The Linnean Society of London ZOOLOGICAL Journal of the Linnean Society

Zoological Journal of the Linnean Society, 2013, **168**, 81–97. With 5 figures

Colour, confusion, and crossing: resolution of species problems in *Bohadschia* (Echinodermata: Holothuroidea)

SUN W. KIM[1]*, ALEXANDER M. KERR[1] and GUSTAV PAULAY[2]

[1]*Marine Laboratory, University of Guam, Mangilao, GU 96923, USA*
[2]*Florida Museum of Natural History, University of Florida, Gainesville, FL 32611, USA*

Received 24 September 2012; revised 17 December 2012; accepted for publication 29 January 2013

Sea cucumbers of the genus *Bohadschia* (Holothuroidea: Echinodermata) are among the larger and more common echinoderms on tropical coral reefs. While the genus is easy to identify and has been recognized for some time, the number and status of species have varied substantially for over a century. The species problem in *Bohadschia* is the result of high intra-specific variability and little inter-specific divergence in the principal, traditional taxonomic characters, the shape and size of microscopic dermal ossicles. We re-evaluate *Bohadschia* primarily based on colour pattern of living animals and mitochondrial sequence data. The character sets are congruent, and both cleanly delineate 11 species and one common hybrid form. The vexing *Bohadschia marmorata* complex is resolved into four species, all of which have available names, although two have not been recognized correctly since their description. A fairly common colour form intermediate between *B. argus* and *B. vitiensis* has mtDNA sequences matching either one or the other species and is interpreted as a hybrid form. Several species occur sympatrically at most locations; however, the most closely related species show evidence of allopatric divergence. Character state reconstructions suggest that the prevalent diurnal burrowing and nocturnal epibenthic feeding behaviour among *Bohadschia* evolved early in the history of the genus. Ossicle shape and size were found to perform poorly in distinguishing species, while colour pattern is diagnostic.

doi: 10.1111/zoj.12026

ADDITIONAL KEYWORDS: aspidochirotida – hybridization – phylogenetics – systematics.

INTRODUCTION

Genetic data are revolutionizing our understanding of species limits, particularly in groups that are morphologically simple and in organisms that do not rely on visual cues for species recognition (Knowlton, 1993). Sea cucumbers are a case in point. Holothuroid anatomy is simple, and was it not for their possession of intricate, endoskeletal ossicles, their taxonomy would be quite challenging. However holothuroid ossicles are often complex, several types usually occur in one animal, and they can be distributed in different organs; they can thus provide diverse characters for species recognition. Ossicles have served as the backbone of holothuroid alpha taxonomy. Yet while ossicles can be very useful and diagnostic at the species level in some clades, they show little inter-specific differentiation or considerable ontogenetic, population-level, or geographical variation in others (Massin, 1994; Wiedemeyer, 1994; Cutress, 1996). It is not surprising that much of holothuroid taxonomy is being reassessed with genetic tools. A recent study by Borrero-Pérez *et al*. (2009) is an excellent example that demonstrates the power of an integrative approach with molecular phylogeny and morphology to resolve taxonomic problems in holothuroids.

*Corresponding author. Current address: Korea Institute of Ocean Science and Technology, 787 Haean-ro, Sangnok-gu, Ansan, Gyeonggi-do 426-744, Korea. E-mail: ksunwk@kiost.ac

One of the greatest taxonomic challenges among sea cucumbers is the holothuriid genus *Bohadschia* Jaeger, 1833. The genus itself is easy to recognize: they are some of the largest, most common, and conspicuous sea cucumbers on coral reefs, with large, loaf-like bodies, often striking colour patterns, and a propensity for discharging copious quantities of Cuvierian tubules under the slightest provocation. They are most similar to the related genus *Actinopyga*, another taxonomically challenging group with loaf-like bodies and similarly simple ossicles, which are readily separated by their anal teeth and lack of Cuvierian tubule discharge. Some species of *Bohadschia* live exposed on reefs; others bury in sediment part of the time. *Bohadschia* only have rosettes and perforated grains in the body wall, two ossicle 'types' that are simply differentiated end members of a continuum of variation, and are of a simple form that show substantial variation within and limited differentiation between species. Anatomical diversity is also insubstantial, but colour pattern is exceedingly variable. This has led to confusion and disagreement about how many species exist and how to differentiate them. Consequently the number of recognized species has waxed and waned substantially over the years.

