

Resurrection of *Bohadschia bivittata* from *B. marmorata* (Holothuroidea: Holothuriidae) based on behavioral, morphological, and mitochondrial DNA evidence

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Abstract

Behavior, color, body size, spicules, and mitochondrial DNA were examined in two morphs from the *Bohadschia marmorata* (Jaeger, 1833) species complex in Micronesia to test whether they are conspecific. This complex consists of eight morphs that have been described as separate species and combined in various ways for over a century. We examined the classic *B. marmorata* type and the type originally described as the species *B. bivittata* (Mitsukuri, 1912); *B. bivittata* was combined with *B. marmorata* by Panning (1944). Several observations and a phylogenetic analysis led us to conclude that *B. marmorata* and *B. bivittata* should return to their status as separate species. First, *B. marmorata* lives in shallow areas with strong currents, and *B. bivittata* lives on open sand between corals in deeper water. Second, the coloration of *B. bivittata* is distinct from *B. marmorata*, and although specimens collected on Yap Island differed from Mitsukuri's original description of *B. bivittata*, no specimens were collected with coloration intermediate between *B. bivittata* and *B. marmorata*. Third, spicules are more highly branched, perforated, and spiked in *B. bivittata* than in *B. marmorata* (and, in our study, spicule complexity did not correlate with body size). Finally, our phylogenetic analysis, based on partial nucleotide sequences of 16s, 12s, and COI mitochondrial genes, resulted in a tree—(*Pearsonothuria graeffei* (*Bohadschia marmorata*) (*B. argus* (*B. bivittata*)))—which shows that *B. marmorata* and *B. bivittata* are not even sister species, with *B. bivittata* more closely related to *B. argus*. Support for the clades for each *Bohadschia* species was strong, but the clade containing *B. argus* and *B. bivittata* had weaker support. Color and spicule examinations made of preserved *B. marmorata*-complex specimens from the Indo-Pacific as well as behavioral observations in the field also support the resurrection of *B. bivittata*.

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Introduction

Morphologically simple marine organisms (such as sea cucumbers) present significant taxonomic challenges. Morphological simplicity means a dearth of available characters to mark breaks between species (Chombard et al., 1998), the numbers of which are reduced further in groups where normally useful characters vary continuously between morphs. Marine animals lack obvious boundaries to dispersal, implying that single species can be found across whole oceans, thus leading to the designation of variable species with vast ranges (Benzie, 1998).

However, recent molecular data from some of these marine “species” have revealed them to be collections of cryptic species not easily recognized by differences in morphology (Van Oppen et al., 2001; Benzie, 1998). Other panmictic species have been shown to have considerable population structure over broad geographic ranges, such as the two sea cucumbers *Holothuria nobilis* and *H. scabra* (Uthicke and Benzie, 2001, 2003). Both these species develop via long-lived planktonic larval stages, so the mechanism behind this structure is not obvious. In the case of *H. nobilis*, its genetic structure is consistent with a rapid population expansion followed by a separation of certain populations during the last ice age (Uthicke and Benzie, 2003). The interplay between past isolation and present gene flow adds the additional complication of hybridization between species, which can be extensive in free-spawning marine organisms (Hatta et al., 1999; Wolstenholme et al., 2003). In this study, we examined the population genetic structure and taxonomic status of two morphs of a sea cucumber species complex with a long history of taxonomic revisions.

Bohadschia marmorata (Jaeger, 1833) has been the subject of extensive taxonomic speculation. After Jaeger’s (1833) original description, Semper (1868) described four closely related species (*similis*, *koellikeri*, *tenuissima* and *vitiensis*). Subsequently, Ludwig, (1875)

described *clemens*, and Mitsukuri (1912) described *bivittata*. Table 1 summarizes the original descriptions of body wall coloration of the type material of these species. Before Mitsukuri’s (1912) publication, synonymizing these species had already been suggested by Théel (1886): he speculated that *marmorata*, *similis*, *koellikeri*, *tenuissima*, *vitiensis* and *clemens* were varieties or juveniles of *B. marmorata*, and he suspected that *B. argus* (Semper 1868) might even be included as a synonym. Mitsukuri mentioned Théel’s proposed synonymy but decided that *marmorata*, *bivittata* and *argus* could be separated on color alone. In Pearson (1913) (unaware of *bivittata*) combined *koellikeri*, *similis*, *tenuissima*, *clemens* and *vitiensis* under the name *vitiensis*. Thus, Pearson shrunk the pool of species to *marmorata*, *vitiensis*, and (by omission) *bivittata*. In Panning (1929) agreed with this step but in 1944 combined *vitiensis*, *bivittata*, and *marmorata* under *marmorata*. In Cherbonnier (1955) separated *marmorata* and *tenuissima* and described a new species, *cousteaui*. Rowe and Doty (1977) maintained Panning’s (1944) single-species taxonomy and suggested that spicule differences between previous types were a by-product of correlations between spicule form and body size. Kerr (1994) followed Rowe and Doty’s synonymy, as did Rowe and Gates (1995). However, Rowe and Gates (1995) mentioned that additional collections and studies may overturn the single-species taxonomy, and several researchers have continued to treat the different morphs as separate species (see Cherbonnier and Ferae, 1984; Massin, 1999; Samyn, 2000; and Lane, in press).

In this paper, we analyze the taxonomy of *B. marmorata* and *B. bivittata* from Micronesia. This study was initiated when one of us (Clouse) noticed that on the Micronesian island of Pohnpei, individuals satisfying the original description of *B. bivittata* (off-white background dorsally with two cloudy, brown transverse stripes) were found on open sand near corals, a habitat distinctly different from the grassflats and mangrove channels where we found individuals matching the

Table 1. Original color descriptions of type material of species currently regarded to belong to the *B. marmorata* species-complex

Name and author	Description
<i>marmorata</i> (Jaeger, 1833)	Dark, marbled spots on a dark, ashy background, which are more prevalent toward the sides
<i>similis</i> (Semper, 1868)	Ventral reddish-brown or gray, dorsum darker and more reddish
<i>koellikeri</i> (Semper, 1868)	Light brown; large, fuzzy brown spots
<i>tenuissima</i> (Semper, 1868)	Ventral yellowish-white, dorsum reddish-brown
<i>vitiensis</i> (Semper, 1868)	Dorsum and ventral light brown
<i>clemens</i> (Ludwig, 1875)	From a juvenile specimen—dorsal light brown with large, washed-out dark spots
<i>bivittata</i> (Mitsukuri, 1912)	Base color of dorsum yellow to brown with two broad, dark-brown bands across the entire breadth, ventral lighter and uniform white to brown
<i>cousteaui</i> Cherbonnier, 1955	Dorsum chocolate brown

