

Molecular Phylogeny of Coral-Reef Sea Cucumbers (Holothuriidae: Aspidochirotida) Based on 16S Mitochondrial Ribosomal DNA Sequence

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Received: 4 February 2004 / Accepted: 5 May 2004 / Online publication: 30 December 2004

Abstract

Members of the Holothuriidae, found globally at low to middle latitudes, are often a dominant component of Indo–West Pacific coral reefs. We present the first phylogeny of the group, using 8 species from the 5 currently recognized genera and based on approximately 540 nucleotides from a polymerase chain reaction–amplified and conserved 3′ section of 16S mitochondrial ribosomal DNA. Parsimony and likelihood analyses returned identical topologies, permitting several robust inferences to be drawn. Several points corroborated the Linnean classification. *Actinopyga* and *Bohadschia* each appear monophyletic and *Pearsonothuria* is sister to *Bohadschia*. Other aspects of our phylogeny, however, were not in accord with the taxonomy of Holothuriidae or previous speculations about the group's evolutionary history. Most notably, the genus *Holothuria* appears paraphyletic. *Actinopyga* and *Bohadschia*, sometimes held to be closely related to one another because of certain morphologic similarities, are only distantly related. The morphologically distinct *Labidodemas*, even thought to warrant separation at the family level, is nested well within *Holothuria*. A maximum parsimony reconstruction of ancestral ossicle form on the phylogeny indicated

that, in addition to a probable bout of elaboration in ossicle form (the modification of rods or rosettes to holothuriid-type buttons), at least 2 rounds of ossicle simplification also transpired in which buttons reverted to rods or rosettes. Cuvierian tubules, defensive organs unique to numerous members of Holothuriidae, were probably present before the initial radiation of the family, but the reconstruction is ambiguous as to their ancestral function.

Key words: phylogeny — 16S rDNA — mtDNA — Holothuriidae — *Isostichopus* — *Stichopus*

Introduction

Sea cucumbers, or holothuroids, in the large, globally distributed family Holothuriidae primarily inhabit the tropical eulittoral, usually coral reefs and adjacent sandy areas, though temperate shallows and deep water (to approx. 1 km) harbor a few species. Holothuriids are primarily deposit feeders, in contrast to the suspension feeding by dendrochirote holothuroids that prevails at higher latitudes. Like nearly all other sea cucumbers, holothuriids have a reduced skeleton of isolated, microscopic ossicles embedded in a pliable body wall. And, like other groups of soft-bodied organisms, their fossil history is threadbare, though button ossicles and articulated calcareous-ring elements characteristic of holothuriids are known from the Upper Jurassic (Gilliland, 1993) and the Middle Triassic (Reich, 2004), respectively. The provenance of these few finds and the family's apparent Tethyan distribution, even at the level of subgenera (Rowe, 1969; Clark and Rowe, 1971; Hendler et al., 1995; Massin, 1999), suggests the antiquity of several groups within the family.

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Table 1. Taxa Used in This Study

<i>Family and Species</i>	<i>Museum voucher number</i>	<i>Genbank accession number</i>	<i>Locality</i>
Holothuriidae			
<i>Actinopyga agassizi</i> (Selenka, 1867)	A110110	AY338413	Long Key, Florida
<i>A mauritiana</i> (Quoy and Gaimard, 1833)	E51760	AY338414	Guam, Mariana Islands
<i>Bohadschia argus</i> Jaeger, 1833	A113273	AY338416	Guam, Mariana Islands
<i>B. marmorata</i> Jaeger, 1833	E51759	AY338417	Guam, Mariana Islands
<i>Holothuria (Platyperona) excellens</i> (Ludwig, 1875)	A113261	AY338418	Guam, Mariana Islands
<i>H. (Mertensiothuria) leucospilota</i> (Brandt, 1835)	E51763	AY338419	Guam, Mariana Islands
<i>Labidodemas semperianum</i> Selenka, 1867	E53083	AY338420	Guam, Mariana Islands
<i>Pearsonothuria graeffei</i> (Semper, 1868)	E51761	AY338421	Guam, Mariana Islands
Stichopodidae			
<i>Isostichopus macroparentheses</i> (HL Clark, 1922)	E47524	AY338415	Guana Island, British Virgin Islands
<i>Stichopus chloronotus</i> Brandt, 1835	E47517	AY338422	Guam, Mariana Islands

