hipp et al., 2004; Yoder et al., 2001), we found no indication that the 16S and COI sequences gave conflicting information ($P = 0.722$), and in subsequent analyses we used both genes together with COI characters coded as missing for *M. lampra*, *M. nereidina*, *M. paucispina*, and *M. robillardi*.

Our analyses of phylogenetic relationships among ophiotrichids emphasized ML methods rather than parsimony methods because our specific goal was to infer branch lengths leading to species with different larval phenotypes under a biologically realistic model of DNA sequence evolution. We used hierarchical likelihood ratio testing in Modeltest (Posada and Crandall, 1998) to choose the appropriate substitution model (six-rate GTR + G, $\alpha = 0.2136$), then estimated the tree topology using this model in PAUP* with stepwise addition of taxa, TBR branch swapping, and no molecular clock. We estimated nodal support for the ML tree as bootstrap percentages in PAUP* (100 replicates) and as the posterior probabilities of clades under the same likelihood model in coalescent simulations using MrBAYES (Huelsenbeck and Ronquist, 2001). We ran this ML simulation for 110,000 generations, and discarded the first 10,000 generations as a burn-in (in preliminary analyses this number was sufficient to avoid sampling trees and parameter values in the non-stationary part of the simulation). We set the model parameter values (six substitution rates, alpha value for among-site rate variation, and nucleotide frequencies) as priors using the "prset" command. We used default values for other Bayesian parameters, including number of chains (4), temperature (0.2), and sampling frequency (100).

Because some branch length comparisons involved species for which we lacked COI sequence data, we estimated these branch lengths (with and without a molecular clock enforced) under the appropriate substitution model identified for 16S alone (five-rate TVM + G, $\alpha = 0.2823$), COI alone (six-rate GTR + G + I, $\alpha = 0.5544$, $I = 0.4301$), and for the combined data (parameters above).

Genetic distances between 16S sequences of conspecific pairs (bold values below) were smallest in *M. lorioli* (0.017) and largest in *M. demessa* (0.042). These distances are less than or comparable to conspecific genetic distances based on 16S sequences of other ophiotrichids (Baric and Sturmbauer, 1999) or other brittlestar families (Sponer et al., 2001). Most among-species distances were larger, but two sister species pairs were separated by genetic distances within this range: *M. leucosticha* and *M. megapoma* (0.030); *M. paucispina* and *M. rhabdota* (0.032). Genetic distances between COI sequences were substantially larger, and reflected more rapid evolution of this gene relative to 16S. COI distances between the outgroup taxa and the ophiotrichids were very large or undefined and are not reported in the table.

In some preliminary phylogenetic analyses, we found very long terminal branch lengths leading to *Ophiarthrum* in comparison to all other internal and terminal branches. This result reflects the unexpectedly large genetic distances between this species and all other sequences. To avoid artifacts associated with this exceptionally long branch, we removed *Ophiarthrum* from the analyses reported in the main text, and we used *Ophionereis* as the sole outgroup.

Genetic distances among brittlestar mtDNA sequences for 16S rDNA (below diagonal) and COI (above diagonal).

