Molecular Phylogeny of Extant Holothuroidea (Echinodermata)

Allison K. Miller¹, Alexander M. Kerr¹, Gustav Paulay², Mike Reich^{3,4,5}, Nerida G. Wilson^{6,7},
Jose I. Carvajal^{6,7}, and Greg W. Rouse⁷

¹University of Guam, Marine Laboratory, UOG Station Mangilao, Guam 96923, USA

²Florida Museum of Natural History, University of Florida, Gainesville, FL, USA

³Bavarian State Collection of Palaeontology and Geology, Richard-Wagner-Str. 10, 80333
München, Germany,

⁴Ludwig-Maximilians-Universität (LMU) München, Department of Earth and Environmental
Sciences, Richard-Wagner-Str. 10, 80333 München, Germany

⁵GeoBio Center^{LMU}, Richard-Wagner-Str. 10, 80333 München, Germany

⁶Western Australian Museum, Locked Bag 49, Welshpool DC, WA 6986, Australia

⁷Scripps Institution of Oceanography, University of California, San Diego, La Jolla, California
92037, USA

Corresponding authors: Allison K. Miller, a33miller@gmail.com and Greg W. Rouse, grouse@ucsd.edu

Abstract:

Sea cucumbers (Holothuroidea) are a morphologically diverse, ecologically important, and economically valued clade of echinoderms; however, the understanding of the overall systematics of the group remains controversial. Here, we present a phylogeny of extant Holothuroidea assessed with maximum parsimony, maximum likelihood, and Bayesian approaches using approximately 4.3 kb of mt- (COI, 16S, 12S) and nDNA (H3, 18S, 28S) sequences from 82 holothuroid terminals representing 23 of the 27 widely-accepted family-ranked taxa. Currently five holothuroid taxa of ordinal rank are accepted. We find that three of the five orders are non-monophyletic, and we revise the taxonomy of the groups accordingly. Apodida is sister to the rest of Holothuroidea, here considered Actinopoda. Within Actinopoda, Elapodida in part is sister to the remaining Actinopoda. This latter clade, comprising holothuroids with respiratory trees, is now called Pneumonophora. The traditional Aspidochirotida is paraphyletic, with representatives from three orders (Molpadida, Dendrochirotida, and Elapodida in part) nested within. Therefore, we discontinue the use of Aspidochirotida and instead erect Holothuriida as the sister group to the remaining Pneumonophora, here termed Neoholothuriida. We found four well-supported major clades in Neoholothuriida: Dendrochirotida, Synallactida, Molpadida and a new taxon, Persiculida. The mapping of traditionally-used morphological characters in holothuroid systematics onto the phylogeny revealed marked homoplasy in most characters demonstrating that further taxonomic revision of Holothuroidea is required. Two time-tree analyses, one based on calibrations for uncontroversial crown group dates for Eleutherozoa, Echinozoa and Holothuroidea and another using these calibrations plus four more from within Holothuroidea, showed major discrepancies, suggesting that fossils of Holothuroidea may need reassessment in terms of placing these forms with existing crown clades.

Keywords:

Holothuroidea; sea cucumbers; phylogeny; nomenclature; molecular clock; transformations

1.1 Introduction

Holothuroids, or sea cucumbers, are a diverse and economically important group of echinoderms. Well known for their many bizarre features (e.g., evisceration [García-Arrarás and Greenberg, 2001], anal suspension feeding [Jaekle and Strathmann, 2013], and body wall shedding [Kropp, 1982]), the 1693 accepted extant species (Paulay and Hansson, 2013) show a varied range of forms (Fig. 1). Adult sizes range from 2mm to >3m, and they can be found in almost every benthic marine environment, including polar regions (O’Loughlin et al., 2010) and the deepest oceanic trench (Gallo et al., 2015). Highest species diversity occurs in the shallow waters of the Indo-West Pacific where hundreds of species are recognized (Paulay and Hansson, 2013). Unsurprisingly, these areas are the most heavily targeted by the *bêche-de-mer*, or *trepang*, fisheries. Dried, cooked, pickled, or raw holothuroids are commonly consumed as culinary delicacies, especially in Asia (Conand, 1989), where high demand has led to global exploitation and skyrocketing prices. *Apostichopus japonicus*, for example, sells for up to US\$2,950 per kg (US\$1,338 per lb) in Hong Kong retail markets (Purcell et al., 2012). At present, there are seven species listed as endangered on the IUCN Red List, and nine listed as vulnerable (accessed 15 Feb 2017).

Despite their diversity, abundance, and economic importance, our understanding of the higher-level relationships within the group remains limited. Multiple incongruences between morphology based taxonomic systems (e.g., Becher, 1909; Kerr and Kim, 2001; Ludwig, 1889-1892; Östergren, 1907; Pawson and Fell, 1965) and limited molecular systematic studies to date (e.g., Arndt et al., 1996; Kerr and Kim, 1999; Lacey et al., 2005; Littlewood et al., 1997), leave many questions regarding holothuroid evolution unanswered. To provide a more in-depth understanding of the higher-level phylogenetic relationships of extant Holothuroidea, we sequenced DNA fragments of six nuclear and mitochondrial genes from 82 holothuroid terminals, representing 23 of the 27 currently accepted family-ranked taxa. Maximum likelihood, Bayesian inference, and maximum parsimony phylogenetic analyses were applied to concatenated gene datasets. Based on these results, we propose a revised taxonomy for the major holothuroid clades. We then mapped four morphological traits onto our molecular phylogenetic results to assess their transformations and utility in systematics. We also conducted time-tree analyses for the group with holothuroid fossils.

1.1.1 Taxonomic history

Various authors have presented reviews of the systems and phylogenies produced by holothuroid taxonomists in the 1800's and early 1900's (Kerr and Kim, 2001; Ludwig, 1889-1892; Samyn, 2003; Smirnov, 2012). To summarize, many early holothuroid taxonomists (Haeckel, 1896; Ludwig, 1891; Perrier, 1902; Semper, 1867-8), used only a few selected morphological characters (e.g., respiratory trees and tubefeet) to support their phylogenetic theories of the group (Fig. 2A-D). It was only in the beginning of the 1900's that workers (e.g. Becher, 1909; Cuénot, 1948; MacBride, 1906; Östergren, 1907) began to incorporate multiple morphological characters (e.g., tentacle morphology, tentacle ampullae, respiratory trees, and muscle bands) into their systems (Fig. 2E-H).

Until recently, the most widely-used classification of Holothuroidea was that of Pawson and Fell (1965) (Fig. 2I). This was primarily based on tentacle morphology, body morphology, and, partially, on the shape of the calcareous ring. Following several earlier authors (Deichmann, 1930; Haeckel, 1896; Ludwig, 1889-1892), Pawson and Fell (1965) used five previously erected ordinal-ranked taxa (Apodida, Elasipodida, Aspidochirotida, Molpadida, and Dendrochirotida), and also named a sixth, Dactylochirotida, based on the group's digitiform to digitate tentacles and plated (imbricate) body walls (i.e., a "test"). Pawson and Fell (1965) incorporated Dactylochirotida with Dendrochirotida as a new subclass, Dendrochirotacea, which they recognized along with the subclass Aspidochirotacea (Elasipodida + Aspidochirotida) and Apodacea (Molpadida + Apodida) (Fig. 2I). Their text supported the views of past taxonomists (e.g., Théel, 1886) and that of later paleontologists (e.g., Boczarowski, 1997; Boczarowski, 2001; Haude, 1995, 2002; Jell, 2010) who postulated that the earliest holothuroid ancestors resembled some extant Dendrochirotida.

Despite the wide use of Pawson and Fell's (1965) taxonomic system, various authors offered alternate views about the relationships of its higher levels. For instance, Hansen (1975) dismissed Aspidochirotacea, and while Haude (1992) produced a classification (Fig. 2J) that largely matched that of Pawson and Fell's (1965), he questioned grouping Molpadida with Apodida as Apodacea, and suggested that Molpadida might belong in Dendrochirotacea. Haude (1992), as with other authors, (Cuénot, 1891; Huxley, 1878; Östergren, 1907; Seilacher, 1961; Semon, 1888; Semper, 1867-8), did not agree with Pawson and Fell's (1965) hypothesis of a dendrochirote-like ancestor for Holothuroidea, and instead proposed an apodan-like ancestor.

Dactylochirotida is now generally considered as part of Dendrochirotida (Paulay and Hansson, 2013; Smirnov, 2012).

While higher-level relationships of holothuroids have been verbally discussed at length, few phylogenetic studies have addressed their evolution. Arndt et al. (1996) and Littlewood et al. (1997) (later interpreted by Smith, 1997) provided limited initial molecular phylogenies of seven and four genera (Fig. 2K). Kerr and Kim (2001) presented the first broad phylogenetic analysis based on morphological data from all recognized families (Fig. 2L). Lacey et al. (2005) published the only broad molecular phylogeny to date based on 18S sequence data from 15 genera representing four of the currently recognized orders (Fig. 2M). All of these studies supported the hypothesis of Apodida as sister to Elasipodida + remaining holothuroids. Reich (2010a) added fossil evidence to previous morphological and molecular evidence and agreed with this hypothesis, postulating a close relationship of Elasipodida to Apodida owing to the presence of wheel-like ossicles and statocysts (Fig. 2N).

Smirnov (2012) published the most recent broad holothuroid taxonomic system (Fig. 2O) after considering the views of Becher (1909,) Kerr and Kim (2001), and the results from previously published (Lacey et al., 2005; Smith, 1997) and unpublished (Solís-Marín, 2003) molecular phylogenetic and paleontological findings (Reich, 2010a; Reich, 2010c). Partially following Becher's (1909) phylogeny, Smirnov (2012) proposed that one group (Synaptacea) include holothuroids with wheel-shaped ossicles and without respiratory trees, and that a second group (Holothuriacea + Elpidiacea) include holothuroids with both respiratory trees and secondarily-lost respiratory trees. He also erected Mesothuriidae to separate *Mesothuria* and *Zygothuria* from Synallactidae, following earlier suggestions of similarities of this group with Holothuriidae (Becher, 1909; Sluiter, 1901a), and raised four subfamilies to family status within the Dendrochirotida.

1.2 Material and methods

1.2.1 Sample acquisition

Targeted holothuroids were collected by SCUBA diving, snorkeling, dredging, trawling, submersible, or remote operated vehicle (ROV) from diverse localities (Table 1). Holothuroid representatives from Pawson and Fell's (1965) six orders and from 23 of the 27 widely-accepted extant holothuroid families (Paulay and Hansson, 2013) were included. Most are deposited at

either the Scripps Institution of Oceanography Benthic Invertebrate Collection (SIO-BIC), or the Florida Museum of Natural History Invertebrate Zoology Collection (UF). The rest are deposited at the Museum Victoria Marine Invertebrates Collection (NMV), Muséum National d'Histoire Naturelle (MNHN), National Institute of Water and Atmospheric Research (NIWA), National Oceanography Center, Southampton, Discovery Collections (NOCS-DC), Museum Zoologicum Bogoriense (MZB), and Korea South Pacific Ocean Research Center (KSORC). Targeted specimens that could not be directly collected for this study were acquired from museum collections and DNA extractions were attempted. Unfortunately, only a few extractions were viable; most were degraded and unusable due to their age, or exposure to formalin. An echinoid and an asteroid served as outgroup taxa, with the asteroid as the root. Echinoidea is sister to Holothuroidea forming Echinozoa, with Asteroidea part of Asterozoa, the sister group to Echinozoa (Reich et al., 2015; Telford et al., 2014). All six genes for the outgroups (*Patiria miniata* and *Strongylocentrotus purpuratus*) were downloaded from GenBank. A total of 84 terminals, both novel and published (GenBank), were used in this study (Table 1).