^aNote: E indicates U.S. National Museum of Natural History echinoderm collection; A, American Museum of Natural History invertebrate tissue collection.

Holothuriidae is the second largest family of sea cucumbers, with about 185 species, or about 11% of the diversity of living holothuroids (Smiley, 1994). The family comprises 5 genera, the largest of which is *Holothuria*, with about 150 species. This diverse genus has dominated comprehensive reviews of the family (Pearson, 1914; Panning, 1929–1935; Deichmann, 1958; Rowe, 1969) because the remaining genera are small, well delimited, and easily diagnosed. Consequently, little attention has been paid to the evolutionary relationships in the family as a whole. Pearson (1914) believed that 2 genera, *Actinopyga* and *Bohadschia*, were closely related and primitive compared with *Holothuria*. Deichmann (1958) suggested that evolution in *Holothuria* had proceeded via progressive simplification of the ossicles from tables and buttons characteristic of many *Holothuria* to less elaborate rods or rosettes, like those found in the *Holothuria* subgenera *Selenkothuria* and *Semperothuria*. In contrast, Rowe (1969) proposed that the genus had proceeded via the opposite trend, from simple ossicles to complex ones, and on the basis of this trend, he provided a tentative phylogeny of subgenera in *Holothuria* that would require the genus to be monophyletic. More recently, Levin (1999), in partial agreement with Deichmann (1958), argued that the evolution of Holothuriidae proceeded from a form similar to the burrowing *Labidodemas*, which has tables and sometimes buttons, to vagile-exposed forms and to suspension feeders, some of which have rods and rosettes.

In this study we tested the utility of a conserved 3' section of 16S mitochondrial ribosomal DNA to clarify relationships between the genera in Holothuriidae, as well as performing an initial test for monophyly of the largest genus *Holothuria*. We then

used the estimate of the relationships to make inferences about the evolution of morphology and ecology in the group.

Materials and Methods

DNA Preparation. We sampled one specimen from one to 2 species in each of the 4 small and morphologically well-circumscribed genera of holothuriids. The fifth and largest genus *Holothuria* consists of several subgenera, from which we selected 2 putatively divergent (morphologically disparate) species. As outgroups to root the holothuriid tree, we used 2 species from different genera within Stichopodidae (Table 1). This latter family was selected largely because of its sister status to the study group Holothuriidae as inferred from a maximum parsimony analysis of morphologic characters (Kerr and Kim, 2001).

To prepare DNA for sequencing, approximately 100 mg of gonad, muscle, or tentacle tissue preserved in ethanol or DMSO was air-dried and ground to a powder in liquid nitrogen, then whole genomic DNA was extracted using the CTAB method of Arndt et al. (1996) or the EZ-DNA genomic DNA isolation reagent (Morwell MD Biosciences). We amplified a portion of approximately 540 nucleotides from the conserved 3' end of the mitochondrial gene coding for the 16S-like, large ribosomal RNA subunit using the echinoderm-specific universal primers 16Sar (5'-CGCCTGTTTATCAAAAACAT-3') and 16Sbr (5'-CTCCGGTTTGAAGCTCAGATCA-3'), then performed double-stranded polymerase chain reaction (PCR) under standard conditions (an initial 30-seconds denaturation at 95°C, followed by 40 cycles of 95°C for 30 seconds, 50° to 55°C for 30 seconds, and 72°C for 1 minute, then a final 4-minute extension at 72°)