Species with author names in parentheses were originally placed in the genus *Holothuria*.

original description of *B. marmorata* (Jaeger, 1833) (gray background dorsally with sharply demarcated, dark brown blotches). Moreover, there were no individuals with an appearance intermediate between *B. marmorata* and *B. bivittata*. Consequently, *B. bivittata* forms were not included in the behavioral study of *B. marmorata* by Clouse (1997b). The second phase of this project was a study of spicules from *B. marmorata* and *B. bivittata* specimens collected around Pohnpei and vouchered in the National Museum of Natural History, Washington, DC, USA (NMNH). In the third phase, we collected tissue samples and digital images from *B. bivittata* from Yap Island and *B. bivittata* and *B. marmorata* from Pohnpei Island. We sequenced three mitochondrial loci and with the sequences reconstructed a phylogeny of these exemplars.

Materials and methods

Phase I: habitat preference and size difference

All Pohnpeian specimens were collected from the southeastern side of Pohnpei Island, around the islet of Temwen, which is located within the barrier reef. Pohnpei and Temwen are surrounded by thick mangrove forests, leading to grassflats, then open sand and patch coral, and finally the barrier reef. The mangrove regions between Temwen and Pohnpei contain numerous shallow water channels cut by the currents that flow between the reef and Madolenihmw Bay. On the north side of Temwen, a deep-water channel extending from the bay to a reef opening at the north-east corner of the Nan Madol ruins surrounds extensive grassflats. Detailed descriptions were made of habitats in which various populations of *Bohadschia marmorata* and *bivittata* were found.

Since it was observed that individuals matching the original description of *B. marmorata* were much smaller and darker when occurring in the grassflats than in the mangroves, specimens from each habitat were examined to find an explanation for this difference. *Holothuria atra* populations with different average body sizes can result from differences in reproductive mode—the smaller population engaging primarily in fission (Chao et al., 1994). However, *B. marmorata* has only been observed reproducing sexually (spawning; Clouse, 1997b). Given the taxonomic difficulty in the *B. marmorata* complex, two hypotheses are proposed: firstly, that the grassflats- and mangrove-dwelling individuals were actually different species; or alternately that individuals inhabiting grassflats, although numerous (about 20 per m²), were not in an optimal habitat. The hypothesis that grassflats and mangrove populations were different species was tested by inspecting their

spicules separately in Phase II (below). The second hypothesis—that mangrove habitats provide better nutrition—was tested by resettling ten individuals on October 2, 1994, from the grassflats population into the mangroves. Their color patterns were recorded (thus allowing future identification of individuals), and they were weighed (drained of respiratory water) using a Pesola hand scale. Weights were checked periodically until May 23, 1995, when only three individuals were found. Thus our weight analysis is based on the last time, February 8, 1995, when at least half of the sample was found, i.e., 130 days after their transfer.

Phase II: spicule variation

In December 1995, 16 specimens agreeing with the original description of *B. marmorata* were collected from mangrove channels, 10 more were collected from grassflats, and two specimens corresponding to the original description of *B. bivittata* specimens were collected from open sand around Temwen. From February to May, 1998, an additional 11 *B. bivittata* specimens were collected from sandy areas around Temwen. Spicule samples were taken from the dorsal body wall (about 1 cm anterior to the anus), from the mid-ventral body wall, and from the tentacles of all specimens. For dorsal body wall spicules, both the number of branches and holes were counted; for ventral body wall spicules, the number of holes were counted; and for tentacle spicules, the protuberances (“spikes” or “thorns”) on the shaft and ends were counted. Counting was done by taking a random view of each slide and noting the average number of branches (or other features of interest) of the spicules in view. Dorsal coloration and spicule characteristics were also noted for all specimens that had previously been determined to be any one of the *B. marmorata* morphs in the collection of the National Museum of Natural History, Washington, DC, USA (NMNH).

Phase III: phylogenetic analysis

During late November and early December, 2002, specimens were collected from Micronesia for a phylogenetic analysis (Table 2). From Yap, eight specimens closely matching Mitsukuri's (1912) original description of *B. bivittata* were collected from grassflats in Manguuy Bay, near the causeway between Maap and Tomil-Gagil islands. On Pohnpei, one *B. bivittata* specimen was collected from open sand between the mangroves and outer reef, and three *B. marmorata* specimens were collected from a mangrove channel near Temwen. Two specimens agreeing with the original description of *B. argus* were collected, one from Yap and one from Pohnpei, and one specimen of

Table 2. Specimens collected in micronesia during late November and early December 2002 for the phylogenetic analysis (Phase III)

Main island	Species	Locale	Habitat	No. collected
Yap	<i>B. argus</i>	Wanyan village	On sandy substrate between corals at the edge of a sinkhole; depth approximately 3 m	1
Yap	<i>B. bivittata</i>	Manguuy bay near the causeway between Maap and Tomil-Gagil islands	Grassflats	8
Chuuk	<i>Pearsonothuria graeffei</i>	Chuuk lagoon	On coral	1
Pohnpei	<i>B. argus</i>	Reef patch outside Madolenihmw Bay	On sandy substrate between corals in narrow channel	1
Pohnpei	<i>B. bivittata</i>	Between Temwen and Joy islets inside barrier reef	On sandy substrate with seagrass	1
Pohnpei	<i>B. marmorata</i>	Between Temwen and Pohnpei islands	Among <i>Halimeda</i> algae and seagrass at bottom of mangrove channel	3

Pearsonothuria graeffei was collected from Chuuk lagoon. Each specimen was photographed live, and, at the end of this study, these images were uploaded to Morphobank.org. Two tissue samples were removed from each specimen and preserved in the field in 100% EtOH and buffered DMSO. Whole animals were preserved in either ethyl or isopropyl alcohol.

We hypothesized that if *B. bivittata* and *B. marmorata* are different species, then *B. bivittata* from Pohnpei would appear in a clade with *B. bivittata* from Yap, and *B. marmorata* and *B. bivittata* would cluster into two clades but remain sister taxa with respect to *B. argus*. Conversely, if *B. marmorata* and *B. bivittata* are merely morphological variants of a single species, then we expected individuals to cluster based on collecting locality and independent of body coloration and spicule form. Stated another way, if *B. marmorata* and *B. bivittata* are a single species, then we expected that distinct Yapese and Pohnpeian clades could be reconstructed with *B. marmorata* and *B. bivittata* morphs present within each clade.