1.2.2 DNA preparation and sequencing

Tentacles or tube feet were subsampled from live animals whenever possible, placed in 95% ethanol, and stored at 4°C. Total genomic DNA (gDNA) was extracted from subsampled tissues following the Qiagen DNeasy protocol for animal tissues. Six gene fragments were sequenced; three mitochondrial: 16S rRNA (16S, partial 671 bp), 12S rRNA (12S, partial 474 bp), and Cytochrome c oxidase subunit I (COI, partial 690 bp), and three nuclear: 18S rRNA (18S, partial 1860 bp), Histone H3 (H3, partial 335 bp), and 28S rRNA (28S, partial 995 bp). PCR reactions were performed using three different reagent mixes; either 12.5 µl GoTaq Green Master Mix (Promega, Inc), 1.0 µl of each primer, 1.0 µl template DNA, and 9.5 µl H₂O; or Illustra puReTaq Ready-To-Go PCR Beads (GE Healthcare, Inc), 1.0 µl of each primer, 1.0 µl template DNA, and 22.0 µl H₂O; or 12.5 µl REDtaq DNA Polymerase (Sigma-Aldrich, Inc), 1.0 µl of each primer, 2.0 µl template DNA, and 8.5 µl H₂O. Most PCR amplifications (Supplementary Material Table 1) used the following profile: 40 cycles of denaturation at 95°C for 30s, annealing at 45°C to 55°C for 40s, and extension at 72°C for 1 min. Following PCR, the PCR product size was confirmed (via 1.5% agarose gel electrophoresis), and the PCR product was purified (2.0 µl ExoSAP-it PCR Product Clean-up with ExoSAP-it thermocycler protocol).

The cleaned products were sequenced using cycle sequencing technology (dideoxy chain termination/cycle sequencing) on ABI 3730XL sequencers (Eurofins sequencing, www.eurofinsgenomics.com). Sequence data were edited with Geneious R6.1.6 (Kearse et al., 2012) and deposited in GenBank (Table 1).

1.2.3 Alignment and Partitioning

Sequences were aligned using MAFFT 7 (Katoh and Standley, 2013) with the E-INS-i strategy and each alignment was manually inspected with Geneious and Mesquite 3.10 (Maddison and Maddison, 2015). To ensure that the most robust phylogenetic inferences were made, several refinements were made on the aligned matrices. All of the rDNA alignments were first run through the Gblocks online server 0.91b (Talavera and Castresana, 2007) to remove poorly-aligned and highly-divergent areas of alignment, using the least stringent settings. Additionally, protein-coding gene alignments were analyzed with DAMBE 5.3.57 (Xia and Xie, 2001; Xia et al., 2003) to assess for substitution saturation. The Gblocks online server removed multiple positions from the original alignments (18S = 89% retained, 12S = 80%, 16S = 74%, 28S = 82%) and DAMBE recommended the removal of the 3rd positions from the COI alignment. The two concatenated alignments, referred to here as ‘complete’ (dataset with nothing excluded) and ‘reduced’ (dataset excluding the positions removed by the Gblocks analyses and also excluding the COI 3rd positions), are available in TreeBASE (www.treebase.org/). Each dataset was partitioned by both gene and codon position (for protein-coding genes: COI and H3), which resulted in nine (with COI 3rd excluded) or ten partitions. jModelTest 2.1.4 (Darriba et al., 2012) was used to determine the best-fit models for the Bayesian inference (BI) analyses from 56 competing models, using Akaike Information Criterion (AIC) calculations. In each case the best-fit model for the BI analyses was GTR + I + Γ .

1.2.4 Phylogenetic analyses

Maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) estimators were conducted on all datasets, first on the individual gene partitions and then on the concatenated gene datasets. MP analyses were performed with PAUP* 4.0a142 (Swofford, 2002) with all characters weighted equally. Heuristic searches were conducted using random stepwise addition of the terminals (1000 replicates) with the tree bisection re-connection (TBR)

permutation algorithm and with maximum zero-length branches collapsed. Nodal support was assessed via parsimony jackknifing (1000 replicates, 10 random additions per iteration, 37% deletion) analyses (Farris et al., 1996). ML analyses were performed with RAxML GUI 1.5 (Silvestro and Michalak, 2012; Stamatakis, 2014) using the GTR + Γ model of substitution as recommended by the program's author. The data was partitioned as outlined above and non-parametric bootstrapping was conducted using the same model conditions with 1000 bootstrap pseudo-replicates. BI analyses were executed using MrBayes 3.2.2 (Ronquist and Huelsenbeck, 2003). Four iterations of 50,000,000 generations were run on four chains, sampling every 1000 generations on the CIPRES Science Gateway 3.3 (Miller et al., 2010). Default priors, unlinked parameter estimates, and GTR + I + Γ for each partition were used for each iteration. Convergence and mixing of the Markov chain Monte Carlo (MCMC) runs were assessed with Tracer 1.6.0 (Rambaut et al., 2013). A majority-rule consensus tree was created from the runs, after a 10% burn-in.

Constraint analyses were conducted after the phylogenetic analyses to demonstrate statistical support for (1) the results against two existing classifications and hypotheses for Holothuroidea and (2) the ten non-monophyletic family-ranked clades recovered within Holothuroidea. The Pawson and Fell (1965) and Smirnov (2012) classifications and hypotheses were transformed into explicit trees and then used in RAxML to make constrained tree topologies (Supplementary Material Figures 1A and 1B) for the terminals used in the current study. The best ML tree topology from the reduced dataset (selected as the primary dataset, see results below) was compared to the best constrained ML trees (i.e., the best Pawson and Fell constraint tree and the best Smirnov constraint tree) from the same dataset with the approximately unbiased (AU) test (Shimodaira, 2002) in PAUP* 4.0a142 (Swofford, 2002). The best ML tree and best ML-constrained trees were then imported into PAUP*, and the AU *p*-values were recovered with the RELL method with 100,000 bootstrap replicates (Kishino et al., 1990). Constraint analyses were also performed on ten family-ranked taxa that were recovered as non-monophyletic (Synaptidae, Laetmogonidae, Synallactidae, Caudinidae, Molpadiidae, Stichopodidae, Cucumariidae, Phyllophoridae, Sclerodactylidae, and Psolidae). These were performed separately with each family constrained as a clade (Supplementary Material Figure 1 C-L) and the constrained tree topologies generated in RAxML with the same parameters as

outlined previously. These constrained family trees were then compared to the best ML tree topology from the reduced dataset using the AU test as explained above.

1.2.5 Divergence dating

Divergence dating of the holothuroid lineages was conducted on the reduced dataset. Eleutherozoa, Echinozoa, and a series of holothuroid nodes (recovered by the current phylogenetic analyses) were constrained as clades and assigned minimum- or stem-age estimates. Age constraints were then applied to generate two datasets: a three-estimate dataset, referred to here as “3-age” (Table 2, A-C), which represented well established echinoderm dates, and a six-estimate dataset, referred to here as “6-age” (Table 2, A-F), which incorporated three lesser-known holothuroid fossil dates as well as the “3-age” dates. Initially, only one dataset was generated (6-age) using the lesser-known fossil dates, however, this led to rate discrepancies in the output trees, and analyses using well-known calibration points from outside Holothuroidea (3-age) were later performed. The BEAST v2.3.3 package (Bouckaert et al., 2014) was used to assess holothuroid divergence times using a relaxed molecular clock method (Drummond et al., 2006) and the same data partitions and conditions from the above BI analyses. BEAUti from the BEAST2 package was used to create ‘.xml’ files with the following parameters: uncorrelated log-normal model of rate change, GTR + I + Γ model of substitution (partitioned), Yule model of divergences, and either three (3-age) or six (6-age) fixed divergence dates (Table 2) (normal distribution) based on fossil evidence (see below). BEAST2 executed the .xml files using a random starting tree with 50 million generations for 10 separate MCMC analyses. Results were assessed for stationarity, convergence, and effective sample size with Tracer 1.6.0. The trees for the 10 runs (95% burnin each) were combined with Log Combiner (Beast package), and then Tree Annotator (BEAST2 package) was used to generate maximum-clade credibility majority rule consensus trees with mean ages for unconstrained nodes.

Table 2 summarizes the dates for the constrained nodes used in the divergence dating analyses. Constrained ages followed a lognormal distribution around a median age and the 95% distribution of each age is indicated.

The earliest echinoderms are reported from the Early Cambrian (Terreneuvian, ~530 Ma) (Smith, 2005; Sumrall and Wray, 2007; Zamora et al., 2013). The first were likely bilateral forms, as represented by the middle Cambrian *Ctenoimbricata* (Zamora et al., 2012), which gave

rise to asymmetrical cinctans and solutes. Radial symmetry ensued after the evolution of the water vascular system, with triradial helicoplacoids and later by pentaradial helicocystoids (Zamora and Rahman, 2014). Crinoids appeared soon after this rapid burst of morphological diversification, between the "Middle" Cambrian (513–524 Ma) and the Early Ordovician (Tremadocian, ~479–488 Ma) (Guensburg and Sprinkle, 2001; Paul and Smith, 1984). The first eleutherozoans in the record were asterozoan-like (e.g., *Maydena* and *Eriaster*) and appeared in the Tremadocian (Jell, 2014) and Floian (both Lower Ordovician, ~485 to 477 and ~470 Ma respectively) (Blake and Guensburg, 2005; Lefebvre et al., 2013). Hence, 481 Ma was used with a lognormal distribution (480–488 Ma) for the crown Eleutherozoa, constrained node A, in the 3-age and 6-age analyses (Table 2, A).

The earliest Holothuroidea is dated to the Middle Ordovician *P. polonicus* conodont zone (Darriwilian, ~464 Ma, Reich, 2001; Reich, 2010a) and the earliest Echinoidea is dated to the slightly younger *P. serra* conodont zone (Reich in Lefebvre et al., 2013; ~462 Ma, Pisera, 1994). From this, a median age of 464 Ma with a lognormal distribution (463–465 Ma) was used for the crown Echinozoa, constrained node B (Table 2, B). The extinct “snake box”-like ophiocistoids were not considered during the dating of the Echinozoa node, since the Ordovician record for the extinct Ophiocistioidea is currently under debate (i.e., *Volchovia* spp. from the Dapingian and Darriwilian strata) (Reich in Lefebvre et al., 2013; Reich, 2010b; Reich and Haude, 2004; Reich and Kutscher, 2014). The date of origin for the crown group Holothuroidea, constrained node C, remains somewhat uncertain due to the group’s depauperate fossil record. In the current work, the crown group Holothuroidea is identified by the presence of a calcareous ring (multiple calcareous pieces circularly arranged and connected with tentacles) and the stem-group Holothuroidea (e.g., *Palaeocucumaria*), in contrast, is delimited by the lack of a distinct calcareous ring, the presence of 40–50 lobate-like arranged tentacles (or “mouth tube feet”), and a single external madreporic plate (Smith and Reich, 2013). Consequently, the oldest reported crown group holothuroid fossils to date are apodan calcareous ring elements reported from early Silurian strata (Llandovery, ~435 Ma Reich, 2015), thus, 435 Ma was used as the median age (431–449 Ma) for crown Holothuroidea, constrained at node C (Table 2, C).

The oldest potential elasipodan calcareous ring elements (‘Pannychiidae’), date to the late Silurian (Ludfordian, ~425 to 423 Ma Reich, 2015), however, the oldest distinctly elasipodan wheel ossicles are known from younger Middle Devonian strata (late Givetian, ~387 to 382 Ma)

(described as myriostrochid in Boczarowski, 2001), thus, 387 Ma was used as the minimum age with a lognormal distribution (384-394 Ma) for the stem Elasipodida, constrained at node D (Table 2, D). The earliest articulated calcareous rings for Holothuriidae, constrained at node E, date only to early Middle Triassic (late Anisian, ~247 to 242 Ma, Reich, 2004; Smith et al., 2013), as do the earliest holothuriid buttons (*Calclamnella regularis*; Stefanov, 1970). Fragmentary table ossicles that may have belonged to Holothuriidae are known from the early Late Permian (earliest Wuchiapingian, ~259 to 254 Ma); however, the placement of Holothuriidae with Mesothuriidae in our current analyses suggests that these table ossicles could have been from a stem group. Therefore, 246 Ma was used as a minimum age with a lognormal distribution (244–257 Ma) for the stem Holothuriidae group (Table 2, E). The oldest Caudinidae + Molpadiidae, constrained at node F, are described from the early Late Permian (Wuchiapingian, ~259 to 254 Ma Mostler and Rahimi-Yazd, 1976); however, these isolated body wall ossicles were probably of stem members. Body wall ossicles are also reported from the early Middle Triassic (Anisian, ~247 to 242 Ma Speckmann, 1968), yet the earliest known undisputable Caudinidae + Molpadiidae calcareous ring material is dated to the Lower Jurassic (Sinemurian, ~199 to 190 Ma, Gilliland, 1992). Since even extant Caudinidae and Molpadiidae body ossicles (ranging from “table” to “anchor” in shape, Östergren, 1897) can sometimes be confused with those from other distant families, the undisputable calcareous ring age date, 198 Ma, was used with a lognormal distribution (196-201 Ma) for stem Caudinidae + Molpadiidae group (Table 2, F).