Table 2. Uncorrected (below diagonal) and K2P (above diagonal) Mean Percentage Distances

Species	1	2	3	4	5	6	7	8	9	10
<i>A. agassizi</i>	—	9.79	25.0	20.6	20.1	17.8	19.4	20.6	19.7	23.7
<i>A. mauritiana</i>	9.09	—	23.4	16.2	17.5	18.1	19.0	20.8	21.5	23.5
<i>I. macroparentheses</i>	21.2	20.0	—	25.2	26.0	21.9	23.2	27.1	26.8	17.2
<i>B. argus</i>	17.7	14.3	21.3	—	4.35	14.5	16.4	14.5	12.9	25.6
<i>B. marmorata</i>	17.3	15.3	21.9	4.12	—	16.0	16.5	14.8	13.8	26.5
<i>H. excellens</i>	20.6	21.2	25.6	16.5	18.3	—	17.7	17.3	17.1	20.3
<i>H. leucospilota</i>	16.9	16.6	19.9	14.6	14.7	20.5	—	9.55	19.7	25.8
<i>L. semperianum</i>	17.7	17.8	22.6	13.0	13.3	20.0	8.81	—	19.8	25.3
<i>P. graeffei</i>	17.1	18.3	22.5	11.7	12.5	20.0	17.0	17.1	—	30.1
<i>S. chloronotus</i>	20.3	20.1	15.3	21.6	22.2	23.8	21.8	21.4	24.6	—

on a Hybaid or PerkinElmer thermocycler. Amplified products were isolated by starch-gel electrophoresis, purified with QIAquick gel extraction kit (Qiagen), and directly sequenced both ways on an ABI 3700/3100 DNA sequencer (Applied Biosystems). Sequences were deposited in GenBank under accession numbers AY338413 to AY338422 (Table 1).

Phylogenetic Analysis. Sequences were aligned using CLUSTAL W 1.5 (Thompson et al., 1994) under default parameters. The resulting alignment for use in the phylogenetic analyses, cropped of overhangs and primer sequence, was 544 sites in length. A phylogeny was estimated in PAUP* 4.0b10 (Swofford, 2000) using 2 criteria: maximum parsimony and maximum likelihood. The maximum parsimony analyses were done under the branch-and-bound setting. We used 2 character-weighting schemes, equal weighting and one in which transitions were down-weighted relative to transversions based on a transition-to-transversion (ti/tv) ratio estimated iteratively from the data via maximum likelihood. To find the simplest model of sequence evolution for use in the maximum likelihood analyses, we compared, using a likelihood-ratio test, the likelihood scores of a branch-and-bound search using the shortest parsimony tree to scores obtained from a general time-reversible model with invariant sites plus among-site rate heterogeneity (GTR+I+ Γ) successively estimated from the data. The winning model and parameters were then used in an unconstrained likelihood search in a second and third round of parameter fitting and model selection. Likelihood options were set to heuristic search and empirical base frequencies and ti/tv ratio was used. For both methods data quality was assessed by bootstrapping using 500 replicates under the conditions used to attain the optimal trees. For the parsimony analyses we also examined the “hierarchical signal” as skewness of the tree-length frequency distributions derived from an exhaustive search (Hillis and Huelsenbeck, 1992).

Character reconstructions were performed on MacClade 4.03 (Maddison and Maddison, 2001) under both accelerated and delayed transformations of character change while allowing unordered multi-state characters (Fitch parsimony). Though differing in several ways, rosettes in the outgroup family Stichopodidae are potentially homologous to the rosettes in Holothuriidae and are coded as such. To test whether the shortest tree obtained by maximum parsimony was preferable to a longer tree with an alternative placement of a selected subtree, we compared them with a one-tailed, normal approximation of a Wilcoxon signed-rank (“Templeton’s”) test statistic, H , as well as with a binomial sign test of winning sites as implemented in PAUP*.