	bel	*cae*1	*cae*2	*dem*1	*dem*2	*koe*1	*koe*2	*lam*	*leu*	*lon*1	*lon*2	*lor*1	*lor*2	*meg*	*ner*1	*ner*2	*pau*	*pro*	*rha*	*rob*	*csp*	*tri*	*por*	*pic*
belli		0.658	*	0.308	*	0.654	*	*	0.544	0.529	*	0.572	*	0.564	*	*	*	0.467	0.585	*	0.697	0.863	*	*
*caenosa*1	0.236		*	0.695	*	0.388	*	*	0.259	0.262	*	0.591	*	0.253	*	*	*	0.638	0.523	*	0.980	0.859	*	*
caenosa2[a]	0.240	**0.028**		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
*demessa*1	0.263	0.321	0.307		*	0.842	*	*	0.612	0.592	*	0.571	*	0.659	*	*	*	0.593	0.569	*	0.684	0.655	*	*
demessa2[a]	0.265	0.316	0.293	**0.042**		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
*koehleri*1	0.269	0.172	0.163	0.371	0.322		*	*	0.429	0.369	*	0.707	*	0.443	*	*	*	0.809	0.620	*	1.018	1.072	*	*
koehleri2[a]	0.257	0.146	0.137	0.334	0.331	**0.041**		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
lampra[a]	0.297	0.277	0.276	0.291	0.311	0.341	0.286		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
leucosticha	0.291	0.141	0.149	0.415	0.361	0.185	0.162	0.362		0.268	*	0.495	*	0.017	*	*	*	0.647	0.576	*	0.741	0.759	*	*
*longipeda*1	0.260	0.113	0.119	0.321	0.317	0.128	0.110	0.325	0.148		*	0.485	*	0.258	*	*	*	0.612	0.457	*	0.671	0.872	*	*
longipeda2[a]	0.273	0.124	0.114	0.321	0.297	0.145	0.121	0.316	0.157	**0.020**		*	*	*	*	*	*	*	*	*	*	*	*	*
*lorioli*1	0.245	0.214	0.226	0.319	0.333	0.259	0.234	0.149	0.267	0.258	0.247		*	0.465	*	*	*	0.644	0.157	*	0.622	0.727	*	*
lorioli2[a]	0.249	0.239	0.234	0.334	0.351	0.244	0.217	0.148	0.269	0.255	0.247	**0.017**		*	*	*	*	*	*	*	*	*	*	*
megapoma	0.251	0.106	0.126	0.356	0.350	0.158	0.126	0.309	0.030	0.113	0.120	0.224	0.233		*	*	*	0.643	0.564	*	0.688	0.832	*	*
*nereidina*1[a]	0.214	0.259	0.259	0.326	0.340	0.280	0.237	0.344	0.282	0.283	0.244	0.311	0.302	0.230		*	*	*	*	*	*	*	*	*
nereidina2[a]	0.223	0.272	0.270	0.362	0.377	0.269	0.236	0.314	0.288	0.249	0.208	0.268	0.267	0.235	**0.030**		*	*	*	*	*	*	*	*
paucispina[a]	0.324	0.287	0.277	0.400	0.400	0.261	0.241	0.185	0.340	0.319	0.295	0.068	0.057	0.296	0.370	0.344		*	*	*	*	*	*	*
propinqua	0.410	0.416	0.345	0.408	0.408	0.424	0.422	0.356	0.527	0.458	0.450	0.418	0.386	0.500	0.519	0.487	0.385		0.609	*	0.541	0.720	*	*
rhabdota	0.308	0.316	0.285	0.393	0.356	0.260	0.245	0.180	0.344	0.325	0.318	0.073	0.063	0.307	0.379	0.352	0.032	0.314		*	0.709	0.773	*	*
robillardi[a]	0.220	0.223	0.191	0.287	0.249	0.190	0.185	0.269	0.247	0.220	0.204	0.227	0.207	0.225	0.284	0.289	0.272	0.351	0.271		*	*	*	*
caespitosa	0.342	0.410	0.375	0.382	0.360	0.299	0.270	0.390	0.407	0.328	0.301	0.315	0.303	0.372	0.359	0.362	0.311	0.572	0.320	0.365		0.835	*	*
trilineata	0.409	0.417	0.371	0.381	0.344	0.404	0.418	0.435	0.508	0.467	0.467	0.393	0.435	0.450	0.461	0.518	0.425	0.524	0.463	0.381	0.360		*	*
ophionereis	0.797	0.764	0.774	0.817	0.774	0.788	0.757	0.725	0.981	0.802	0.701	0.637	0.648	0.869	0.863	0.809	0.705	0.954	0.715	0.818	0.867	0.751		*
ophiarthrum	1.817	1.807	1.808	1.994	2.048	1.773	1.814	2.019	1.930	1.822	1.687	1.922	1.997	1.732	1.768	1.987	1.973	2.284	1.957	1.782	2.262	2.326	1.863	

Distances between conspecifics are shown in bold face.

Undefined distances are indicated with an asterisk.

[a] 16S sequence only

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