1.2.6 Morphological transformations

Four holothuroid attributes were scored for mapping transformations on our phylogenetic results. These were: 1. Respiratory trees; 2. Skeletal elements in general; 3. “Table” ossicles; 4. Tentacle shape (Supplementary Material Table 2). Four other attributes were analyzed and can be found in the Supplementary Material Table 2: depth, habitat preference, calcareous ring (CR) posterior projections, and CR foramina. The characters were scored either as binary (respiratory trees and table ossicles) or multistate (skeletal elements and tentacle shape). Voucher specimens were directly analyzed and scored for each character (Supplementary Material Table 2) if possible. Otherwise, published accounts and personal observations (listed as footnotes in the

scoring matrices; Supplementary Material Table 2 Nexus file) were relied upon. In a few cases, characters were scored for terminals based on information for closely related taxa.

Characters

1. Respiratory trees: 0 = absent; 1 = present. Respiratory trees are specialized intestinal diverticula that serve as internal gas exchange organs. They lie bilaterally within the coelomic cavity and consist of ramified tubes. Presence or absence of respiratory trees has served as the basis for dividing holothuroids into major clades since Jaeger (1833). Respiratory trees can increase oxygen uptake by 60% in some species (Brown, 1979) and are sometimes used in anal suspension feeding (Jaekle and Strathmann, 2013).
2. Skeletal elements (ossicles): 0 = absent from body wall; 1 = present as separated elements within the body wall; 2 = present as imbricating plates forming an outer test of varied rigidity. Ossicles are commonly found dispersed in the body walls of most holothuroids. A few holothuroids lack ossicles in their body walls, while a few others have enlarged ossicles, usually plates, that are arranged in an imbricating, or overlapping fashion all over their bodies. Holothuroids with such a robust skeleton are often said to have a “test,” a condition that was deemed plesiomorphic by Pawson and Fell (1967). Terminals were only scored absent if body wall ossicles are not known in any developmental stages (some holothuroids lose their ossicles with age [Pawson, 1977]).
3. “Table” ossicles: 0 = absent; 1 = present. “Table” ossicles may be homologous across holothuroids, albeit their variability makes defining them and assessing their distribution somewhat challenging. In general tables have a flattened disk from which a spire, comprised of one to four columns, arises orthogonal to the disk. Tables are arranged as an outer monolayer in the body wall, with the disks lying in the plane of the body wall, and the spires, often with bristled apices (crowns), projecting outward to the surface of the body wall. Here table ossicles are defined as ossicles with this shape and position. A few specialized ossicles, such as dendrochirotid “cup” ossicles and four or three armed crosses with perforated ends, are generally considered to be derived from tables (O’Loughlin et al., 2011; O’Loughlin et al., 2015; Smirnov, 2012) and were so scored.
4. Tentacle Shape: 0 = absent (outgroups); 1 = peltato-digitate (as defined by Clark, 1908); 2 = pinnate; 3 = digitiform; 4 = digitate (2-4 digits); 5 = peltate; 6 = dendritic. Peltato-digitate tentacles have three to nine pairs of digits, no central digit (Clark, 1908) and

digits are typically arranged in a semicircle, giving the tentacle a “palm” shape. Pinnate tentacles have a single terminal digit and paired digits that span much of the tentacle length. Digitiform tentacles are simple, unbranched, and finger-shaped. Digitate tentacles have one or two pairs of digits and lack a central terminal digit. Peltate, or shield-shaped, tentacles have a terminal disk that is either smooth or terminates in numerous short projections (papillated). Dendritic tentacles have an elongate stalk from which numerous side-branches extend and further ramify, decreasing in size toward the apex.

Character transformations were traced onto the reduced dataset ML tree topology (branch lengths included) using a Markov k-state one parameter (Mk1) model (Lewis, 2001) with Mesquite 3.10 (Maddison and Maddison, 2015).

1.3 Results

Sequences were obtained for 85% of the genes targeted across the 84 taxa used. Four to six genes were sequenced from all terminals, except for *Echinocucumis hispida*, *Parastichopus tremulus*, and *Cucumaria miniata* (three missing genes), and *Myriotrochus antarcticus* (five missing genes). The maximum likelihood (ML) tree for the reduced dataset is shown in Figure 3, with corresponding ML, Bayesian inference (BI), and maximum parsimony (MP) node support values where appropriate. Results from the reduced dataset are emphasized, because of saturation and poor alignment in the complete dataset. Topologies for ML, BI, and MP analyses with both the reduced and the complete dataset are shown in Supplementary Material Figure 2, along with summary statistics (Supplementary Material Table 3). Results from all analyses of both datasets were largely congruent.

The ML result of the reduced dataset was assessed with the approximately unbiased (AU) test against the best ML trees constrained with the Pawson and Fell (1965) and Smirnov (2012) hypotheses. The reduced dataset had significantly better likelihood (lnL -52038.81928) than both the Pawson and Fell (lnL -52630.36209) the Smirnov (lnL -52401.23704) constrained trees. Thus the AU test rejected both these classifications, and a new one is implemented here. The ten AU tests of the reduced dataset ML tree against ML trees where the various non-monophyletic families were constrained to be clades, generally showed the unconstrained topology to be significantly better. The reduced dataset ML tree showed significantly ($p < 0.05$) better

likelihood (lnL -52038.81928) than Synallactidae (lnL -52446.55069), Caudinidae (lnL -52210.61349), Molpadiidae (lnL -52256.98088), Cucumariidae (lnL -52132.07824), Phyllophoridae (lnL -52177.12494), Sclerodactylidae (lnL -52189.73852), and Psolidae (lnL -52119.02726) constraint trees. The reduced dataset ML tree did not have significantly better likelihood when Synaptidae, Laetmogonidae, and Stichopodidae were constrained as clades.

Monophyly of extant Holothuroidea was strongly supported in all analyses (Fig. 3). Apodida *sensu* Pawson and Fell (1965), was always highly supported as the monophyletic sister group to all other holothuroids. Within Apodida, Myriotrochidae was also strongly supported as a clade and appeared sister to the remaining Apodida in all analyses. Notably, the branch length for *Myriotrochus* was at least three times greater than any other branch length within the tree (Fig. 3). It appears that the *Myriotrochus* sp. COI sequence was responsible for this disparity (COI and all other genes except for 18S were missing for *Myriotrochus antarcticus*). An additional COI sequence comparison of *Myriotrochus* sp. and an independently sequenced myriotrochid (Smithsonian National Museum of Natural History Invertebrate Zoology Collection, *Myriotrochus ahearnae* paratype) demonstrated that this long branch was not due to contamination (87% identity, Supplementary Material Table 4). Of the other Apodida sampled, Synaptidae was paraphyletic in all analyses, though a constrained monophyletic Synaptidae was not significantly worse than the best tree (see above AU test results). The ML and BI analyses of both datasets and the MP complete dataset recovered *Euapta* as sister to a well-supported chiridotid + *Leptosynapta* clade, while the MP reduced dataset analysis recovered *Leptosynapta* as a sister group to a *Chiridota* + *Euapta* clade. Chiridotidae was only monophyletic in the MP analyses and even *Chiridota* was not recovered as a clade in several of the analyses.

Apodida was sister to a uniformly well-supported clade comprising the remaining holothuroids, here termed Actinopoda following Ludwig (1889-1892) and denoting the presence of podia (Fig. 4). Within this clade, the deep-sea taxon Elasipodida (*sensu* Théel, 1882), as traditionally circumscribed, was polyphyletic in all analyses (Fig. 3, purple), with Deimatidae separated from the other Elasipodida families (further results below). The remaining terminals, here referred to as Elasipodida (Laetmogonidae, Psychropotidae, Elpidiidae, and Pelagothuriidae), formed a well-supported clade that was sister to all other Actinopoda (Fig. 4, purple). Within this group, *Benthogone abstrusa* was always recovered as sister to the pelagothuriid *Eynyniastes eximia* and the two *Pannychia* terminals consistently formed a group

sister to all other Elasipodida, thus rendering Laetmogonidae paraphyletic (Fig. 3), though a constrained monophyletic Laetmogonidae was not significantly worse than the best tree (see AU test results). All representatives of Elpidiidae and Psychropotidae formed a well-supported clade; however, their placement within Elasipodida was poorly supported.

The sister group to Elasipodida was a consistently well-supported clade (Fig. 3), here termed Pneumonophora following Selenka (1867) and denoting the presence of respiratory trees (Fig. 4). Within Pneumonophora, a clade containing representatives from all of the currently accepted genera of Holothuriidae and Mesothuriidae was well-supported in most analyses and was sister to the remaining terminals. This clade is referred to here as Holothuriida (Fig. 4). The only major topological difference between the complete and reduced datasets regarding Holothuriida was that, in the MP complete analysis, Holothuriida formed a grade with respect to the rest of Actinopoda. This result is discounted here since all other analyses recovered Holothuriida as a clade with reasonable to high support, and since this relationship has been previously suggested based on morphology (see above). Within Holothuriida, the two mesothuriid terminals always formed a group sister to Holothuriidae. Holothuriidae appeared monophyletic in all analyses, with *Holothuria hilla*, *H. leucospilota*, and *Labidodemas* cf. *pertinax* always forming a well-supported clade of vermiform holothuriids. The placement of this vermiform clade varied across analyses; the MP analyses of the complete and reduced datasets showed a *Bohadschia koellikeri* + *Pearsonothuria graeffei* clade as sister to the vermiform-shaped group, while the ML and BI analyses of both datasets (Supplementary Material Figure 2) recovered a “bread loaf”-shaped clade of *Actinopyga varians* (*Bohadschia koellikeri* + *Pearsonothuria graeffei*) sister to the vermiform clade.

Holothuriida was sister to a well-supported clade of the remaining Pneumonophora, which we refer to as Neoholothuriida (Fig. 4). Neoholothuriida contained two main groups in all analyses: Dendrochirotida (*sensu lato*), and all remaining taxa. All representatives from Dendrochirotida and Dactylochirotida were recovered in the Dendrochirotida (*sensu lato*) group with high support by all analyses (Fig. 3). It is referred to here as Dendrochirotida (Fig. 4). Most of the deeper nodes within the clade, however, were poorly supported and the clade’s topology varied among the analyses (Supplementary Material Figure 2), with the MP topologies differing markedly from the ML and BI analyses of each dataset. Nevertheless, in all three analyses dactylochirotid terminals (all Ypsilothuriidae) were nested within Dendrochirotida, and four

(Psolidae, Cucumariidae, Sclerodactylidae, and Phyllophoridae) of the seven represented Dendrochirotida families were consistently non-monophyletic across analyses and datasets (Fig. 4).

The other Neoholothuriida group comprised the well-supported clades Persiculida, Molpadida and Synallactida, but was poorly supported and we choose not to name it here. The new clade Persiculida included genera formerly assigned to Synallactidae (*sensu* Ludwig, 1894) and Gephyrothuriidae (often placed in Molpadida [e.g., Haeckel, 1896]) (Fig. 4, orange). Synallactidae was always recovered as polyphyletic in all analyses, with some terminals in Persiculida, and others intermixed with sequence representatives from Stichopodidae (*sensu* Haeckel, 1896). With the exception of *Gephyrothuria*, Molpadida included all the other molpadidan terminals used in this study (representing Caudinidae and Molpadiidae), but neither family was recovered as monophyletic in any of the analyses (Fig. 4, red). Synallactida included members from Synallactidae and Stichopodidae (*sensu* Haeckel, 1896) and, intriguingly, Deimatidae (*sensu* Théel, 1882) (Fig. 4, green), a result that was recovered in all analyses of each dataset. Within Deimatidae, *Deima validum* was always recovered as sister to *Oneirophanta setigera* + *Orphnurgus glaber* with high support. The Synallactidae terminals (except those that grouped with *Gephyrothuria* as Persiculida) along with all the Stichopodidae terminals formed a well-supported sister group to Deimatidae. Most stichopodids formed a clade in almost all analyses, except for *Apostichopus*, which nested among the paraphyletic grade of synallactids. The MP analyses for the complete dataset recovered a similar topology, except that Stichopodidae did form a clade (Supplementary Material Figure 2C).