Results

Of the 544 sites in the analyzed alignment, 325 were invariant, and 65 variable characters were parsimony-uninformative, leaving 154 parsimony-informative sites. Pairwise distances as percentages of sequence dissimilarity between holothuroid taxa ranged from a minimum of 4.12% between *Bohadschia argus* and *B. marmorata* and a high of 24.6% between *Pearsonothuria graeffei* and *Stichopus chloronotus* (Table 2). Ti/tv ratios were greater than 2 for 6 of 45 pairwise species comparisons, from a maximum of 6.50 between *B. argus* and *B. marmorata*, with one high value (4.43) between members of different genera, *Holothuria leucospilota* and *Labiododemas semperianum*, to a low of 0.67 between *Actinopyga agassizi* and *Stichopus chloronotus*. For the combined sequences there was a slight mean excess of A residues (29.0%), while C residues were least frequent (23.1%) (Table 3). Across all species, GC content averaged 47.8%, from a low of 46.1% for *B. argus* to a high of 49.6% for *L. semperianum* (Table 3).

The equally weighted maximum parsimony analysis, which produced a shortest tree of length 407, is shown in Figure 1 (A). The consistency index (CI) for this tree was 0.700, the CI excluding unin-

Table 3. Percentage of Bases in Amplified Partial 16S-like mitochondrial rDNA

Species	Base (%)				GC content (%)	Base count
	A	C	G	T		
<i>A. agassizi</i>	31.2	24.2	23.8	20.8	48.0	433
<i>A. mauritiana</i>	30.8	22.8	24.7	21.7	47.5	429
<i>I. macroparentheses</i>	28.1	22.0	24.7	25.2	46.7	437
<i>B. argus</i>	30.7	21.8	24.3	23.2	46.1	358
<i>B. marmorata</i>	30.0	22.8	24.7	22.6	47.5	430
<i>H. excellens</i>	30.0	23.2	24.5	22.3	47.7	429
<i>H. leucospilota</i>	28.2	23.8	23.8	24.1	47.6	432
<i>L. semperianum</i>	26.7	24.1	25.5	23.7	49.6	431
<i>P. graeffei</i>	30.6	23.0	23.7	22.7	46.7	431
<i>S. chloronotus</i>	25.5	24.5	24.8	25.2	49.3	440
Mean	29.0	23.1	24.4	23.4	47.8	425

formative characters was 0.641, the rescaled CI was 0.386, and the retention index was 0.552. The number of unambiguous changes on internal branches ranged from 5 to 24. The frequency distribution of tree lengths was highly left skewed, with a $g1$ score of -1.055 , which is well beyond the $P < 0.01$ significance level and indicates that at least part of the tree contains considerable hierarchical signal, ostensibly owing to genealogic similarity (Hillis and Huelsenbeck, 1992). Bootstrap percentages for in-group nodes ranged from a high of 99% to a low of

54%. The ti/tv ratio estimated via maximum likelihood was 1.515. Using this to weight the characters in a second parsimony analysis returned a tree with a topology identical to the equal weighted tree and had similar branch lengths (via a Wilcoxon test: $N = 18$, $H = 2.00$, $P > 0.10$). The likelihood ratio test indicated that the simplest model of sequence evolution fitting the data given the tree topology was Kimura's 2-parameter form. After 2 further rounds of tree and parameter estimation, the maximum likelihood analysis returned a tree ($-\ln L = 2509.670$) whose