We assembled a sequence data set covering three loci: 16S, 12S, and COI from the mitochondrial genome. Although we sequenced DNA from many individual sea cucumbers, sequence data from all loci of interest were not obtained from all exemplars. Thus we used those data that were available for most exemplars (Table 3).

DNA preparation

DNA was extracted from one tissue sample per specimen using Qiagen DNA easy Tissue Kit #69506 (Qiagen, Inc., Valencia, CA) following manufacturer's protocol.

DNA amplification was done via the polymerase chain reaction (PCR) using the primers listed in Table 4. PCR was performed using Amersham Biosciences puReTaq™ Ready-To-Go™ PCR Beads (Amersham Biosciences, Piscataway, NJ). Reagent and template DNA “cocktails” of 25 µl were prepared for each primer pair and template DNA containing Ready-To-Go™ PCR Bead, 21 µl reverse osmosis purified or commercial RNase free water, 1.0 µl forward primer, and 1.0 µl reverse primer.

Thermocycling was conducted with an initial denaturation at 94 °C for 5 min then 40 cycles of denaturation at 94 °C for 20 s, annealing at 49 °C for 20 s, and extension at 72 °C for 20 s followed by a 2 min final extension time at 72 °C in MJ Research Tetrad and Dyad Thermal cyclers (MJ Research, Watertown, MA). In some cases annealing temperatures were lowered to 45 °C. PCR products were purified with TeleChem ArrayIt™ PCR Purification Kit (TeleChem International Inc., Sunnyvale, CA) according to manufacturer's protocol. Purification was performed on a Biomek 2000 robot.

Direct Sequencing was performed using Big Dye™ Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) in MJ Research Tetrad and Dyad Thermal cyclers (MJ Research, Watertown, MA) according to manufacturer's instructions. Isopropanol and EtOH precipitations were used to purify sequencing reactions. Both strands from each sample were sequenced. An Applied Biosystems 3700 DNA analyzer was used to sequence products of the above procedures. Chromatograms were analyzed and contiguous fragments were assembled in Sequencer (v 4.0.5). All assembled sequences were verified as holothuroid

Table 3. Genbank and Morphobank accession numbers for exemplars and loci used in the phylogenetic analysis

Collection information			Genbank accession number			Morphobank accession number	
Species	Specimen number	Location	16S mtDNA	COI mtDNA	12S mtDNA	Dorsal image	Ventral image
<i>B. argus</i>	14	Yap	AY574869			M25015	M25016
<i>B. argus</i>	57	Pohnpei	AY574870	AY574878	AY574861	M25027	
<i>B. bivittata</i>	05	Yap	AY574871		AY574862	M25009	M25010
<i>B. bivittata</i>	06	Yap	AY574872	AY574879	AY574863	M25011	M25012
<i>B. bivittata</i>	07	Yap	AY574873	AY574880	AY574864	M25013	M25014
<i>B. bivittata</i>	36	Pohnpei	AY574876			M25023	M25024
<i>B. marmorata</i>	34	Pohnpei	AY574874	AY574881	AY574865	M25019	M25020
<i>B. marmorata</i>	35	Pohnpei	AY574875	AY574882	AY574866	M25021	M25022
<i>B. marmorata</i>	37	Pohnpei	AY574877	AY574883	AY574867	M25025	M25026
<i>Pearsonothuria graeffei</i>	28	Chuuk	AY574868		AY574860	M25017	M25018

Morphobank accession numbers can be used to find dorsal and ventral images of these specimens at Morphobank.org. Multiple images may be retrieved at once by entering a range of accession numbers separated by a dash; the following url will retrieve all images used in this study: <http://morphobank.org/quick?M25009-M25027>.

sequences by use of the BLAST program against Genbank. Final extracted sequences were deposited in Genbank (Table 3).

Search strategy

Unaligned DNA sequences were analyzed in POY software (version 3.05; Wheeler et al., 2003; <http://research.amnh.org/scicomp/projects/poy.php> or <ftp://ftp.amnh.org/pub/molecular/poy>) using direct optimization. In direct optimization sequences are aligned anew each time a tree is built and refined (Wheeler, 1996). The calculations were performed in parallel in a cluster of 5 workstations with dual Intel P4 cpus running the LINUX version 2.4.18–14 smp operating system (Janies and Wheeler, 2001). In POY, evolutionary base substitution events in sequences are treated with cost functions just as in multiple alignment. However, one key difference between direct optimization and multiple

alignment is that in direct optimization evolutionary differences in sequence length are accommodated by allowing insertion–deletion events as transformations between ancestral and descendant sequences rather than via the use of gap characters (Wheeler, 1996). In combined analysis of sequences of various loci in POY, homology statements of DNA sequence alignments are co-optimized to produce a best total evidence tree. POY has been successfully used to lower incongruence among data partitions with respect to traditional methods of multiple alignment followed by phylogenetic analysis (Wheeler, 1998).

Sequence data from individual sea cucumbers were analyzed with each molecular locus on its own and all data combined. We performed direct optimization searches across a parameter space of several models of evolution to test the sensitivity of the results to choices of relative weights of insertion–deletion events,

Table 4. The following oligonucleotides were used for PCR primers

COIaug5	HCGMATGAAHAAHATGAGHT
COIaug3	CATKGCRTANACCATNCC
LCO	GGTCAACAAATCATAAAGATATTGG
HCOOUTOUT	GTAAATATATGRTGDGCTC
16SA	CGCCTGTTTATCAAAAACAT
16SB	CTCCGGTTTGAACCTCAGATCA
16Spri519	GWAATACCGCGGCKGCTG
16Spri915	GCCCCGYCAATTCCT
16Spri1391	GACGGGCGGTGTGTRCA
12SF	CTTAATTGACAAAGCAAAGCACTGA
12SR	AACCTCCTTCCTTTAATTTACAAGA
12SAI	AAACTAGGATTAGATACCCTATTAT
12SBI	AAGAGCGACGGGCGATGTGT

transversions, and transitions (Wheeler, 1995). The command scripts for combined runs are as follows:

- `poy -molecularmatrix g1tv1ts1.txt -trailinggap 1 bohad16.sept.tot coipri.sept.tot bohad12.sept.tot -parallel -multirandom -replicates 20 -slop 5 -check-slop 10 -oneasis -buildmaxtrees 2 -maxtrees 2 -holdmaxtrees 24 -sprmaxtrees 2 -tbrmaxtrees 2 -nodiscrepancies> bohadtotg1tv1ts1septnlex.out 2> bohadtotg1tv1ts1septnlex.err`
- `poy -molecularmatrix g1tv2ts1.txt -trailinggap 1 bohad16.sept.tot coipri.sept.tot bohad12.sept.tot -parallel -multirandom -replicates 20 -slop 5 -check-slop 10 -oneasis -buildmaxtrees 2 -maxtrees 2 -holdmaxtrees 24 -sprmaxtrees 2 -tbrmaxtrees 2 -nodiscrepancies> bohadtotg1tv2ts1septnlex.out 2> bohadtotg1tv2ts1septnlex.err`
- `poy -molecularmatrix g2tv1ts1.txt -trailinggap 1 bohad16.sept.tot coipri.sept.tot bohad12.sept.tot -parallel -multirandom -replicates 20 -slop 5 -check-slop 10 -oneasis -buildmaxtrees 2 -maxtrees 2 -holdmaxtrees 24 -sprmaxtrees 2 -tbrmaxtrees 2 -nodiscrepancies> bohadtotg2tv1ts1septnlex.out 2> bohadtotg2tv1ts1septnlex.err`
- `poy -molecularmatrix g2tv2ts1.txt -trailinggap 3 bohad16.sept.tot coipri.sept.tot bohad12.sept.tot -parallel -multirandom -replicates 20 -slop 5 -check-slop 10 -oneasis -buildmaxtrees 2 -maxtrees 2 -holdmaxtrees 24 -sprmaxtrees 2 -tbrmaxtrees 2 -nodiscrepancies> bohadtotg2tv2ts1septnlex.out 2> bohadtotg2tv2ts1septnlex.err`
- `poy -molecularmatrix g4tv2ts1.txt -trailinggap 7 bohad16.sept.tot coipri.sept.tot bohad12.sept.tot -parallel -multirandom -replicates 20 -slop 5 -check-slop 10 -oneasis -buildmaxtrees 2 -maxtrees 2 -holdmaxtrees 24 -sprmaxtrees 2 -tbrmaxtrees 2 -nodiscrepancies> bohadtotg4tv2ts1septnlex.out 2> bohadtotg4tv2ts1septnlex.err`

The tree lengths resulting from these searches are presented in Table 5. The optimality criterion of minimum incongruence as measured by the MFES

(Mickevitch and Farris, 1981) statistic was used to choose the best tree. This method aims to measure the degree of character conflict among multiple data sets by quantifying the number of extra steps forced upon the individual data sets when they are combined. MFES is calculated as follows:

$$\text{MFES} = (\text{tree length resulting from analysis of combined data} - \text{tree lengths resulting from analysis of each locus on its own}) / \text{tree length resulting from analysis of combined data.}$$

We performed jackknife replicates on most congruent trees as specified by the following commands: `-random 10000 -jackboot -jackfrequencies all -jacktree -notbr -spr`. Jackknifing (Farris et al., 1996), is a technique for estimating the confidence in an internal branch of a tree by resampling the original data set without replacement. The jackknife values for the branch reported in Fig. 1 are the percentage of 10,000 resampled replicates that recover the branch. The jackknife run was executed as follows:

```
poy3e -parallel bohad16.sept.tot coipri.sept.tot bohad12.sept.tot -molecularmatrix g1tv2ts1.txt -trailinggap 1 -multirandom -replicates 10000 -jackboot -jackfrequencies all -jacktree -notbr -spr -topofile bohadtotallmodels.jack.topo> bohadtotallmodels.-jackknife.10000.out 2> bohadtotallmodels.jackknife.10000.err
```

Bremer searches (Bremer, 1988) were performed as series of POY searches to find the shortest tree not including the group of interest. Bremer support indices are the number of steps needed to dismantle an internal clade, so higher indices indicate more support for a clade. To accomplish this, a group inclusion matrix for consensus of the best tree from all models was calculated using the program `jack2hen` and encoded in the file `bohadtotallmodels.jack`. The script used in this search is as follows:

```
poy3e bohad16.sept.tot coipri.sept.tot bohad12.sept.tot -random 10000 -molecularmatrix g1tv2ts1.txt -trailinggap 1 -bremer -constrain bohadtotallmodels.jack>
```

Table 5. Tree lengths under various evolutionary models

Gap: change ratio	Model description		Tree lengths				MFES × 1000
	Transversion: transition ratio	Trailing gap	12S	16S	COI	Total	
1	1	Na	151	179	267	592	1498
1	2	1	188	248	296	723	1492
2	1	1	164	199	278	631	1496
2	2	3	300	336	549	1160	1504
4	2	7	509	482	975	1989	1477

The models are described in the first three columns, tree lengths for each locus alone (12S, 16S, and COI) are in the next three columns, then for all loci together, and finally the MFES statistic.