1.3.1 Molecular clock analyses

The 3-age (Fig. 5) and 6-age (Fig. 6) Beast v2.3.3 analyses closely resembled the tree topology recovered from the ML and BI analyses (Fig. 3), apart from minor differences within Apodida, Synallactida, and Dendrochirotida on some poorly-supported branches. Substitution rates and divergence times for some key nodes, however, were markedly different between the 3-age and 6-age analyses. In general, recovered substitution rates were slower in deeper branches and faster in terminal branches in the 3-age analysis. An exception to this trend was between constrained nodes B and C, which showed more than twice the substitution rate of anywhere else on the tree in the 3-age analysis (Fig. 5). The 6-age analysis also showed an extremely high

substitution rate between constrained nodes B and C and between C and D (Fig 6). Overall this resulted in some marked differences in age estimates at these nodes that were constrained in the 6-age analyses and left free to vary in the 3-age analyses. The elasipodid stem age, used at Node D for Actinopoda, was constrained at ~387 Ma in the 6-age analysis (Fig. 6) and was recovered with a much younger age of ~281 Ma in the 3-age analysis (Fig. 5). Stem Holothuriida (Node E) was constrained at ~246 Ma in the 6-age analysis (Fig. 6), however, it was recovered as ~203 Ma in the 3-age analysis (Fig. 5). Finally, Node F (stem Molpadida) was constrained at ~198 Ma in the 6-age analysis (Fig. 6), but was recovered as ~158 Ma in the 3-age analysis (Fig. 5). Age discrepancies were also notable between the analyses for nodes that were free to vary. For instance, the most recent common ancestor (MRCA) for Apodida was ~282 Ma in the 6-age analysis (Fig. 6) but was ~259 Ma in the 3-age analysis (Fig. 5). Also, the age for the MRCA of Pneumonophora was ~309 Ma in the 6-age analysis (Fig. 6), while the 3-age analysis recovered the node at ~243 Ma (Fig. 5). The MRCA of Dendrochirotida was left free to vary in both sets of analyses, but was recovered at ~182 Ma in the 6-age analysis (Fig. 6) and ~151 Ma in the 3-age analysis (Fig. 5). In summary, constraining several nodes in Holothuroidea (Nodes D-F) in the 6-age analysis led to markedly higher variation in substitution rates and older ages for unconstrained nodes relative to the 3-age analysis (Figs. 6 and 5).

1.3.2 Transformation analyses

The ML transformation on the ML tree topology for the reduced dataset showed that respiratory trees evolved once in the branch leading to Pneumonophora (proportional likelihood >0.95), and were afterwards lost once in Deimatidae (Fig. 7A). Transformation analyses further suggested that free body wall ossicles are plesiomorphic, while a “test” arose several times within Dendrochirotida, and that body wall ossicles were probably lost twice, at the origins of Persiculida (proportional likelihood >0.95) and Pelagothuriidae (represented here by *Enypniastes eximia*) (Fig. 7B).

The evolutionary history of table ossicles appears less clear. It is possible that table ossicles arose once at the origin of Pneumonophora (proportional likelihood ~ 0.5) and underwent multiple independent losses and modifications (Fig. 7C). When the transformation was assessed using a parsimony criterion there were four most parsimonious reconstructions (MPR) of 12 steps. The MPR that maximized the homology of table ossicles showed three

origins (including one below the node for Pneumonophora) and nine losses, while the other MPRs showed up to eight origins and fewer losses.

Tentacle shape is a longstanding diagnostic character in holothuroid taxonomy. The plesiomorphic condition for Holothuroidea was ambiguous (Fig 7D). Peltate tentacles at the base of Actinopoda and peltato-digitate tentacles at the base of Apodida had high proportional likelihood support. Dendritic tentacles were strongly supported at the base of Dendrochirotida (proportional likelihood >0.95), as were two independent origins of digitate tentacles (with two to four digits) in Molpadida and in *Gephyrothuria alcocki*.

1.4 Discussion

This is the first broad-scale molecular phylogenetic analysis of Holothuroidea allowing for a reassessment of the relationships of these highly derived echinoderms. The approximately 4.3 kb of sequence data obtained from mitochondrial (COI, 16S, 12S) and nuclear (H3, 18S, 28S) sources, was analyzed under a variety of methods which consistently recovered a series of well-supported nodes. This allows for the broad-scale taxonomy of Holothuroidea to be revised with some confidence. Apodida is a well-established taxon that was recovered as a clade with a high level of support and little revision is required. Elasipodida, Dendrochirotida and Molpadida are three other traditionally used names that are reformulated here to accommodate three well-supported clades recovered in the current study. Old names that have long been disregarded, namely Actinopoda and Pneumonophora, are reintroduced, as well as several new major taxa: Holothuriida, Neoholothuriida, Persiculida and Synallactida. Linnaean ranks are not applied to these more inclusive taxon names as there are not enough ranks to sufficiently reflect even the broad-scale topology of the tree (see Pleijel and Rouse, 2003). The reader is referred to Figure 4 and to the indented naming system below that reflects the hierarchy of names used here (not including family or genus-ranked taxa; see Figure 3).

1.4.1 Holothuroidea

- Apodida

- Actinopoda

 - Elasipodida

 - Pneumonophora

Holothuriida
Neoholothuriida
 Dendrochirotida
 Persiculida
 Molpadida
 Synallactida

1.4.1.1 Apodida

The Apodida terminals formed a well-supported clade in all analyses (Fig. 3, yellow), and, as proposed by numerous authors (Becher, 1909; Cuénot, 1948; Haude, 1992; Kerr, 2001; Lacey et al., 2005; Littlewood et al., 1997; MacBride, 1906; Reich, 2010a; Semper, 1867-8; Smirnov, 2012), was recovered as sister to all other holothuroids. Although Smirnov (2012) named this clade Synaptida (Cuénot, 1891), we use the older (derived from Brandt, 1835) and widely-accepted (Paulay and Hansson, 2013) name, Apodida, here. Within Apodida, Myriotrochidae was sister to the remaining Apodida taxa as long recognized (Kerr, 2001; Östergren, 1907; Smirnov, 1998; Smirnov, 2012). The long branch leading to Myriotrochidae (Fig. 3) was caused by the highly divergent *Myriotrochus* sp. COI sequence, as none of the other four gene sequences (18S, 28S, 16S, or H3) demonstrated major branch length differences relative to the other holothuroid taxa. The *Myriotrochus* sp. COI sequence was validated in comparison with a COI sequence from *Myriotrochus ahearnae*, which also showed high divergence from other Holothuroidea but close similarity to *Myriotrochus* sp. (Supplementary Material Table 4). Further investigation is warranted as to why these myriotrochid COI sequences, which also result in markedly different amino acid sequences, show such differences from the rest of Holothuroidea. Lastly, the paraphyletic relationship of synaptids relative to chiridotids is surprising and warrants further phylogenetic analysis.

1.4.1.2 Actinopoda

The presence or absence of podia (pedicels and papillae) has long been recognized as a key character for Holothuroidea (e.g., Brandt, 1835; Jaeger, 1833; Ludwig, 1889-1892). Few authors have applied a name for the holothuroid clade that is sister to Apodida, a group that was consistently recovered with high support by all analyses in the current study (Fig. 3). Potential

apomorphies include peltate tentacles, radial ambulacral canals, and podia. However, peltate tentacles appear to have undergone subsequent reversals in parts of the clade (e.g., Dendrochirotida and Molpadida) (see below Character Transformations 1.4.3). Brandt (1835) named a group Pedatae for those holothuroids with tube feet, as opposed to his Apodes for those without tube-feet, but the name Pedatae, or Pedata, has rarely been used. Ludwig (1891) also divided Holothuroidea into two major groups Actinopoda and Paractinopoda, which he based on the morphology of the ambulacral system. His Actinopoda included representatives from Aspidochirotida, Elasipodida, Dendrochirotida and Molpadida, thus corresponding to the clade here recognized, unlike the more fluid concept of Brandt (1835). Actinopoda, erected by Calkins (1909), is a name that has been applied at various taxonomic levels for protists including Radiolaria and Heliozoa. These taxa, however, are now considered a polyphyletic assemblage and the name is no longer in common use (Adl et al., 2012; Ishitani et al., 2011). Therefore, the name Actinopoda is here resurrected for the holothuroid sister group to Apodida (Fig. 4).

1.4.1.3 Elasipodida

The traditionally formulated Elasipodida was recovered as polyphyletic in all analyses (Fig. 3), with Deimatidae consistently recovered within Synallactida (Fig. 4), while the remaining Elasipodida terminals formed a well-supported clade. The name Elasipodida is here applied to this latter clade (Fig. 4, purple). Taxonomic relationships among Elasipodida families have varied over the years (see Becher, 1909; Cuénot, 1948; Haeckel, 1896; Ludwig, 1891; MacBride, 1906; Östergren, 1907; Perrier, 1902; Théel, 1882), and a few authors have called attention to the poor fit of deimatids. For instance, Perrier (1902) and Becher (1909) hypothesized Deimatidae as a group within Aspidochirotida, while Ekman (1925) and Hansen (1975) noted differences (e.g., gonad structure) between Deimatidae and the rest of Elasipodida. Recently, Smirnov (2012, 2016) removed Deimatidae from Elasipodida and placed it in Aspidochirotida on the basis of calcareous ring shape, ossicle morphology, and an unpublished molecular phylogenetic analysis (Solís-Marín, 2003).

Within Elasipodida, members of four family-ranked taxa were represented: Elpidiidae, Laetmogonidae, Pelagothuriidae, and Psychropotidae. Elpidiidae and Psychropotidae were always recovered with strong support, but their positions within Elasipodida were poorly resolved. Laetmogonidae, although not statistically non-monophyletic (AU tests), was always

recovered as paraphyletic; *Pannychia* was always sister to the other Elasipodida, and *Benthogone abstrusa* was always sister to the pelagothuriid *Enypniastes eximia*, though with poor support. Consequently, the main morphological characters that currently diagnose Laetmogonidae, namely the absence of lateral papillae and presence of wheel ossicles (Smirnov, 2012), are possibly plesiomorphies and may need reassessment. The systematic position of Pelagothuriidae has long been debated (Cuénot, 1948; Haeckel, 1896; Hansen, 1975; Kerr and Kim, 2001; Östergren, 1907; Perrier, 1902). Our study does not support a relationship between Pelagothuriidae and Psychropotidae (Gebruk, 1990), or that Pelagothuriidae should be removed from Elasipodida (Heding, 1950). It will be interesting to see where the other known pelagothuriid, *Pelagothuria natatrix* (Fig. 1A), the only wholly pelagic echinoderm, falls in future phylogenetic analyses.

1.4.1.4 Pneumonophora

Although respiratory trees have been considered an important character in holothuroid classification since Jaeger (1833), there have been few names applied to the large well-supported respiratory tree-possessing clade that our study recovered as sister to Elasipodida. Early workers divided Holothuroidea into clades with and without respiratory trees, which correspond to Apodida and Pneumonophora here, as Elasipodida was then unknown (e.g., Müller, 1850; Selenka, 1867; Semper, 1867-8). The discovery of Elasipodida by Théel (1879) introduced a group without respiratory trees but with podia, and the relationships of Apodida, Elasipodida, and other clades have been diversely interpreted since. The topology supported here, where Elasipodida is sister to Pneumonophora, was first proposed by Cuénot (1891) and has been supported recently by the phylogenetic analyses of Kerr & Kim (2001) and Lacey et al. (2005). Smirnov (2012) called this clade Holothuriacea in his review; however, the name Pneumonophora is preferred here since respiratory trees are a clear synapomorphy for the group and it is an older name. Two major clades were recovered within Pneumonophora: Holothuriida and Neoholothuriida.

1.4.1.5 Holothuriida

The present study consistently recovered Holothuriidae + Mesothuriidae as a well-supported sister group to all other Pneumonophora (Fig. 4, light blue), thereby warranting the

new name, Holothuriida. The distinctiveness of mesothuriids and their potential relationship to holothuriids has a long history (Becher, 1909; Sluiter, 1901a), but this has not been recognized recently and they have lately been placed in Synallactidae. Solís-Marín's (2003) unpublished molecular study showed that Synallactidae was divided into 'Mesothuriidae,' a clade with gonads in a single tuft, and a clade of all other synallactids possessing paired gonads. Smirnov (2012) formally erected Mesothuriidae, which he placed in his order Aspidochirotida (Fig. 2O). While Synallactidae has long been recognized (Heding, 1940; Koehler and Vaney, 1905; Ludwig, 1894; Perrier, 1898), our results also show that the group is polyphyletic even with the removal of mesothuriids (see below section 1.4.10). The restriction of the gonad to one side of the dorsal mesentery is a synapomorphy of Holothuriida.