Jaeger (1833) erected *Bohadschia* for five species, including two that have not been subsequently identified (*B. albiguttata* and *B. lineolata*), and one (*B. ocellata*) that has been referred to *Holothuria* (but see below). The other two have generally been recognized as valid: *B. marmorata*, which was selected as the type species of the genus by Pearson in 1914, and *B. argus*. While the concept of *B. argus* has been relatively stable in the subsequent literature, *B. marmorata* has been the subject of extensive controversy. Three species were described in the following 45 years: *Holothuria subrubra* Quoy & Gaimard, 1833, currently recognized, but with a messy history (see Massin *et al*., 1999); *Sporadipus* (*Colpochirus*) *ualanensis* Brandt, 1835, which was only once re-examined by Ludwig (1881) and was considered a synonym of *B. marmorata*, a decision not questioned since; and *Holothuria paradoxa* Selenka, 1867, a distinctive Hawaiian endemic generally recognized since. Semper (1868) introduced four species (*Holothuria similis*, *H. koellikeri*, *H. tenuissima*, and *H. vitiensis*), all similar to *marmorata*, starting renewed attention in the genus. Ludwig (1875) described *H. clemens*, another member of the *marmorata* complex, based on a 3-cm juvenile from Samoa. Théel (1886) suggested that *marmorata*, *similis*, *koellikeri*, *tenuissima*, *vitiensis*, and *clemens*, and even the distinctive *argus*, were varieties or juveniles of a single, variable species. Meanwhile Haacke (1880) described *Holothuria utrimquestigmosa*, which Ludwig (1883) considered also a synonym of *B. marmorata*, but Cherbonnier (1952) later synonymized with *B. subrubra*. Subsequently, Mitsukuri (1912) described *H. bivittata*, and mentioned Théel's (1886) proposed synonymies, but decided that *marmorata*, *bivittata*, and *argus* could be separated on colour alone. Similarly, Pearson (1903) and Koehler & Vaney (1908) felt that the Semper species should be united within his *tenuissima*. 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 the following year, Pearson (1914) placed *Bohadschia* as a subgenus of the genus *Mülleria*, and recognized five species: *B. marmorata*, *B. argus*, *B. vitiensis*, *B. paradoxa*, and *B. graeffei*, and designated *marmorata* as the genotype. Panning (1929) initially agreed with this step, but he later added *bivittata* to the list (Panning, 1940). However, Panning (1944) subsequently believed the ossicle differences were too subtle and placed *vitiensis* and *bivittata* under *marmorata* as subspecies. Cherbonnier (1954) separated *marmorata* and *tenuissima* and described a new species, *B. cousteaui*. This and the more recently described species, *B. steinitzi* Cherbonnier, 1963, *B. maculisparsa* Cherbonnier & Féral, 1984, and *B. atra* Massin *et al*., 1999 have not figured as prominently in this controversy. Rowe & Doty (1977) maintained Panning's (1944) single-species taxonomy for the *marmorata* complex, and suggested that previously noted ossicle differences were due to ontogenetic variation. Field workers (e.g. Tan Tiu, 1981; Kerr, 1994; Reyes-Leonardo, 1984) have generally followed Rowe and Doty's synonymy, as did Rowe & Gates (1995). However, the latter authors remarked that additional study may overturn the single-species taxonomy. Massin (1999) provisionally maintained *vitiensis* as distinct from *marmorata*. Most recently, Samyn (2003) has suggested that at least *vitiensis* and *similis* must be regarded as valid species. Several other recent researchers have continued to treat some or all of these morphs, sometimes provisionally, as separate species (Cherbonnier & Féral, 1984; Massin, 1999; Samyn, 2000; Lane, 2004). Initial phylogenetic work (Clouse, Janies & Kerr, 2005) showed that two sympatric forms, recognized as *marmorata* and *bivitatta*, are genetically distinct and spawn at different times. Uthicke, Byrne & Conand (2010) found that the specimens of *Bohadschia* they sequenced fell into four clades, *B. argus* and three species in the *B. marmorata* complex.

These disputes concerning *Bohadschia* centre largely on whether the few traditionally used diagnostic characters relate to species identity or are intra-specifically variable – the result of ontogenetic variation, geographical trends, or stable polymorphisms. Several of the 'forms' of *marmorata* were

described based on differences in colour pattern, without distinguishing ossicle characters. For example, *vitiensis* is uniformly light brown, while *bivittata* is also light, but with dorsolateral transverse dark brown bands. However colour pattern is quite variable, as demonstrated by Rowe & Doty (1977: fig. 6g, h). Unfortunately, most descriptions of body colour, as well as ossicle variation, recorded in the 19th century literature are inadequate to ensure confident diagnosis. As authors frequently did not have access to or consult type specimens, several erroneous species interpretations entered, and were perpetuated, in the literature.