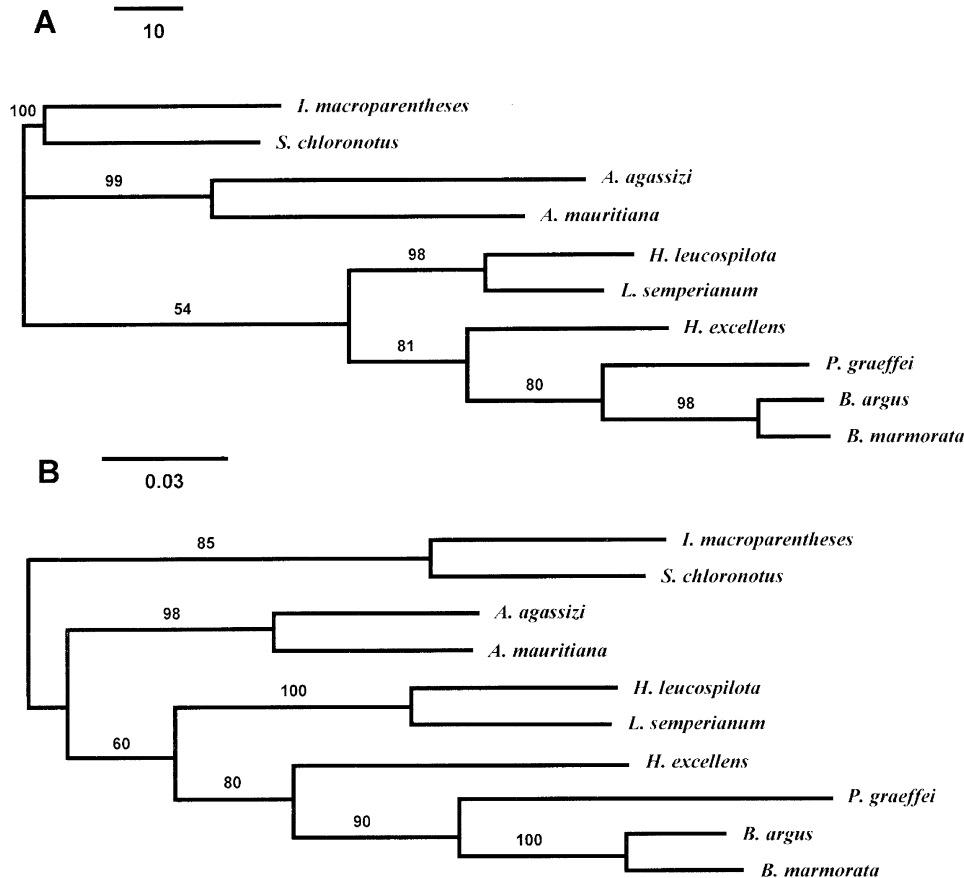


Fig. 1. Phylogenetic trees. **A:** Single most parsimonious tree using equal and transition-transversion weights (length, 407). **B:** Maximum likelihood tree ($-\ln L = 2509.670$).

topology was identical to that from the maximum parsimony analyses (Figure 1, B). This result appeared insensitive to the complexity of the substitution model as use of either a JC69 (equal base frequencies and one substitution rate) or a GTR+I+ Γ model (with 4 categories of rate variation and empirically estimated invariant proportions) also returned the same tree.

In all analyses, *Actinopyga* and *Bohadschia* were each monophyletic with high bootstrap percentages. Additionally, *Pearsonothuria graeffei* was sister to *Bohadschia* spp., and *Labidodemas semperianum* was sister to *Holothuria leucospilota*, in each case with strong bootstrap support. In all trees, regardless of reconstruction method, the genus *Holothuria* appeared paraphyletic. To further test the robustness of this result, we compared the shortest parsimony tree (Figure 1, A) to the shortest tree obtained under the constraint that *Holothuria* is monophyletic (length, 425). The latter tree was significantly longer, and hence not preferred, by a normal approximation of a Wilcoxon signed-rank test ($N = 30$, $H = 93$, $z = -3.268$, $P = 0.0010$) or a binomial test (winning sites, 24; $P = 0.0014$). Similarly, the placement of *Actinopyga* and *Bohadschia* together near the base of the tree (length = 418) was not preferred over their positions in the shortest tree ($N = 31$, $H = 160$, $z = -1.976$, $P = 0.0482$), though the marginal significance of the more conservative binomial test in this instance (winning sites = 21; $P = 0.0725$) indicated that sites contributed somewhat unequally to the probability of the Wilcoxon statistic. The shortest tree with *Labidodemas* arising outside of *Holothuria* (length = 419) was also significantly longer than the shortest tree overall ($N = 14$, $H = 7.5$, $z = -3.207$, $P = 0.0013$; winning sites = 13; $P = 0.0018$).