1.4.1.6 Neoholothuriida

The morphologically-heterogeneous Neoholothuriida, a new taxon erected here, includes members that were previously scattered across other major holothuroid taxa: Aspidochirotida, Dendrochirotida, Dactylochirotida, Molpadida, and Elasipodida. Although Neoholothuriida has only one known uniting character – known members show two gonads, one on either side of their dorsal mesentery –, four morphologically well-defined and well-supported clades (Fig. 4) can be identified within it: Dendrochirotida, Persiculida, Molpadida and Synallactida.

1.4.1.7 Dendrochirotida

The long-recognized Dendrochirotida is still strongly supported and is a morphologically well-defined clade. The group is united by the possession of an introvert: clearly separated retractor muscles are attached to the pharyngeal complex and withdraw the tentacles and anterior end of the animal into the body. Pawson and Fell (1965) distinguished their newly recognized Dactylochirotida by their simplified (sometimes digitiform) tentacles and the development of a "test," which both now appear to be derived conditions that have evolved more than once within Dendrochirotida (see below Character Transformations 1.4.3). Smirnov (2012, 2016) believed that these two characters arose multiple times within Dendrochirotida and did not recognize Dactylochirotida, which he considered polyphyletic. Although Dactylochirotida should clearly be dismissed, the relationships within Dendrochirotida are less obvious; at least four (Psolidae, Cucumariidae, Sclerodactylidae, and Phyllophoridae) of the eight represented families appear

non-monophyletic in our analyses and remain poorly resolved (Fig. 4). Thus, the short internal branch lengths, and correspondingly poor topological support, make taxonomic revision within the clade unwise at this time. Michonneau and Paulay (2014) analyzed partial COI sequence data from several Phyllophoridae and also showed that several genera were non-monophyletic. Their results agree with the results of this study, which together suggest that the morphological characters (often only one or two) currently used to diagnose family and generic-level taxa within Dendrochirotida need reexamination and that a major taxonomic revision of Dendrochirotida is warranted. Smirnov (2012) also recently proposed a major overhaul of the family-level classification of the order, but without a supporting phylogenetic analysis. Thus, the relationships within Dendrochirotida remain unresolved and await more in depth study.

1.4.1.8 Persiculida

A new taxon, Persiculida, is erected for a well-supported clade also characterized by lack of body wall ossicles, presence of a posterior (pygal) furrow, and, potentially, characteristic secondary chemistry (Kalinin et al., 2005; Silchenko et al., 2004). This group includes three genera generally classified in Synallactidae (Aspidochirotida) and one generally classified in Gephyrothuriidae (Molpadida) (Fig. 4, orange). O’Loughlin and Ahearn (2005) identified a “pygal-furrowed” group of Synallactidae, containing *Molpadiodemas* and *Pseudostichopus*, as one lacking body wall ossicles and having a vertical posterior furrow. We consistently recovered a clade with these morphological characters that included these genera in addition to *Paroriza* and *Gephyrothuria* (formerly in Molpadida). We name the clade Persiculida (Latin *persica* = peach, + *culus* = posterior), as the pygal furrow resembles a ‘cleft on a peach’. *Gephyrothuria* (Gephyrothuriidae) has been shuffled between Aspidochirotida and Molpadida since its description by Koehler and Vaney (1905) (see O’Loughlin [1998] for review). Östergren (1907), considered *Gephyrothuria* as sister to Synallactidae, which prompted Heding (1935) to create the monotypic order Gephyrothurioidea, which he characterized as a group that lacked caudal appendages, tentacular ampullae, and paired longitudinal muscles. Later, Heding (1940) moved *Gephyrothuria* back into the order Aspidochirotida when he created his family Gephyrothuriidae and its two subfamilies, Gephyrothuriinae and Benthostichopodinae. These two subfamilies included the genera *Pseudostichopus*, *Paroriza*, *Molpadiodemas*, *Gephyrothuria*, and *Benthothuria*, and thus, Heding’s Gephyrothuriidae corresponds closely to the Persiculida

recognized here, except for the inclusion of *Benthothuria* (a genus we did not sample) that remains to be phylogenetically evaluated. Recently, Smirnov (2012) recognized a separate order (Gephyrothuriida) for *Gephyrothuriida* (*sensu stricto*) (including *Hadalothuria*; see also O’Loughlin [1998]) based largely on Heding’s (1935) characters (e.g., calcareous ring, tentacles, and tentacle ampullae morphology).

Paroriza prouhoi was always well supported as sister to *Gephyrothuria alcocki*, the type taxon of Gephyrothuriidae, and we here recommend that *Paroriza* be considered part of Gephyrothuriidae (Fig. 4, Gephyrothuriidae [2]). With *Paroriza* reassigned to Gephyrothuriidae, and with *Synallactes* (the type taxon for Synallactidae) recovered within Synallactida, the two remaining clades formerly of Synallactidae (*sensu* Pawson and Fell 1965) that now lie within Persiculida also warrant new names (Fig. 3, *Molpadiodemas* and *Pseudostichopus*).

We designate a new family Molpadiodemidae (Fig. 4, Molpadiodemidae) to include members of the genus *Molpadiodemas*, and a new family Pseudostichopodidae (Fig. 4, Pseudostichopodidae) to include members of the genus *Pseudostichopus*. Both of these families can be diagnosed by morphological characters recognized by O’Loughlin and Ahearn (2005) (Appendix A for the formal diagnoses).

1.4.1.9 Molpadida

Despite its uncertain position within Neoholothuriida, the results of the current study reveal Molpadida (*sensu* Pawson and Fell, 1965) as a non-monophyletic group (Fig. 3) and, as discussed above, suggest that *Gephyrothuria* (Gephyrothuriidae) be moved to Persiculida (Fig. 4). Of the three remaining currently accepted families of Molpadida (Paulay and Hansson, 2013), Molpadiidae and Caudinidae were non-monophyletic, yet always strongly recovered as a clade; Eurpyrgidae was not represented. Molpadida without Gephyrothuriidae has always formed a well-recognized grouping. In the past, some taxonomists grouped Molpadida with, or within, Apodida because members of the group lacked obvious tubefeet (Brandt, 1835; Bronn, 1860; Burmeister, 1837; Pawson and Fell, 1965; Perrier, 1902; Théel, 1886). Our analyses clearly placed Molpadida away from Apodida, as part of the well-supported Neoholothuriida. The loss of tubefeet in Molpadida may have been driven when benthic neoholothuriids with respiratory trees switched to an infaunal lifestyle (Shick, 1983), an idea that is supported by fossil evidence; the first appearance of molpadidan sea cucumbers coincides with the general trend of Mid-

Mesozoic marine infaunalization (Kier, 1982; Kier, 1987; Stanley, 1977; Vermeij, 1977). Though clearly not closely related to Apodida, the position of Molpadida within Neoholothuriida remains uncertain. The current study places Molpadida as sister to Synallactida with poor support (Fig. 3); however, the close relationship of Molpadida to Dendrochirotida was and is the favored arrangement by other authors (Clark, 1910; Haeckel, 1896; Kerr and Kim, 2001; Ludwig, 1889-1892, 1891; MacBride, 1906; Smirnov, 2012).

1.4.1.10 Synallactida

We name the clade that forms a polytomy with Persiculida and Molpadida (see Fig. 4 polytomy) Synallactida. It is currently unclear which morphological synapomorphies support Synallactida, although most known species are epibenthic. Many Synallactida (e.g., *Stichopus*, *Isostichopus*, *Synallactes*, and *Bathyplores*) also display a unique defense behavior – they will shed their outer body walls – when they are disturbed or attacked (see Kropp, 1982). Further research might demonstrate that this behavior is prevalent throughout the clade, excluding Deimatidae.

Within Synallactida, the three terminals of Deimatidae were always recovered as sister to a clade comprising stichopodids and synallactids. Since *Synallactes* (the type genus of Synallactidae) was always recovered as the sister group to a strongly-supported clade of other synallactids and stichopodids, the name Synallactidae is only applied to *Synallactes* in this study. This suggests that the other represented synallactid genera, *Bathyplores* and *Paelopatides*, be moved to Stichopodidae (Fig. 4, [1]). As discussed above, we consistently recovered Synallactidae (*sensu* Ludwig, 1894) as polyphyletic, a result that supports Smirnov's (2012) speculation that Synallactidae needs revision. However, the current study suggests that his proposed solution of having two subfamilies, "Pseudostichopodinae" and "Synallactinae," is insufficient. An alternative solution might be to combine both the stichopodid and restricted synallactid taxa within Synallactida under Synallactidae.

1.4.2 Time-tree analyses

The same three nodes (Nodes A-C) were calibrated in each of the divergence time analyses (3-age and 6-age). These were relatively uncontroversial crown group dates for Eleutherozoa (Blake and Guensburg, 2005; Jell, 2014; Lefebvre et al., 2013), Echinozoa

(Lefebvre et al., 2013; Pisera, 1994; Reich, 2001; Reich, 2010a), and Holothuroidea (Reich, 2001; Reich, 2010a). For the 3-age analyses the remaining nodes (all nodes excluding Nodes A-C) were left free to vary. In contrast, for the 6-age analyses a further three additional constraints (Nodes D-F) were applied for clades within Holothuroidea (Table 2). This addition of further constrained dates within Holothuroidea yielded some marked effects, as these nodes were seen as much younger in the 3-age analyses (Figs. 5 and 6). For instance, under the 3-age analyses it appears that two to three holothuroid lineages survived the end-Permian extinction event (~252 Ma), while the 6-age analyses showed at least five survived. All analyses revealed the rate of molecular evolution within a similar range of values across the tree, except for some internodes near the base of the tree. The dramatic increase in the molecular rate of evolution that is seen between nodes B and C in both analyses and between C and D in the 6-age analyses compared to the 3-age analyses does raise concern that the placement of holothuroid fossils as representatives of certain extant lineages (see Materials and Methods 1.2.5) may need reassessment. The most dramatic difference between the two age trees is the 100 Ma discrepancy seen when Actinopoda (all holothuroids besides Apodida) was constrained by stem date “D” (~387 Ma) in the 6-age analyses (Fig. 6), compared to when this node was free to vary in the 3-age constraints analyses (Fig. 5) (~281 Ma). Although several distinct actinopodid ossicles (*Tribrachiodemas ordovicicus*; Reich, 2010c), calcareous ring elements (“pannychiid” radial elements; Reich 2015a), and body fossils (unnamed taxon with deimatid affinities; Reich and Smith, 2011) are recorded from the Early Paleozoic, the major age discrepancy and marked molecular rate of evolution between Nodes C and D suggest that these fossils may need re-examination.

What is consistent in both of the age-constraint analyses is that Apodida and Actinopoda independently transitioned the end-Permian extinction (Figs. 5 and 6). Within Apodida, the unconstrained nodes showed similar age differences in both the 3-age and 6-age analyses, and although the distinctions between stem and crown group myriotrochid and chiridotid fossil wheel ossicles are uncertain, both of the divergence time analyses place the myriotrochid and chiridotid most recent common ancestor (MRCA) within the same approximate age (Permian). Within Actinopoda the unconstrained nodes also showed similar age differences in both the 3-age and 6-age analyses. The late origin of Dendrochirotrida recovered here raises questions about Jell’s (2010) identification of the Silurian body fossil *Porosothyone picketti* as a member of Dendrochirotrida and instead supports Smith and Reich (2013) who placed this taxon as sister to

Apodida. It appears that the detailed structure of the calcareous ring of *Porosothyone*, a central perforation for the radial nerve and three anterior prolongations, was misinterpreted by Jell as a depression and posterior prolongations, respectively. This emphasizes the need for further fossil exploration and anatomical reviews of extant holothuroids (with emphasis on the calcareous ring).

1.4.3 Character Transformations

The results of this study raise interesting issues with regard to the utility of currently employed morphological features diagnosing current holothuroid taxonomy (Fig. 4).