The objective of our study was to evaluate diversity and species limits in *Bohadschia* based on extensive sampling. At the beginning of the project we were able to distinguish 12 forms consistently based on field characters, such as habitus, colour, morphology of papillae, and burrowing behaviour. In addition, we were aware of several colour morphs that did not comfortably fit into these. Sequencing using two mtDNA markers allowed us to evaluate these forms, while examination of a number of available types have led to provisional assignment of names to all but one of them. Here we untangle species limits in the genus based on genetic and morphological evidence. A full taxonomic review of *Bohadschia* is in preparation.

MATERIAL AND METHODS

Collections and observations

Large numbers of *Bohadschia* specimens were observed and recorded across numerous localities in the Indo-west Pacific (IWP) in search of different morphs and to evaluate the range of variation in colour pattern exhibited in populations. Night snorkels and dives allowed finding species that emerge from the sediment at night. Colour patterns and general field appearance were captured by *in situ* or lab photography and representative samples were collected for anatomical and genetic study (Table 1). Specimens are deposited in the Florida Museum of Natural History, University of Florida, Gainesville, USA (UF Echinodermata), the Royal Museum of Central Africa, Tervuren, Belgium (MRAC), Queensland Museum, Brisbane, Australia (QM), and Geoscience Centre of the University of Göttingen, Germany (GZG, Table 1).

Laboratory methods

Genomic DNA was isolated following the DNAzol extraction protocol (Chomszynski *et al*., 1997; Meyer, 2003; Molecular Research Center, Inc., Cincinnati, OH, USA). Fragments of the mitochondrial genes were amplified using PCR. The amplified regions include 656 nucleotides of the cytochrome oxidase subunit I (COI) region with echinoderm-specific primers COIe-F (5′-ATAATGATAGGAGGRTTTGG-3′) and COIe-R (5′-GCTCGTGTRTCTACRTCCAT-3′), and approximately 543 nucleotides of the large ribosomal RNA subunit with the echinoderm-specific primers 16S A-R (5′-CGCCTGTTTATCAAAAACAT-3′) and 16S B-R (5′-GCCGGTCTGAACTCAGATCACGT-3′) (Palumbi *et al*., 1991; Arndt *et al*., 1996). All PCR amplifications were conducted using 1 mM of dNTP mixture (Fisher Scientific, Houston, TX, USA), 2.5 mM $MgCl_2$ (Sigma Aldrich, St Louis, MO, USA), 0.4 mM of each primer, 10× PCR buffer, 0.25 unit of Taq, and approximately 50–100 ng DNA in 25-μL reactions. PCR reactions were conducted with the initial denaturation at 95 °C for 3 min, and 40 cycles of denaturation at 94 °C for 40 s, annealing at 50 °C for 40 s, extension at 72 °C for 1 min, and the last extension for 3 min. After the PCR reaction, PCR products were cleaned using QIAquick PCR purification kit according to the manufacturer's protocol (Qiagen, Valencia, CA, USA). Most amplified products were sequenced at the Integrated Center of Biomedical Research Facility at University of Florida (http://www.biotech.ufl.edu) following the facility's protocol. COI from a few specimens were sequenced as part of the Marine Barcode of Life Project (MarBOL), with extractions through sequencing at the Smithsonian's LAB (Laboratories for Analytical Biology) under the oversight of Chris Meyer.

Post-sequencing procedures and phylogenetic analyses

Forward and reverse strands were assembled and edited with Sequencher v.4 (GeneCodes, Inc., Ann Arbor, MI, USA). The assembled sequences were aligned using MEGA v.4.1 (Tamura *et al*., 2007) with gap-opening and gap-extension penalties of 15, and ClustalW v.1.6 DNA weight matrix (Thompson, Higgins & Gibson, 1994). The aligned sequence data were then tested for the most appropriate nucleotide substitution model using MrModelTest v.2.3 (Nylander, 2004). According to Akaike's information criterion (AIC; Akaike, 1974) implemented in MrModelTest, the General Time Reversible (Tavaré, 1986) model with gamma distribution across sites and a proportion of invariable sites was the most suitable model for both COI and 16S sequence data.