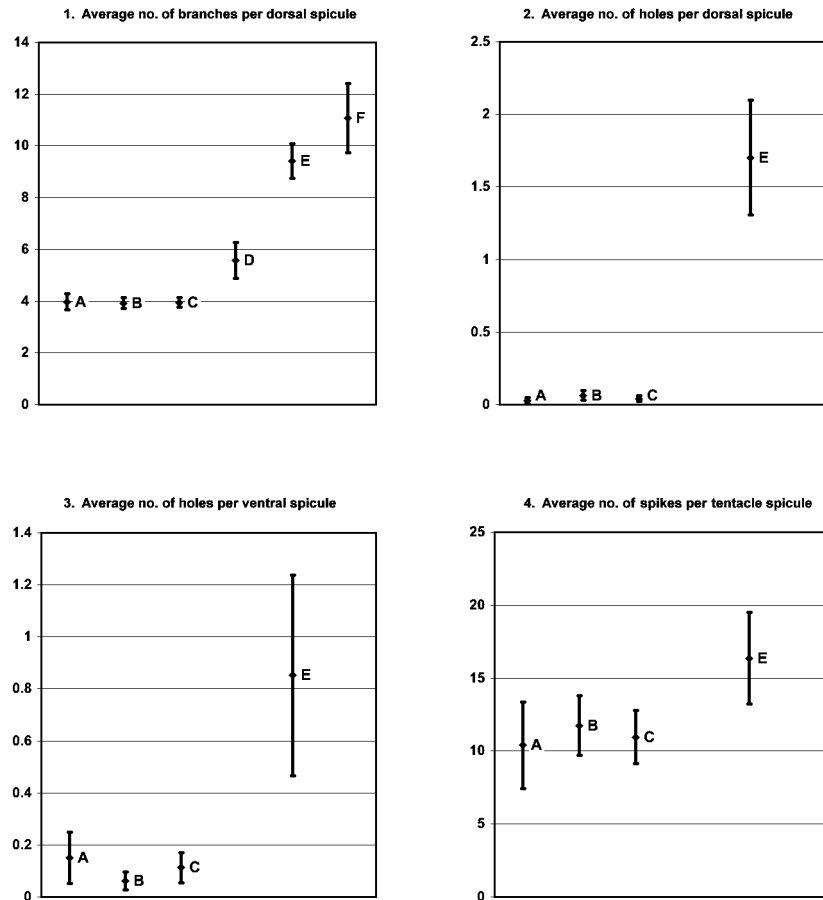


Fig. 1. Averages and 95% confidence intervals for (1) the average number of branches on dorsal body wall spicules, (2) holes in dorsal body wall spicules, (3) holes in ventral body wall spicules, and (4) spikes on tentacle spicules from (A) a mangrove population of *B. marmorata* on Pohnpei ($n = 15$), (B) a grassflats population of *B. marmorata* on Pohnpei ($n = 11$), (C) the combination of mangrove and grassflats (A and B) *B. marmorata* populations ($n = 26$), (D) NMNH *B. marmorata* specimens ($n = 8$), (E) *B. bivittata* specimens from Pohnpei ($n = 13$), and (F) NHMH specimens of *B. bivittata* ($n = 2$).

bohadtotalmodels.bremer.10000.out 2> bohadtotalmodels.bremer.10000.err

Results

Phase I: habitat preference and size difference results

On Pohnpei, specimens corresponding to the original description of *B. marmorata* appear to prefer habitats characterized by strong currents during tide changes and complete exposure at low tide. In the mangroves, the species occurs in shallow channel intersections on sandy substrates with patches of *Halimeda* algae; in the grassflats, it occurs only in areas exposed at low tide and thus without coral. *Bohadschia bivittata* types on Pohnpei are found exclusively in deeper water beyond grassflats characterized by open sand interspersed with coral heads, a habitat similar to that of *B. argus*, although usually not as deep nor with as much coral. On

Yap, *B. bivittata* occurs in a habitat of open sand with sparse *Halimeda* algae and dead coral rubble.

On Pohnpei, samples of mangrove-dwelling *B. marmorata* individuals were much larger than those in the grassflats (378.8 ± 105.5 g (mean \pm one std. dev.), $n = 45$, versus 101.8 ± 46.2 g, $n = 51$; t -test; $P < 0.001$). Of the 10 individuals transferred from the grassflats to the mangroves, the 5 that were recovered after 130 days had increased from an average weight of 153.0 ± 29.1 g to 238.0 ± 67.5 g, a figure significantly higher than their initial weight ($n = 5$; paired t -test; $P < 0.05$) and that of the original grassflats population (t -test; $P < 0.001$), but still lower than the original mangrove population (t -test; $P < 0.01$).

Phase II: spicule variation results

Grassflats- and mangrove-dwelling *B. marmorata* specimens were not significantly different for each spicule comparison ($P > 0.10$), and thus their spicule

data are combined for comparisons with *B. bivittata*. For dorsal body wall spicules, *B. bivittata* specimens had more than twice the number of branches per spicule (9.42 ± 1.37 , $n = 13$, versus 3.95 ± 0.50 , $n = 26$; t -test; $P < 0.001$) (Fig. 1) and more than 40 times the number of holes per spicule (1.70 ± 0.80 versus 0.04 ± 0.05 ; t -test; $P < 0.001$) than *B. marmorata* specimens. Likewise, *B. bivittata* individuals had more than seven times the number of holes per spicule from their ventral body wall (0.85 ± 0.79 versus 0.11 ± 0.16 ; t -test; $P < 0.01$) and more spikes on spicules from their tentacles (16.36 ± 6.42 versus 10.96 ± 4.93 ; t -test; $P < 0.005$) than *B. marmorata* specimens. Since only two *B. bivittata* specimens had live weights measured (those collected in 1995), spicule-weight regressions were done with *B. marmorata* specimens only; these showed weak correlations at best, none of which were significant (live weight versus branches of dorsal spicules, $R^2 = 0.0006$ and $P = 0.9$; versus holes of dorsal spicules, $R^2 = 0.1322$ and $P = 0.07$; versus holes ventral spicules, $R^2 = 0.0724$ and $P = 0.18$; and versus spikes of tentacle spicules, $R^2 = 0.0756$ and $P = 0.17$).

In the NMNH collection (Table 6), two specimens had coloration matching the original description of *B. bivittata*; they were identified as “*B. cousteau*” and “*B. bivittata*” and were collected from the Marshall Islands and the Philippines, respectively. Eight specimens had coloration resembling the original description and drawing of *B. marmorata*; they were variously identified as *B. marmorata*, *B. tenuissima*, and *B. bivittata*, and they were collected from Thailand, the Philippines, Hawaii, and the Marshall Islands. Of the remaining specimens, two (E23997 from Saudi Arabia and E21582 from Kenya) had long dorsal body wall spicules with side branches not previously described for *Bohadschia*, and one specimen (E17081 from the Marshall Islands) had “prickly” rosettes. Among the NMNH specimens, the average number of branches per dorsal body wall spicule was greater for specimens attributable to *bivittata* than those attributable to *marmorata* (11.07 ± 0.97 versus 5.57 ± 1.00 ; t -test; $P < 0.02$; Fig. 1).

Phase III: phylogenetic analysis results

Sufficient DNA was sequenced to do a phylogenetic analysis for the following specimens: three *B. bivittata* and one *B. argus* from Yap, one *Pearsonothuria graeffei* from Chuuk, and three *B. marmorata*, one *B. bivittata*, and one *B. argus* from Pohnpei. The best tree (Fig. 2) across all parameter values outlined in Table 5 supported the hypothesis that *B. bivittata* is a monophyletic group, as the *B. bivittata* from Pohnpei clustered with those from Yap and not the *B. marmorata* from Pohnpei. However, the tree did not support the hypothesis that *B. marmorata* and *B. bivittata* are sister

species: *B. bivittata* appears to be more closely related to *B. argus* than its former conspecific. Bremer and jackknife values both showed strong support for the monophyly of each species and weaker support for the monophyly of *B. argus* and *B. bivittata*.