1.4.3.1 Respiratory tree

The origin of holothuroid respiratory trees and their systematic significance has long been debated (Brandt, 1835; Jaeger, 1833; Müller, 1850; Selenka, 1867). Recent systematic, morphological, and molecular works (Kerr and Kim, 1999; Kerr and Kim, 2001; Lacey et al., 2005; Smirnov, 2012) (Fig. 2 L, M, and O), as well as the results of the current study (Fig. 7A), support one of the oldest theories (Selenka, 1867; Semper, 1867-8) that suggested that respiratory trees are derived and evolved once within Holothuroidea. The current study places this derivation as an apomorphy for Pneumonophora, with one obvious loss, occurring in Deimatidae. This loss in Deimatidae was hypothesized recently by Smirnov (2012, 2016) and contradicts the current widely-accepted view that Deimatidae be placed within Elasipodida (e.g., Paulay and Hansson, 2013). It is unclear why deimatids lost their respiratory trees. However, transformations of general holothuroid shape and habitat preference (Supplementary Material Table 2) suggest that oxygen availability and body wall thickness might be guiding factors. Holothuroids with respiratory trees receive approximately half of their oxygen through these structures, and the rest through diffusion across their body surface (Brown, 1979). Shick (1983) suggested that the direct uptake of oxygen by respiratory trees and a thick body wall with a reduced skeleton may have allowed holothuroids to occupy “crevicular” habitats unavailable to most testaceous echinoderms. Possibly the reverse conditions transpired in Deimatidae; their remarkably thin body walls (hydrostatic skeleton comprised of large compact plates, mentioned above), low metabolic demands (many reside in the deep sea where it is cold and oligotrophic), and their open epibenthic habits may have decreased their need for respiratory trees.

1.4.3.2 Skeletal elements

A reduced skeleton of body wall ossicles is plesiomorphic for Holothuroidea (Fig. 7B), and it was from this state that the “absent” and “testate” forms derived independently several times. Holothuroids without ossicles in their body walls may have ossicles in their tube feet, tentacles, gonads, pygal lobes, and respiratory trees (O’Loughlin and Ahearn, 2005). According to the transformation analysis (Fig. 7B), such an “absent” skeletal type is an apomorphy for Persiculida and possibly Pelagothuriidae (the only other pelagothuriid, *Pelagothuria natatrix*, lacks body wall ossicles [Ludwig, 1894], but was not represented here). The lack of ossicles in some other holothuroids (e.g., *Achiridota*, *Kolostoneura*) not represented here, are likely independent and derived losses, and it is likely that more genera with the “absent” skeletal type (e.g., *Hadalothuria*, *Benthothuria*, *Hansenothuria*, and *Meseres*) may belong to Persiculida. Holothuroids without body wall ossicles are rare and understudied, thus the reasons for their lack of ossicles are poorly understood; however, it could be that these species are hindered by ossicles or that they have replaced them with alternative structures. For example, ossicles could have been lost in *Pelagothuria natatrix* and *Enypniastes eximia* – “pelagic” and “benthopelagic” (respectively) species which spend a majority of their time in the water column (Miller and Pawson, 1990) – because they added weight and were disadvantageous. *Enypniastes eximia* also has bioluminescent granular bodies in their body walls that they can “slough off” when they are physically disturbed (Robison, 1992); it is possible that ossicles might have interfered with this defensive technique. Some *Pseudostichopus* (Persiculida) species cover themselves with sponge spicules, foraminifera, or other hard debris (O’Loughlin and Ahearn, 2005) that may act as alternatives to ossicles. Interestingly, Heding (1935) also mentions a “conspicuous thick layer of cartilaginous-like tissue between the cutis and the transverse-muscles” of *Molpadiodemas acaudum* (= *M. atlanticus*), which may stiffen the body wall in the same manner as ossicles. Although they do not lack body wall ossicles entirely, many members of Molpadida will transform their ossicles into phosphatic deposits as they age (Pawson, 1977; Pawson et al., 2001). It has been speculated that such an alternative phosphatic skeleton may be more suitable in the low-oxygen environments where many of these species reside (Rhoads and Morse, 1971). More research is needed to test these theories.

Holothuroids with a “testate” skeleton tend to have large imbricating plate ossicles in their body walls. Pawson and Fell (1965) and Haude (1992; 1995) suggested that extant holothuroids with isolated ossicles arose from heavily-plated or testaceous holothuroids, while both Kerr and Kim (1999) and Smirnov (2015) suggested that the “test” represents a later innovation. Our results support the latter hypothesis and clearly indicate that the “test” skeletal type arose once or several times (e.g., Haude, 2002) within Dendrochirotida (Fig. 7B).

1.4.3.3 Table ossicles

Much has been written about relationships among diverse ossicle types in sea cucumbers, but homologies and evolution of ossicle types remains poorly constrained. Tables are one of the more widespread and recognizable ossicles, and are considered an important diagnostic character for many holothuroid groups (Smirnov, 2012). Gilliland (1993) and Smirnov (2012) postulated that various types of table ossicles appeared and developed independently across Holothuroidea, with Smirnov further believing that “lung holothurians” (Smirnov’s subclass Holothuriacea = our clade Pneumophora Fig. 4) had the “ability to develop a spire from several closely spaced 2-4 pillars.” In contrast, Kerr and Kim (2001) suggested that a clade of holothuroids with table ossicles diverged from elasipodan-like ancestors by the Middle Devonian. The table ossicle transformation in the current study (Fig. 7C) was ambiguous and does not resolve this controversy. Table ossicles were found to either be an apomorphy for Pneumophora with subsequent losses, or to have evolved multiple times within derived Holothuroidea (Fig. 7C).

1.4.3.4 Tentacle shape

Tentacles are an apomorphy for Holothuroidea; however, the plesiomorphic tentacle form remains unclear. Most tentacle types, such as digitiform, digitate, and dendritic, appear to have evolved from a general peltate tentacle form (Fig. 7D), and certain tentacle morphologies appear as apomorphies for certain clades (e.g., Dendrochirotida). Although few studies have investigated the functionality of holothuroid tentacles (e.g., Roberts and Bryce, 1982; Sun et al., 2015), it is thought that tentacle shape corresponds with food type (Smirnov, 2016) and habitat preference, and that this “resource partitioning” might help avoid competition for food (Roberts and Moore, 1997). For example, Roberts and Moore (1997) suggested that the peltate and digitate tentacles of some deep-sea deposit-feeding holothuroids (e.g., *Oneirophanta mutabilis*,

Psychropotes longicauda, and *Paroriza pallens*) may be adaptations to the specific sediments on which they feed, and that their selected diets may limit their competition for food in occasionally oligotrophic environments.

1.4.3.5 Other morphological characters

Although we did not perform transformation analyses for them, one traditional and two novel characters appear as clear apomorphies for clades recovered by the current study. Many classical holothuroid systematists gave precedence to the presence or absence of tubefeet above all other recognized characters (e.g., Brandt, 1835; Burmeister, 1837; Lampert, 1885). Brandt (1835) made the first holothuroid system using this character; however, Ludwig's (1889-1892) system fits closest to the tree topologies recovered here. Given their presence in Echinoidea and other echinoderms, tubefeet are plesiomorphic for Holothuroidea and their absence in Apodida and their reductions (mid-body tube feet) in Molpadida are likely apomorphic conditions.

In addition to the traditionally used characters discussed above, the current phylogeny suggests two novel morphological apomorphies for two main holothuroid clades. The first is the presence of gonads on one side of the dorsal mesentery, which supports Holothuriida, and the second is the presence of a posterior vertical furrow, which supports Persiculida. More research is needed to assess the evolution of these features, as well as clarify which morphological apomorphies may characterize some of the large clades identified here (e.g., Neoholothuriida and Synallactida). With the well-supported holothuroid higher-level phylogeny presented here, transformation analyses of other morphological features may prove rewarding. For example, some have proposed that the morphology of the calcareous ring may have high systematic value (Pawson and Fell, 1965; Reich, 2015; Reich and O'Loughlin, 2010; Smirnov, 2012).

1.5 Conclusion

This is the first phylogenetic study to assess the higher-level relationships of Holothuroidea using a multi-gene approach, including molecular data from multiple difficult-to-access (deep-sea and polar) taxa. The results of the current study complement and extend those of recent molecular (Lacey et al., 2005) and morphological (Kerr and Kim, 2001) phylogenetic analyses, as well as taxonomic works incorporating these findings (Smirnov, 2012). However, systematic revisions for a series of well-supported clades are also introduced in this study

because it was revealed that a large-scale revision of Holothuroidea was necessary. Extant holothuroids fall into two main clades: Apodida and Actinopoda. Within the latter taxon, Pneumonophora and Elasipodida are recognized, as well as a series of smaller clades within Pneumonophora: Holothuriida and Neoholothuriida (containing Molpadida, Persiculida, Synallactida, and Dendrochirotida). Although there is strong support for most of the clades across all analyses, low support for a few (e.g., Molpadida + Synallactida + Persiculida) makes it clear that more data are necessary. Fortunately, advances in next-generation sequencing methods allow for the fast and relatively easy processing of much more data than presented here (e.g., Liang et al., 2013; O'Hara et al., 2014; Ozsolak and Milos, 2010). Holothuroids have a series of traits that are homoplastic, such as ossicles, tentacles, and tubefeet and the current study demonstrates the limited utility of some of these in systematics. Time-tree analyses also suggest that assignment of several holothuroid fossils may need reassessment.

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1.8 Appendix A

A.1 Family diagnoses

Diagnoses for three families within the clade Persiculida. Apomorphies for the below families are indicated by italics.

Clade Persiculida: *Posterior vertical furrow present. Body wall ossicles absent.*

Remarks: No formal rank is assigned to Persiculida here. The clade contains three families:

Gephyrothuriidae, Molpadiodemidae fam. nov., and Pseudostichopodidae fam. nov.

Gephyrothuriidae was placed as sister group to Molpadiodemidae in the current phylogenetic analyses, though the node was poorly supported. Other genera (e.g., *Hadalothuria*, *Benthothuria*, *Hansenothuria*, and *Meseres*) possibly belong within this clade and might be added to these families when more data become available for them.

Family Gephyrothuriidae (Koehler and Vaney, 1905)

Diagnosis: Persiculida (above) characterized by: cylindrical body with rounded terminal ends; body surface covered in numerous scattered small papillae and tube feet that give the body surface a granular look, tube feet are concentrated towards the anterior and posterior ends; tube feet and papillae are more prominent closer to radials; *long (4-10mm) filiform papillae present*; tentacle ampullae and retractor muscles absent; large often 16-20 digitate or digitate-cupped tentacles, one polian vesicle; respiratory trees with two branches and connect at a common base; longitudinal muscles undivided; gonad tubules on both sides of dorsal mesentery; calcareous ring comprised of 10 thick plates, radial plates of the calcareous ring possess an anteriorly opening central hole or notch, radial and interradial plates largely uniform in size.

Type genus: *Gephyrothuria* (Koehler and Vaney, 1905)

Type species: *Gephyrothuria alcocki* (Koehler and Vaney, 1905)

Other included species: *Paroriza prouhoi* (Hérourard, 1902)

Remarks: Koehler and Vaney (1905) erected Gephyrothuriidae for their monotypic genus *Gephyrothuria*. Since then, the placement of the family within Holothuroidea has been greatly debated (see O'Loughlin, 1998). Most recently, Smirnov (2012) resurrected Heding's (1935) order Gephyrothuriida and placed Gephyrothuriidae (containing *Gephyrothuria* and

Hadalothuria) within it. Our phylogenetic results (main text Fig. 3) support the close relationship of *Gephyrothuria* with *Paroriza* within Persiculida, thus we reassign *Paroriza* to Gephyrothuriidae and place it within Persiculida here. Interestingly, Massin (1987) also placed *Paroriza* in Gephyrothuriidae (Koehler and Vaney, 1905) after analyzing the general anatomy of his new *Paroriza verrucosa* specimens. We also remove *Hadalothuria* (Hansen, 1956) from Gephyrothuriidae until more data become available for it and it can be reassigned confidently.