Discussion

Systematics. These results comprise the first phylogenetic test of the classification of the Holothuriidae. Parsimony and likelihood analyses returned identical topologies, each with high bootstrap support (Figure 1). Our phylogeny corroborated some aspects of the currently used Linnean classification. Both *Actinopyga* and *Bohadschia* appear to be good genera. These taxa are relatively small (approx. 16 and 11 species, respectively), morphologically well circumscribed, and easily diagnosable by a set of characters from several organ systems that are unique to each genus. Within the limited sample of taxa used in this study, each genus is monophyletic, and both are supported by high bootstrap percentages. Additionally, the finding of a close relationship

between *Pearsonothuria graeffei* and *Bohadschia* echoes the previous placement of the former species in the latter genus (Panning, 1929; Rowe, 1969). The single species of *Pearsonothuria* has since been placed in its own genus in recognition of its distinctive spicules and secondary chemistry (Levin et al., 1984).

Other aspects of our phylogeny, however, were not in accord with the taxonomy of Holothuriidae or with previous speculations about its evolutionary history. Most notably, the genus *Holothuria* appears paraphyletic. Rowe (1969) provides a tentative phylogeny based on ossicle similarity that shows the group as monophyletic. The results presented here (Figure 1), however, indicate that *Holothuria* is not a single lineage, but that it also gives rise to *Pearsonothuria*+*Bohadschia* and *Labidodemas*. The paraphyly has strong bootstrap support regardless of the reconstruction method used. Additionally, the shortest parsimony tree in which *Holothuria* is monophyletic is significantly longer than, and hence not preferred over, the maximum parsimony tree pictured in Figure 1(A). The paraphyletic status of this genus is perhaps not unanticipated. *Holothuria* is a large morphologically diverse family with approximately 150 species and a complex nomenclatural history. Although much of *Holothuria* can be diagnosed by distinctive ossicles (holothuriid tables and buttons), their absence in numerous other members could well be secondary. Moreover, there are no anatomic characters fixed within the genus. Rather, only overlapping subsets of these traits are possessed by any one subgenus, and they are sometimes possessed by non-*Holothuria* holothuriids (e.g., shape and proportion of the esophageal calcareous ring elements, presence and shape of Cuvierian tubules, thickness of body wall, form and arrangement of dorsal papillae, gross shape, enlargement and calcification of anal papillae, and arrangement of tube feet).

Another surprise is the evolutionary distance between *Actinopyga* and *Bohadschia*. Although these genera are each distinctive on several accounts, they also have similar ossicles, body walls, and calcareous rings as compared to other holothuriids and, as a result, have long been considered closely related (e.g., Pearson, 1914; Panning, 1940). In this study *Actinopyga* and *Bohadschia* were separated by 25 unambiguous changes on 3 branches, 2 with strong bootstrap support (Figure 1A). As well, uniting the genera or considering them as a paraphyletic unit at the base of the ingroup requires a significantly longer tree (418 vs. 407), though the P values of the sample statistics hovering around the acceptable error rate of 0.05 suggest caution. Finally,