Additional observations

Some of the eight *B. bivittata* specimens collected from Yap for tissue samples had coloration quite different from *B. bivittata* specimens collected from Pohnpei and from Mitsukuri's (1912) original description. Although all *B. bivittata* specimens, as expected, had the pair of cloudy, transverse bands on a cream background dorsally, four also had brown blotches in a similar position on their ventral surface as well as other dark, well-defined markings, usually around the mouth. One of these Yapese *B. bivittata* specimens with unexpected ventral markings was further distinguished by having both its dorsal bands and ventral blotches sharply defined like the dorsal blotches on *B. marmorata* specimens (Fig. 3). Dorsal and ventral images of specimens used in the phylogenetic analysis may be found by searching the accession numbers in Table 3 at the web site Morphobank.org. Multiple images may be retrieved at once by entering a range of accession numbers separated by a dash; the following url will retrieve all images used in this study: <http://morphobank.org/quick?M25009-M25027>.

Bohadschia bivittata specimens from both Pohnpei and Yap were highly prone to discharging Cuvierian tubules. Thus, five of the nine *B. bivittata* specimens but none of the *B. marmorata* specimens collected on Yap and Pohnpei during Phase III released tubules while being photographed.

Discussion

Our various examinations all support Mitsukuri's original assertion that *B. bivittata* is a distinct species from *B. marmorata*, but admittedly we are only providing clarification between two of the more clearly described and consistent morphs in this complex. Although we did not examine Mitsukuri's types, we do feel that the morph we are resurrecting as *B. bivittata* is the same species as in Mitsukuri's original description and not one of the other six types. Other morphs have been mentioned as having highly branched spicules—*similis* (Semper, 1868; Cherbonnier, 1954), *vitiensis* (Semper, 1868; Clark and Rowe, 1971), *koellikeri* (Semper, 1868), and *tenuissima* (Semper, 1868)—and other morphs have been described as having transverse bands on a light background—*similis* (Cherbonnier, 1954) and *clemens* (Cherbonnier and Feral, 1984)—but

Table 6. National Museum of Natural History, Washington, DC, USA, (NMNH) specimens in the *B. marmorata* species-complex

NMNH ID number	Original ID	Dorsal coloration	Collection locale
E08026	<i>vitiensis</i>	White	Micronesia (Ifaluk Atoll)
E16581	<i>marmorata</i>	Uniform orange brown	Philippines
E16779	<i>marmorata</i>	Like original <i>marmorata</i>	Hawaii
E17081	<i>marmorata</i>	Uniform gray-brown (also with “prickly” rosette-shaped spicules)	Marshall Islands
E17082	<i>marmorata</i>	Like original <i>marmorata</i>	Marshall Islands
E21582 (1 of 4)	<i>marmorata</i>	Uniform black (also with long, branching spicules)	Kenya
E21582 (2 of 4)	<i>marmorata</i>	Uniform black (also with long, branching spicules)	Kenya
E21582 (3 of 4)	<i>marmorata</i>	Uniform black (also with long, branching spicules)	Kenya
E21582 (4 of 4)	<i>marmorata</i>	Uniform black (also with long, branching spicules)	Kenya
E23112	<i>marmorata</i>	Small white spots on chocolate background	Maldives Islands
E23116	<i>marmorata</i>	Uniform dark chocolate brown	Maldives Islands
E23121	<i>marmorata</i>	Small white spots on chocolate background	Sumatra
E23135	<i>marmorata</i>	Close to <i>bivittata</i>	Maldives Islands
E23997	<i>marmorata</i>	Uniform gray (also with long, branching spicules)	Saudi Arabia
E24494	<i>marmorata</i>	Like original <i>marmorata</i>	Philippines
E24531 (1 of 2)	<i>marmorata</i>	Like original <i>marmorata</i>	Philippines
E24531 (2 of 2)	<i>marmorata</i>	Like original <i>marmorata</i>	Philippines
E24595	<i>marmorata</i>	Like original <i>marmorata</i>	Thailand
E24601	<i>marmorata</i>	Small white spots on chocolate background	Maldives Islands
E27337	<i>bivittata</i>	Like original <i>bivittata</i>	Philippines
E28261	<i>marmorata</i>	Small white spots on chocolate background	Andaman Sea
E34340 (1 of 2)	<i>tenuissima</i>	Close to <i>bivittata</i>	Thailand
E34340 (2 of 2)	<i>tenuissima</i>	Like original <i>marmorata</i>	Thailand
E34342 (1 of 2)	<i>tenuissima</i>	Uniform gray	Egypt
E34342 (2 of 2)	<i>tenuissima</i>	Uniform gray	Egypt
E34343	<i>marmorata</i>	Three white blotches on chocolate background (one around the anus and two anteriorly)	Papua New Guinea
E35336	<i>marmorata</i>	White	Micronesia (Bikini Atoll, Marshall Islands)
E47070 (1 of 2)	<i>marmorata</i>	Whitish-beige	Somalia
E47070 (2 of 2)	<i>marmorata</i>	Whitish-beige	Somalia
E47072	<i>cousteaui</i>	Like original <i>bivittata</i>	Micronesia (Bikini Atoll, Marshall Islands)
E8023	<i>bivittata</i>	White	Micronesia (Ifaluk Atoll)
E9153	<i>bivittata</i>	Uniform light brown	Micronesia (Saipan, Marianas Islands)
No number	<i>bivittata</i>	Like original <i>marmorata</i>	Philippines

only Mitsukuri’s description of *bivittata* (1912) has both characters in the original description. In addition, our understanding of Mitsukuri’s color description is supported by Cherbonnier and Feral’s (1984) description of *B. bivittata* specimens and personal communication from F.W.E. Rowe.