References:

Koehler and Vaney (1905) p. 78; Heding (1935) p. 77; Massin (1987) pp. 116-118; O’Loughlin (1998) pp. 493-497; Smirnov (2012) p. 825

Family Molpadiodemidae fam. nov., Miller et al., 2017

Diagnosis: Persiculida (above) characterized primarily by the characters diagnosed by O’Loughlin and Ahearn (2005) for the *Molpadiodemas*: cylindrical body with rounded terminal ends; body surface covered in small discrete tube feet; *large prominent tube feet and papillae absent from the paired radii*; tentacle ampullae and retractor muscles absent; often 18-20 peltate tentacles; one large ventral polian vesicle; respiratory trees comprised of two unequal clustered branches connected at a common base near the cloaca; longitudinal muscles are undivided and *sit flat against the inner body wall*; *gonad tubules branch out from a common gonoduct base* and are positioned posteriorly on each side of the dorsal mesentery; calcareous ring comprised of 10 thick plates; radial plates of the calcareous ring possess an anteriorly opening central hole or notch, and have two pairs of posterior “teeth” or protrusions; interradial plates of the calcareous ring are set lower than the radial plates and have one large anterior tooth and a smooth concaved posterior surface; ossicles are not found in the body walls or tube feet; *branched rod ossicles can be present in tentacles of which the side branches and terminals commonly collide to form networks*; body surface sometimes inhabited by epibionts that can occasionally cause dermal cavities.

Type genus: *Molpadiodemas* (Heding, 1935)

Type species: *Molpadiodemas atlanticus* (Perrier, 1898)

Other included species: *Molpadiodemas villosus* Théel, 1886 and *Molpadiodemas involutus* (Sluiter, 1901b)

Remarks:

Heding (1935) suggested that his newly erected genus, *Molpadiodemas*, seemed closely related to *Gephyrothuria*, but that there were such great differences that they must be a separate genus. He later decided to group them both in his family Gephyrothuridae (Heding, 1940). O’Loughlin and Ahearn (2005) completed a thorough rediagnosis of *Molpadiodemas* and made *Platystichopus* (Heding, 1940) a junior synonym. Our phylogenetic results (main text Fig. 3) agree with Heding’s (1935) idea of a close relationship between *Molpadiodemas* and *Gephyrothuria*, though we recovered *Paroriza* as closest to *Gephyrothuria*. We combine O’Loughlin and Ahearn’s (2005) key morphological characters for *Molpadiodemas* (body surface covered with small tubefeet, tube feet and papillae missing from the paired radii, branched gonad tubules arise from a common base, longitudinal muscles are fixed flat to the body wall, and ossicles not found in tube feet) with our molecular evidence and place *Molpadiodemas* (*sensu* O’Loughlin and Ahearn, 2005) in its own family within Persiculida.

References:

Heding, (1935) pp. 77-80; Heding, (1940) pp. 356-357; Deichmann, (1940) pp. 208-211; O’Loughlin (1998) p. 497; O’Loughlin (2002) pp. 303-315; O’Loughlin and Ahearn, (2005) pp. 149-153.

Family Pseudostichopodidae fam. nov., Miller et al., 2017

Diagnosis: Persiculida (above) characterized mainly by the genus, *Pseudostichopus*, diagnosis of O’Loughlin and Ahearn (2005): cylindrical body with rounded terminal ends; *large prominent tubefeet or papillae are positioned along the paired radii*; tentacle ampullae and retractor muscles absent; often 18-20 peltate tentacles; one polian vesicle is ventrally positioned; respiratory trees comprised of two unequal clustered branches connected at a common base near the cloaca; *longitudinal muscles are round in shape and protrude slightly from the inner body wall*; *gonad tubules branch separately from the gonoduct (not from a common base)* and are positioned on each side of the dorsal mesentery; the calcareous ring is comprised of 10 thick plates; radial plates of the calcareous ring possess an anteriorly opening central hole or notch, and have two pairs of posterior “teeth” or protrusions; interradial plates of the calcareous ring are set lower than the radial plates and have one large anterior tooth and a smooth concaved posterior surface; ossicles are sometimes found in papillae and tube feet; *rod ossicles can be*

present in tentacles and are often unbranched; body surface often inhabited by epibionts or covered by hard debris (e.g., sponge spicules and forams).

Type genus: Pseudostichopus (Théel, 1886)

Type species: Pseudostichopus mollis (Théel, 1886)

Remarks: Théel (1886) erected *Pseudostichopus* for *Pseudostichopus mollis* (Théel, 1886) and *Pseudostichopus villosus* (Théel, 1886), though the latter is now accepted as *Molpadiodemas villosus*. O’Loughlin and Ahearn (2005) rediagnosed the genus with a clear set of morphological characters and placed it within Synallactidae. Our phylogenetic results (main text Fig. 3) recovered *Pseudostichopus* as the sister group to *Molpadiodemas* and *Gephyrothuria* + *Paroriza* within Persiculida but with low support. Here we consider O’Loughlin and Ahearn’s (2005) key morphological characters for *Pseudostichopus* (tube feet and papillae often on the paired radii, non-branched gonad tubules arise singularly in a line from the gonoduct, longitudinal muscles are convexly fixed to the body wall, and ossicles are sometimes found in tube feet and papillae) with our molecular evidence and place *Pseudostichopus* (*sensu* O’Loughlin and Ahearn, 2005) in its own family within Persiculida.

References:

Théel, (1886) p. 169; Massin, (1987) pp. 116-118; O’Loughlin and Ahearn, (2005) pp. 149-169.

Figure legends

Fig. 1. Morphological diversity of Holothuroidea. (A) *Pelagothuria natatrix*. Photo © D.L. Pawson. (B) *Ypsilothuria* cf. *bitentaculata*. Photo © G.W. Rouse. (C) *Paracaudina chilensis*. Photo © G. Paulay. (D) *Lissothuria nutriens*. Photo © G.W. Rouse. (E) *Enypniastes eximia*. Photo © G. Paulay. (F) *Colochirus robustus*. Photo © A.K. Miller.

Fig. 2. Previous hypotheses of extant holothuroid phylogenetic relationships. (A) Semper (1867-8). (B) Ludwig (1889-1892). (C) Haeckel (1896). (D) Perrier (1902). (E) MacBride (1906). (F) Östergren (1907). (G) Becher (1909). (H) Cuénot (1948). (I) Phylogenetic interpretation of Pawson and Fell's (1965) Linnaean classification. (J) Haude (1992). (K) Smith's (1997) interpretation of data from Littlewood et al. (1997). (L) Kerr and Kim (2001). (M) Lacey et al. (2005). (N) Reich (2010a). (O) Phylogenetic interpretation of Smirnov's (2012) system. Taxon designations are from Pawson and Fell (1965) and asterisks (*) indicate alternative positions of Molpadida.

Fig. 3. Maximum likelihood (ML, lnL – 50494.61) and Bayesian Inference (BI, lnL - 48725) tree inferred from the concatenated six-gene dataset (reduced dataset, nine partitions). Symbols adjacent to nodes refer to ML bootstrap scores (BS), BI posterior probability (PP), and maximum parsimony (MP) parsimony jackknife (JK) majority rule consensus tree values in that order (BS/PP/JK). Branch lengths for the outgroup terminals and the ingroup were shortened (/) for clarity. A space () indicates BS or JK values < 50% or PP values < 0.70. A hyphen (-) indicates that a node was not found in the respective analysis. An asterisk (*) indicates a node value with BS or JK > 90 and PP > 0.95. Colored boxes surround members of Pawson and Fell's (1965) orders: yellow = Apodida; purple = Elasipodida; green = Aspidochirotida; red = Molpadida; blue = Dendrochirotida; and orange = Dactylochirotida.

Fig. 4. Summary of holothuroid relationships based on BI and ML analyses of the reduced dataset with the clade and group names proposed in this study. Only nodes with >50% BS maximum likelihood values are shown. Asterisks (*) indicate that the type taxon of the indicated family is present within the group and roman numerals (I, II, or III) indicate that a paraphyletic or polyphyletic family group may require a new name. Of these roman numeral groups the non-

monophyly of Laetmogonidae and Synaptidae was not significantly supported by AU tests (Shimodaira, 2002). The number one [1] distinguishes genera (*Bathyploetes* and *Paelopatides*) newly placed within Stichopodidae. The number two [2] distinguishes a new genus (*Paroriza*) within Gephyrothuriidae. The two newly erected families (Molpadiodemidae and Pseudostichopodidae) are in bold. The colored boxes surround members of the large groups recovered by the current study.

Fig. 5. Chronogram of Holothuroidea. Three nodes (green circles with letters A-C) were used as fossil-based time constraints (refer to text and Table 2). Estimated mean divergence times are listed adjacent to the nodes. Purple lines through nodes represent the 95% confidence interval for the dates. Tree branch colors (following color spectrum located in the top left corner) represent the relative rate of overall substitutions per site per million years; brown branches represent the slowest rates, green intermediate, and purple represent the fastest. The dark blue bars underneath the stratigraphic divisions indicate ‘species’-level diversity of Holothuroidea through time (based on Reich, 2013).

Fig. 6. Chronogram of Holothuroidea. Six nodes (green circles with letters A-F) were used as fossil-based time constraints (refer to text and Table 2). Estimated mean divergence times are listed adjacent to the nodes. Purple lines through nodes represent the 95% confidence interval for the dates. Tree branch colors (following color spectrum located in the top left corner) represent the relative rate of overall substitutions per site per million years; brown branches represent the slowest rates, green intermediate, and pink represent the fastest. The dark blue bars underneath the stratigraphic divisions indicate species ‘species’-level diversity of Holothuroidea through time (based on Reich, 2013).

Fig. 7. Maximum likelihood transformations for (A) respiratory trees (B) body wall skeletal elements excluding table ossicles (C) table ossicles and (D) tentacle shape. Numbers adjacent to nodes refer to proportional likelihood estimations. Multiple scores, if present, are separated by a forward slash and listed in order of most likely states. An asterisk (*) indicates that a node has an estimated proportional likelihood of >95%. A “(c)” indicates that a terminal is part of a complex. The colored boxes around the clades represent the large clades recovered by the current study

(refer to text and Fig. 4).

Table 1. Voucher information and GenBank accession numbers for specimens used in the molecular analyses. New sequences are in bold. An exclamation mark (!) indicates the taxon was identified to genus by DNA. Depositories: SIO-BIC, Scripps Institution of Oceanography Benthic Invertebrate Collection; UF, Florida Museum of Natural History Invertebrate Zoology Collection; NMV, Museum Victoria Marine Invertebrates Collection; MNHN, Muséum National d'Histoire Naturelle; NIWA, National Institute of Water and Atmospheric Research; NOCS-DC, National Oceanography Center, Southampton, Discovery Collection; MZB, Museum Zoologicum Bogoriense; KSORC, Korea South Pacific Ocean Research Center. One asterisk (*) denotes that only a subsample voucher exists. Two asterisks (**) denotes that another specimen, NOCS-DC 13369#1, is from a nearby station on the same cruise.

Taxa	Location	Voucher	18S	16S	CO1	H3	12S	28S
<i>Abyssocucumis abyssorum</i>	USA, California	SIO-BIC E5610	KX856836	KX856767	KX874335	KX874442	KX856686	
<i>Acaudina molpadioides</i>	Australia, West Coast	NMV-MIC F112144	KX856820	KX856743	KX874336	KX874455	KX856677	KX856891
<i>Actinopyga varians</i>	Micronesia, Chuuk State	KSORC 0315	KX856851	KX856787	KX874345	KX874409		
<i>Afrocucumis africana</i>	Mariana Islands, Guam	SIO-BIC E6844	KX856841	KX856780	KX874348	KX874451		KX856908
<i>Amperima robusta</i>	Antarctica	NIWA 37990	KX856798	KX856728	KX874381	KX874457	KX856702	
<i>Apostichopus californicus</i>	varies	varies	DQ777084	DQ777096	HM542319			DQ777087
<i>Apostichopus parvimensis</i>	USA, California	no voucher	KX856808	KX856750	KX874373	KX874417	KX856666	
<i>Aslia pygmaea</i>	USA, Florida	SIO-BIC E6812	KX856838	KX856774	KX874339	KX874450	KX856685	DQ777091
<i>Astichopus multifidus</i>	USA, Florida	FLMNH-IZC 3985	KX856809	KX856753	KX874376	KX874422	KX856665	
<i>Bathyplores</i> sp. 1	Antarctica	SIO-BIC E5111	KX856812	KX856748	KX874358	KX874415	KX856667	KX856881
<i>Bathyplores</i> sp. 2	North Scotia Ridge	SIO-BIC E6356	KX856802	KX856747	KX874357	KX874416	KX856668	KX856882
<i>Benthogone abstrusa</i>	Australia, NW Coast	NMV-MIC F146599	KX856795	KX856733	KX874374	KX874466		KX856874
<i>Bohadschia koellikeri</i>	Indonesia/Australia	No Voucher	KX856852	KX856785	JX683894	KX874411		
<i>Chiridota albatrossii</i> complex 1	USA, California	SIO-BIC E5611	KX856859	KX856788	KX874397	KX874471	KX856718	KX856870
<i>Chiridota albatrossii</i> complex 2	USA, California	SIO-BIC E5612	KX856860		KX874398	KX874472	KX856717	
<i>Chiridota laevis</i>	Greenland	SIO-BIC E5602	KX856858		KX874399	KX874473	KX856719	
<i>Chiridota rigida</i>	Mariana Islands, Guam	SIO-BIC E6841	KX856857		KX874401	KX874469	KX856721	KX856868
<i>Colochirus robustus</i>	Indonesia, Raja Ampat	SIO-BIC E6626	KX856832	KX856781	KX874347		KX856695	
<i>Crucella scotiae</i> 1	Scotia Arc	SIO-BIC E5296	KX856829	KX856761	KX874366	KX874433		KX856901
<i>Crucella scotiae</i> 2	Antarctica	NMV-MIC F193785	KX856826	KX856760	KX874367	KX874434	KX856690	KX856897