we note that the morphologically distinct genus *Labidodemas* arises from within *Holothuria*. Among holothuriids the 8 members of *Labidodemas* are unique in the possession of an undulating, ribbon-like calcareous ring. Because of this character, Rowe (1969) considers *Labidodemas* distinct from the *Holothuria*, even suggesting that it may warrant separation at the family level. James (1981) goes further and assigns the genus to its own family, Labidodematidae. Levin (1999) also implies that the position of *Labidodemas* lies outside *Holothuria* and is near the base of the holothuriid tree, arguing that the genus displays the most primitive characters of the family. Most recently, Massin et al. (2004) argue that possession of *Holothuria*-like table ossicles, as well as their discovery of Cuvierian tubules in one species, suggest that *Labidodemas* could be most closely related to *Holothuria* or even "a part of it." In this study *Labidodemas* was indeed nested well within *Holothuria* and was sister to *H. leucospilota* with very high bootstrap support (Figure 1). As well, placement outside *Holothuria* required a significantly longer tree (419 vs. 407). The sequences for *Labidodemas* and *H. leucospilota* were also under-saturated for transversions ($ti/tv = 4.43$), which further suggests a close relationship via a relatively recent divergence (or, less probably, a more distant relationship but a considerably arrested base substitution rate across a potentially large, inclusive lineage not sampled here).

Character Evolution. The phylogeny also permitted several tentative inferences about the evolution of morphology and ecology within the Holothuriidae. Both Pearson (1914) and Rowe (1969) suggest that the family has evolved along the lines of increasing ossicle complexity, from those bearing "simple" rosette and rod ossicles in the body wall to those with more complex table and button elements. Conversely, Deichmann (1958) and Levin (1999) speculate that tables and buttons are ancestral, the group only later evolving rosettes and spiny rods. A parsimonious ancestral state reconstruction on our phylogeny (Figure 2, A, B), though, indicated that, in addition to an elaboration of ossicle form, at least 2 rounds of simplification also transpired. The first occurred when *Actinopyga* diverged from the rest of Holothuriidae and apparently lost the table ossicles found in many other holothuriids and most non-holothuriids of the order Aspidochirotida. Then, along the branch leading to all holothuriids sans *Actinopyga*, buttons evolved, and later tables were again lost, perhaps by way of the intermediate state displayed by *Pearsonothuria*, which possesses 3- and 4-pillared pseudotables, as well as "racquet" ossicles