The unexpected result from the phylogenetic analysis that *B. bivittata* is more closely related to *B. argus* than to *B. marmorata* fits well with the findings in other marine invertebrate species-complexes that such complexes can hide novel taxa and unforeseen relationships (as mentioned in the *Introduction*). In addition, when

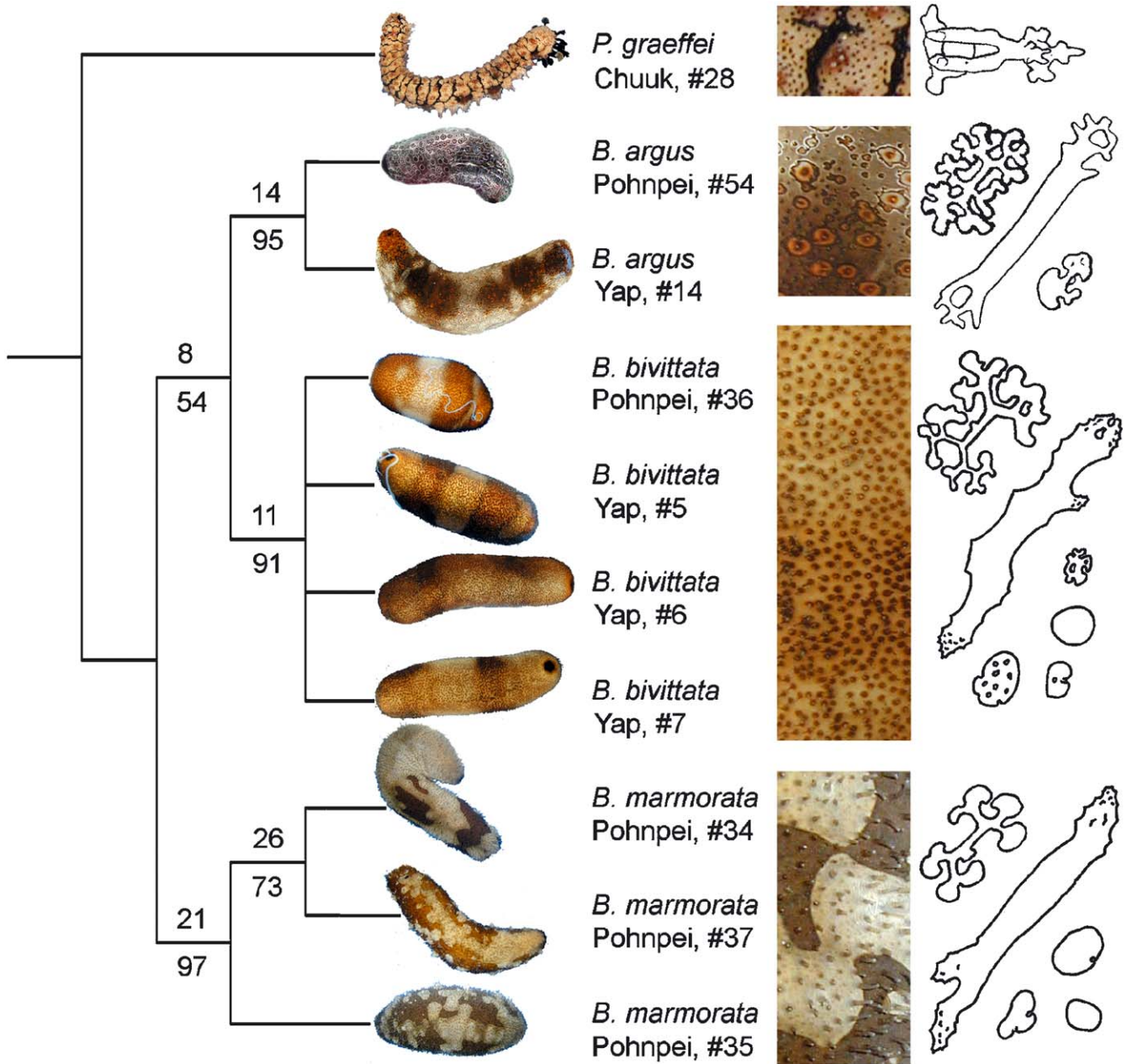


Fig. 2. The best tree across all parameters for ten specimens from which was sequenced 16s, 12s, and COI mitochondrial DNA. Specimens clustered into four clades (*Pearsonothuria graeffei*, *B. argus*, *B. bivittata*, and *B. marmorata*) regardless of island of origin (Chuuk, Yap, or Pohnpei). Bremer support indices are above each node, and jackknife percentages (based upon 10,000 pseudoreplicates) are below. To the right of each species is a sample of their skin coloration and spicules. *Pearsonothuria graeffei* is characterized by its distinct “rocket-shaped” spicule, which, along with the spicules of *B. argus*, are reproduced from Semper (1868). The *B. marmorata* and *B. bivittata* spicules are drawn from Pohnpeian specimens collected during Phase II; all spicules are shown for shape only and not drawn to relative scale.

one considers behavioral, ecological and morphological characters, a close relationship between *B. bivittata* and *B. argus* is not so odd: both are notoriously prone to releasing Cuvieran tubules, unlike *B. marmorata*, which rarely discharges tubules when handled extensively; the former two live in deeper habitats among coral, while *B. marmorata* lives in shallow areas exposed at low tide;

and although all three have rosette-shaped spicules, those of *B. argus* and *B. bivittata* are extremely branched.

Spicules of *Bohadschia* species are very similar in form and hence appear fairly useless in resolving relationships within the *B. marmorata* species-complex. However, when considered in greater detail, they can, to some