Taxa	Location	Voucher	18S	16S	CO1	H3	12S	28S
<i>Cucumaria miniata</i>	varies	varies	DQ777082		HM542157			DQ777092
<i>Deima validum</i>	Northeast Atlantic	Lost**	KX856815	KX856744	KX874364	KX874426	KX856679	
<i>Echinocucumis hispida</i>	Antarctica	NIWA 37845	KX856835		KX874396	KX874438		
<i>Echinocucumis</i> cf. <i>hispida</i>	Antarctica	SIO-BIC E6674	KX856833	KX856779	KX874395	KX874437	KX856714	KX856899
<i>Enypniastes eximia</i>	New Zealand	SIO-BIC E6670	KX856792	KX856730	KX874383	KX874465	KX856708	KX856875
<i>Euapta tahitiensis</i>	Micronesia, Chuuk State	KSORC 0253	KX856861		KX874402	KX874475	KX856723	KX856869
<i>Euthyonidiella huwi</i>	Antarctica	SIO-BIC E5709	KX856840	KX856782	KX874371	KX874448	KX856698	KX856910
<i>Gephyrothuria alcocki</i>	New Zealand	NIWA 45733	KX856856	KX856738	KX874377	KX874406	KX856671	KX856893
<i>Heteromolpadia tridens</i>	Australia, West Coast	NMV-MIC F112138	KX856823	KX856740	KX874362	KX874431	KX856673	KX856890
<i>Heterothyone alba</i>	New Zealand	SIO-BIC E6666	KX856831	KX856765	KX874390	KX874444	KX856693	KX856898
<i>Holothuria hilla</i>	Mariana Islands, Guam	SIO-BIC E6842	KX856854	KX856783	KX874337	KX874407	KX856663	KX856892
<i>Holothuria leucospilota</i>	varies	varies	AY133472	AY338419	FJ971394		DQ777101	DQ777093
<i>Isostichopus badiotus</i>	USA, Florida	FLMNH-IZC 4703	KX856819	KX856756	KX874354	KX874425		
<i>Labiodemas</i> cf. <i>pertinax</i>	Mariana Islands, Guam	No Voucher	KX856850	KX856784	KX874338	KX874408		
<i>Leptosynapta clarki</i>	USA, Massachusetts	SIO-BIC E6815	KX856864		HM542252	KX874476		KX856872
<i>Lissothuria nutiens</i>	USA, California	SIO-BIC E6675	KX856845	KX856775	KX874341	KX874443	KX856700	
<i>Massinium magnum</i>	Papua New Guinea	FLMNH-IZC 14866	KX856849	KX856778	KX874351	KX874467		
<i>Mesothuria oktanemus</i>	French Polynesia	FMNH-IZC 9746	KX856824	KX856734	KX874394	KX874429	KX856710	KX856894
<i>Molpadia arenicola</i>	USA, California	SIO-BIC E6830	KX856821	KX856741	KX874344	KX874413	KX856676	KX856895
<i>Molpadia musculus</i> complex	Antarctica	SIO-BIC E5295	KX856816	KX856739	KX874386	KX874412	KX856674	KX856889
<i>Molpadiodemas involutus</i>	South Pacific Ocean	NIWA 49857	KX856801	KX856736	HM196530	KX874404		KX856878
<i>Molpadiodemas villosus</i>	varies	varies	DQ777086	DQ777098	AF486436	KY019184		DQ777088
<i>Myriotrochus antarcticus</i>	Antarctica	NMV-MIC F168643	KX856866					
<i>Myriotrochus</i> sp.	Antarctica	NIWA 37812	KX856865	KX856789	KX874403	KX874468		KX856867

Taxa	Location	Voucher	18S	16S	CO1	H3	12S	28S
<i>Oneirophanta setigera</i>	French Polynesia	FMNH-IZC 9706	KX856813	KX856745	KX874363	KX874427		KX856885
<i>Orphnurgus glaber</i>	Australia, NW Coast	NMV-MIC F146594	KX856814	KX856746	KX874361	KX874428	KX856678	KX856883
<i>Pachythyone rubra</i>	USA, California	SIO-BIC E6676	KX856847	KX856770	KX874387	KX874446	KX856684	
<i>Paelopatides</i> sp. 1 !	USA, California	SIO-BIC E4365	KX856806	KX856757	KX874356	KX874418	KX856670	KX856884
<i>Paelopatides</i> sp. 2	Mexico, Gulf of California	SIO-BIC E5609	KX856807		KX874355	KX874419	KX856669	
<i>Pannychia</i> cf. <i>moseleyi</i>	USA, California	SIO-BIC E5614	KX856794	KX856732	KX874379	KX874464	KX856706	KX856876
<i>Pannychia moseleyi</i>	USA, California	SIO-BIC E4625	KX856793	KX856731	KX874380	KX874463	KX856707	
<i>Paracaudina chilensis</i>	USA, Oregon	FLMNH-IZC 11429	KX856822	KX856742	KX874343	KX874414	KX856675	
<i>Paracucumis turricata</i>	Antarctica	NMV-MIC F169314	KX856828	KX856762	KX874368	KX874439	KX856691	KX856902
<i>Paradota</i> sp.	Antarctica	SIO-BIC E5604	KX856862		KX874400	KX874470	KX856722	
<i>Parastichopus tremulus</i>	Norway	FLMNH-IZC 4863	KX856804	KX856752	KX874359			
<i>Paroriza prouhoi</i>	Northeast Atlantic	NOCS-DC 13369#2	KX856855	KX856737	KX874378	KX874405	KX856672	
<i>Pearsonothuria graeffei</i>	Indonesia, Raja Ampat	Donated to MZB	KX856853	KX856786	EU848285	KX874410	AY574860	
<i>Peniagone diaphana</i>	Northeast Atlantic	NOCS-DC 13369#2	KX856800	KX856725	KX874384	KX874459	KX856705	
<i>Peniagone</i> sp.	Mexico, Gulf of California	SIO-BIC E5608	KX856799	KX856726	KX874385	KX874460	KX856704	KX856877
<i>Pentactella leonina</i>	Chile	SIO-BIC E6672	KX856846	KX856768	KX874369	KX874440	KX856699	KX856906
<i>Pentactella</i> sp.	Burdwood Bank East	SIO-BIC E6671	KX856834	KX856769	KX874372	KX874441	KX856687	KX856903
<i>Pentamera calcigera</i>	Greenland	SIO-BIC E5613	KX856843	KX856773		KX874452	KX856688	KX856905
<i>Phyrella mookiei</i>	Mariana Islands, Guam	FLMNH-IZC 10336	KX856842	KX856777	KX874346	KX874477	KX856711	KX856909
<i>Placothuria squamata</i>	New Zealand	SIO-BIC E6668			KX874391	KX874445	KX856692	
<i>Protelpidia murrayi</i>	Antarctica	SIO-BIC E5110	KX856796	KX856727	KX874382	KX874456	KX856703	
<i>Pseudostichopus</i> sp. 1	Antarctica	SIO-BIC E6363	KX856811	KX856758	KX874388	KX874453	KX856715	KX856880
<i>Pseudostichopus</i> sp. 2	Antarctica	SIO-BIC E5708	KX856810	KX856759	KX874389	KX874454	KX856716	KX856879
<i>Psolidium dorsipes</i>	Antarctica	SIO-BIC E6673	KX856837	KX856766	KX874350	KX874435	KX856694	KX856900

Taxa	Location	Voucher	18S	16S	CO1	H3	12S	28S
<i>Psolidium whittakeri</i>	Antarctica	SIO-BIC E5136	KX856827	KX856763	KX874349	KX874432	KX856689	
<i>Psycheotrepes exigua</i>	Antarctica	NIWA 39018	KX856791		KX874392	KX874462	KX856712	KX856873
<i>Psychropotes longicauda</i>	USA, California	SIO-BIC E4074	KX856790	KX856724		KX874461	KX856713	
<i>Rhipidothuria racovitzai</i>		MNHNP IE-2009-4932	KX856797	KX856729		KX874458		
<i>Sclerodactyla briareus</i>	USA, Florida	SIO-BIC E6814	KX856844	KX856771	KX874342	KX874447	KX856683	KX856904
<i>Sigmodota contorta</i>	Antarctica	SIO-BIC E5135	KX856863			KX874474	KX856720	KX856871
<i>Stichopus chloronotus</i>	Mariana Islands, Guam	SIO-BIC E6843*	KX856818	KX856755	KX874352	KX874424	KX856701	
<i>Stichopus vastus</i>	Micronesia, Chuuk State	KSORC 0321	KX856817	KX856754	KX874353	KX874423		KX856886
<i>Synallactes</i> sp.	Mexico, Gulf of California	SIO-BIC E5607	KX856803	KX856749	KX874365	KX874420	KX856680	KX856888
<i>Thelenota anax</i>	Micronesia, Chuuk State	KSORC 0317	KX856805	KX856751	KX874375	KX874421	KX856681	KX856887
<i>Thyone</i> sp.	Papua New Guinea	FLMNH-IZC 14936	KX856839	KX856776	KX874360		KX856696	
<i>Thyonella gemmata</i>	USA, Florida	SIO-BIC E6813	KX856848	KX856772	KX874340	KX874449	KX856682	KX856907
<i>Ypsilothuria</i> cf. <i>bitentaculata</i>	USA, California	SIO-BIC E4760	KX856830	KX856764	KX874370	KX874436	KX856697	
<i>Zygothuria oxyclera</i>	France, Guiana	FLMNH-IZC 17117	KX856825	KX856735	KX874393	KX874430	KX856709	
Outgroups								
<i>Patiria miniata</i>	varies	varies	DQ060777	DQ297074	HM542106	DQ676897	AY370695	DQ060004
<i>Strongylocentrotus purpuratus</i>	varies	varies	L28056	X12631	HM542410	NM214544	X12631	XM790710

Table 2. Node age constraints (minimum ages) used to estimate divergence times or stem lengths. All nodes were constrained as monophyletic. The 3-age dataset included nodes A-C. The 6-age dataset included nodes A-F.

Node	Median Age Used	95% Age Distribution (Ma)	Node	Source
A	481	480-488	Crown Eleutherozoa	(Blake and Guensburg, 2005)
B	464	463-465	Crown Echinozoa	(Lefebvre et al., 2013; Pisera, 1994)
C	435	431-449	Crown Holothuroidea	(Reich, 2010a; Reich, 2015)
D	387	385-394	Stem Elasipodida	(Boczarowski, 2001)
E	246	244-257	Stem Holothuriidae	(Reich, 2004)
F	198	196-201	Stem Molpadida	(Gilliland, 1992)

Maximum likelihood transformations for the respiratory tree (A) and skeletal elements in body wall (B). Transformations were generated using the Mk1 model and the topology shown in Fig. XX. Estimated proportional likelihood values are displayed near nodes: an asterisk (*) indicates a node with an estimated proportional likelihood of >95%, and scores <95% are listed in order of most likely states. Multiple scores are separated by a forward slash (/). Colors correspond to the clades seen in Fig XX. <- proposed nomenclature tree.

Maximum likelihood transformations for table ossicles (C) and tentacle shape (D).

Transformations were generated using the Mk1 model and the topology shown in Fig. XX. Estimated proportional likelihood values are displayed near nodes: an asterisk (*) indicates a node with an estimated proportional likelihood of >95%, and scores <95% are listed in order of most likely states. Multiple scores are separated by a forward slash (/). Colors correspond to the clades seen in Fig. XX. <- proposed nomenclature tree.