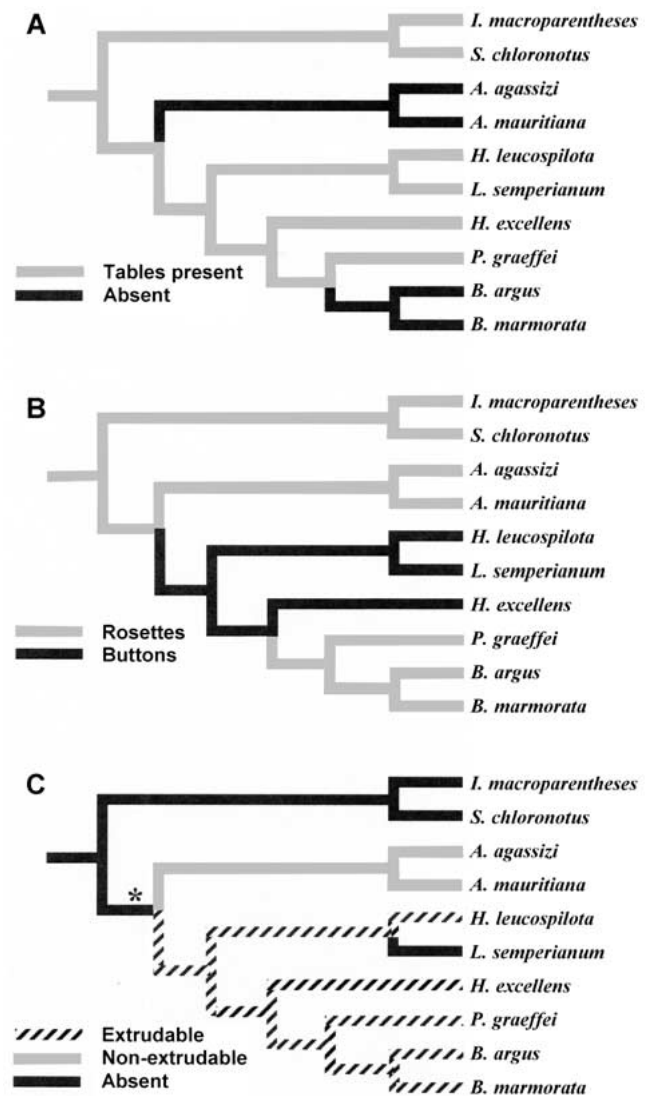


Fig. 2. Maximum parsimony ancestral reconstructions of selected morphologic traits. **A:** Table ossicles in the body wall. **B:** Button ossicles in the body wall. **C:** Cuvierian tubules. See "Materials and Methods" for justification of state assignments. Ancestral states resolved with an accelerated transformation of character change. Asterisk in tree C indicates that under a delayed transformation the underlying branch manifests the "extrudable" state.

that may be highly modified tables (Levin et al., 1984). Later still, button ossicles reverted to rosettes and rods below *Bohadschia*. This picture of ossicle evolution within Holothuriidae indicates the minimum number of reversions that likely occurred and is less straightforward than previously speculated. We expect that the secondary loss of holothuriid tables and buttons is even more widespread, such that some members of groups joined by the absence of these features will prove to be only distantly related. This further predicts, as did Deichmann (1958 in Rowe, 1969) and Levin (1999), that suspension

feeding with peltato-dendritic tentacles will be a derived feature of the family, as it occurs most often in subgenera lacking buttons or tables.

Finally, the phylogeny presented here permits inferences about the evolution of an organ unique to the Holothuriidae. Cuvierian tubules are multiple, elongate structures attached to the base of the left respiratory tree of numerous species of holothuriids (Smiley, 1994). Typically, the tubules, which become sticky when extruded, are expelled through the cloaca, extended, and autotomized when the animal is disturbed (Vander Spiegel and Jangoux, 1987). Recently Cuvierian tubules have been reported (Massin et al., 2004) from a single species of *Labidodemas* and extrusion of sticky tubules has been observed (A.M. Kerr, unpublished observation) in *Pearsonothuria graeffei*. However, in *Actinopyga* and *Holothuria* (*Microthele*) spp., the tubules do not become sticky and are not extended or autotomized (Vanden Spiegel and Jangoux, 1993; Vanden Spiegel, 1995). An ancestral reconstruction (Figure 2, C) suggests that the organs were present before the initial radiation of the family and subsequently lost in some members of *Holothuria* such as *H. (Semperothuria) flavomaculata*. Our reconstruction is ambiguous as to the ancestral function of Cuvierian tubules, since one lineage, *Actinopyga*, leading from the basal node of Holothuriidae has small, nonsticky tubules that are rarely evulsed, while the other lineage often exhibits long, adhesive, and readily extruded organs used in defense. This raises the possibility that if the small, nonautotomized tubules in *Actinopyga* represent the original state, rather than a secondary condition, then Cuvierian tubules may have originally evolved in a role other than defense.

Acknowledgments

We thank C. Ahearn, A. Arndt, G. Paulay, D. Pawson, L. Jarecki, E. Laso-Wasem, R. Woollacott, and Keys Marine Laboratory for access to specimens and technical support; and W. Appletans, C. Kerr, C. Massin, G. Paulay, and F. Rowe for valuable criticism; and we acknowledge the Ernst Mayr Grant (Museum of Comparative Zoology), Lerner-Gray Fund (American Museum of Natural History), The Falconwood Corporation, and a STAR Fellowship (U.S. Environmental Protection Agency) to A.M.K.; a NASA (Fundamental Biology Program) grant, NAG-1399, to D.A.J.; and an NSF grant, DEB 9806570, to J.K. Y.S. acknowledges the use of unpublished data from a joint project with F. Rowe (Hoxne, Suffolk, U.K.); for this project F. Rowe was funded by Access to Belgain Collections, Brussels.

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