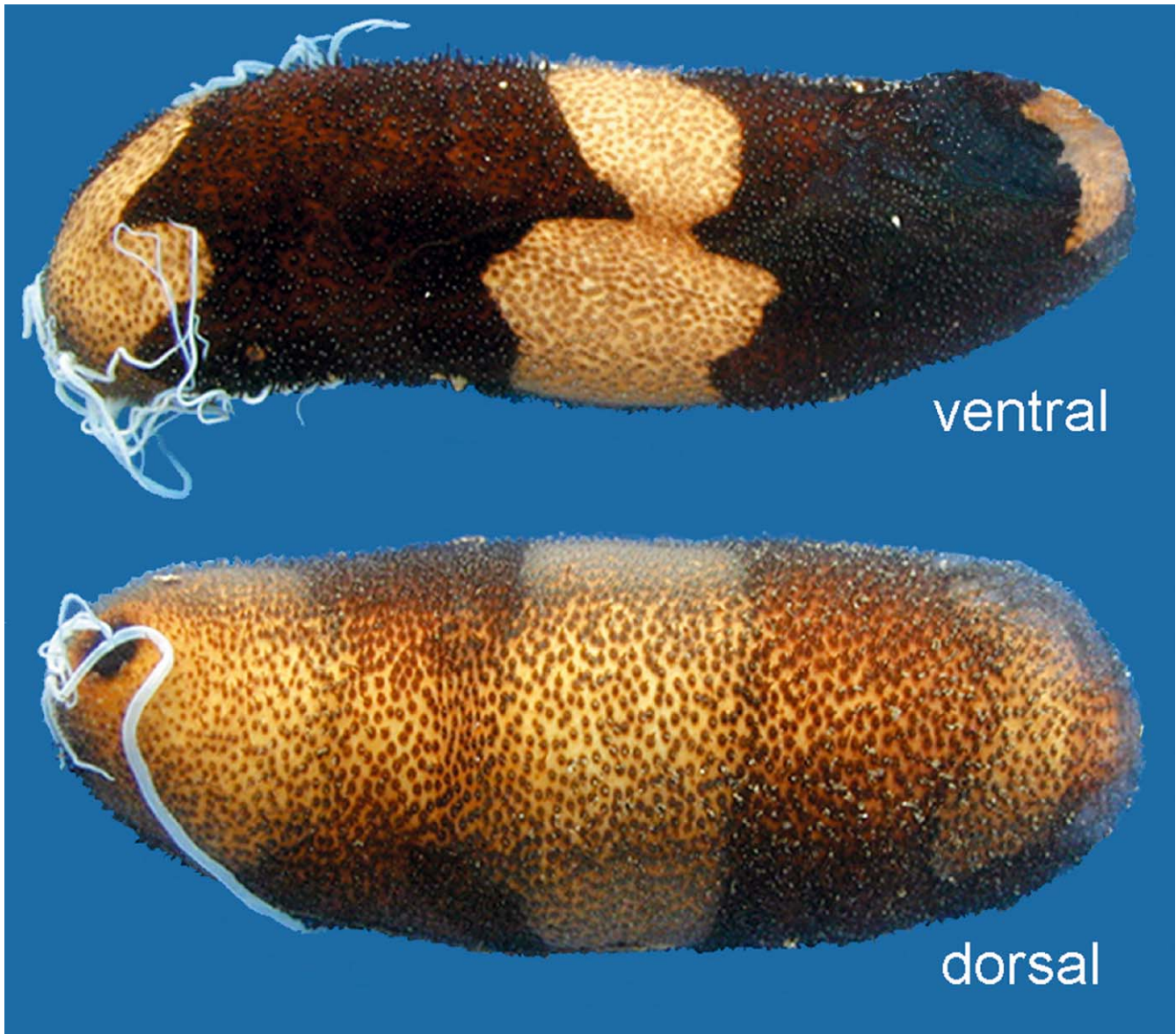


Fig. 3. A strange *B. bivittata* specimen from Yap. Although clustering with other *bivittata* specimens (see Fig. 2), this specimen and others from Yap differed from the original description (Mitsukuri, 1912) in having sharply demarcated, dorsal, transverse bands that continue to the ventral side (Mitsukuri described the venter as being lighter than the dorsum, usually a uniform white or brown).

extent, be used to separate species. In addition, there are still other basic spicule observations that need to be documented in this complex: e.g., Clouse (1997a) has already reported that *B. bivittata* specimens studied to date lack end-plates in their tube feet (unlike *B. marmorata*), and the *B. marmorata*-complex specimens in the NMNH collection from East Africa/Arabia included a species distinguished by a uniform dark shade and long spicules with side branches. Although juvenile and adult sea cucumber spicules may differ (Cutress, 1996), and there is evidence that *Bohadschia* spicule variations do correlate with body size (Massin

et al., 1999), our failure to find a correlation between spicule complexity and body size does not support such observations for this complex, especially with the extreme body size difference between grassflats and mangrove populations of *B. marmorata* on Pohnpei.

One potential problem with doing statistical comparisons of spicule characters is seen in the data from NMNH specimens (Fig. 1): branches of dorsal spicules in *B. marmorata* specimens we collected were significantly different from those of *B. marmorata*-like specimens in the NHMH collection. Although it is conceivable that Pohnpeian specimens have especially

weakly branched spicules, NMNH specimens are from both East and West of Pohnpei (Southeast Asia, the Philippines, and the Marshall Islands). The difference between NMNH *B. marmorata* and *B. bivittata* spicules is large enough that this anomaly is of no consequence here, but it does hint at methodological problems in counting spicule branches and a need for standardizing and reducing human error in the application of these techniques.

The taxonomy of *Bohadschia marmorata*—especially from the Indian and Southwestern Pacific Oceans—still remains quite unresolved, and we cannot say where other *B. marmorata* types lie in relation to our re-established *marmorata-bivittata* split. Indeed, there remains the possibility that some morphs described prior to *bivittata* (and thus, whose names have priority)—*similis*, *koellikeri*, *tenuissima*, *vitiensis* (Semper, 1868) and *clemens* (Ludwig, 1875)—will prove to be both distinct from *marmorata* and conspecific to *bivittata*, and this resurrected species will be buried yet again. In addition, we suspect that two species, *ualanensis* (Brandt, 1835) and *utrimquestigmosa* (Haacke, 1880), also belong to the *B. marmorata* species-complex. However, their color descriptions are vague and unaccompanied by drawings, and they have never been considered in subsequent discussions of the complex. *Bohadschia ualanensis* (originally in the genus *Sporadipus*) (Brandt, 1835) is described as “wholly dark, or dark mixed with yellow,” and *B. utrimquestigmosa* supposedly has a dorsal coloration of “bright coffee-brown” and a “white” ventral surface (Haacke, 1880). Selenka (1867) mentions “*ualensis*” (with no description and only a reference to Brandt), which seems to be a misspelling, since this species was named for “Ualan” Island (today called “Kosrae,” one of the main islands of the Federated States of Micronesia). Haacke (1880) at least also mentions the spicules in his description of *utrimquestigmosa*, but only says that they resemble those of *B. argus*.

The application of molecular phylogeny is a much-needed approach in the study of the systematics of the *B. marmorata* species-complex. Color and spicule morphology have generally been more a source of confusion rather than tools for answering taxonomic questions in this complex, and the degree to which their variation indicates species breaks or can be used to identify hybrids is not known (except now, and only to some degree, i.e., for *B. bivittata* and *B. marmorata* in Micronesia). We recommend that color and spicules still be recorded and described for specimens from this species-complex, for we have shown here that, when considered in greater detail than what is usually required in sea cucumber taxonomy, they can indicate monophyletic groups. However, now that sequence data and molecular tests for hybridization are increasingly accessible, it may be more expedient to bypass color

and spicule morphology in *B. marmorata* systematics. In fact, we envision mapping classic color and spicule characters onto molecular phylogenies to understand why their usefulness breaks down among these morphs.

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