Phylogenetic trees were estimated using PAUP*4b10 (Swofford, 2003), Garli v0.951 (Zwickl, 2006), and MrBayes v.3.2.1 (Ronquist & Huelsenbeck, 2003) for maximum-parsimony (MP), maximum-likelihood (ML), and Bayesian inference (BI) analyses, respectively. MP analyses were conducted with all characters weighted equally. Heuristic searches

Table 1. List of examined *Bohadschia* specimens in this study

Species	Sample locality	Voucher	Taxa ID	GenBank accession numbers	
				COI	16S
B. argus	Guam	UF7074	S469	JN543443	JN543427
B. argus	Kosrae	UF6949	S423	JN543440	
B. argus	Kosrae	UF6966	S453	JN543441	JN543426
B. argus	Kosrae	UF6980	S457	JN543442	
B. argus	Line Island	UF5752	FLMNH109G12	JX683892	
B. argus	Lizard Island		EU848263	(EU848263)	
B. argus	Lizard Island		EU848277	(EU848277)	
B. argus	Majuro	UF7086	S368	JN543438	
B. argus	Majuro	UF7087	S393	JN543439	JN543421
B. argus	Moorea	UF5299	FLMNH110G05	JX683880	
B. argus	New Caledonia		EU848251	(EU848251)	
B. argus	New Caledonia		EU848268	(EU848268)	
B. argus	Philippines	UF6901	N314	JN543434	JN543411
B. argus	Pohnpei		AY574878	(AY574878)	
B. argus	Vanuatu	UF5553	S054	JN543436	JN543417
B. argus	Vanuatu	UF5563	S055	JN543437	JN543418
B. argus	Vanuatu	UF5565	S049	JN543435	
B. atra	Kenya	MRAC1989	C073	JN543447	
B. atra	Mayotte	No voucher	FLMNH001F03	JX683878	
B. atra	Madagascar	UF7258	A093	JN543445	JN543402
B. atra	Madagascar	UF7372	A094	JN543446	JN543403
B. atra	Madagascar	UF7574	A087	JN543444	JN543398
B. atra	Mayotte	UF7012	FLMNH001F04	JX683887	
B. cousteaui	Madagascar	UF7328	A091	JN543450	JN543401
B. cousteaui	Tanzania	MRAC1871	C070	JN543451	
B. hybrid	Guam	UF4696	G098	JN543452	
B. hybrid	Guam	UF4747	G170	JN543453	
B. hybrid	Guam	UF7142	N448	JX838802	
B. hybrid	Guam	UF7144	N447	JX838803	
B. hybrid	Guam	UF7145	S472	JN543488	JN543428
B. hybrid	Kosrae	UF6807	S452		JN543425
B. hybrid	Kosrae	UF6956	S420	JN543487	
B. hybrid	Lizard Island		EU848296	(EU848296)	
B. hybrid	Majuro	UF7140	S388	JN543486	JN543419
B. hybrid	Okinawa	UF3956	A083		JN543397
B. hybrid	Palau		EU848256	(EU848256)	
B. koellikeri	Guam	UF4705	G099	JN543470	
B. koellikeri	Guam	UF4744	G171	JN543471	
B. koellikeri	Kiribati	UF5768	A102	JN543468	JN543405
B. koellikeri	Lizard Island	UF8305	FLMNH036F09	JX683894	
B. koellikeri	Moorea	UF5019	A103	JN543469	
B. koellikeri	Moorea	UF5273	FLMNH110F06	JX683884	
B. koellikeri	Moorea	UF5274	FLMNH110G06	JX683879	
B. koellikeri	Moorea	UF5338	FLMNH110B08	JX683893	
B. marmorata	Guam	UF4748a	G093	JN543454	
B. marmorata	Kosrae	UF6921	S430	JN543464	JN543423
B. marmorata	New Caledonia	UF0956	G145	JN543455	
B. marmorata	New Caledonia		EU848249	(EU848249)	
B. marmorata	Philippines	UF5578	N301	JN543456	
B. marmorata	Philippines	UF5724	N348	JN543457	JN543413
B. marmorata	Pohnpei		AY574881	(AY574881)	
B. marmorata	Pohnpei		AY574882	(AY574882)	
B. marmorata	Pohnpei		AY574883	(AY574883)	
B. marmorata	Vanuatu	UF5558	S042	JN543458	
B. marmorata	Vanuatu	UF5559	S053	JN543463	JN543416
B. marmorata	Vanuatu	UF5561	S044	JN543459	
B. marmorata	Vanuatu	UF5562	S047	JN543462	
B. marmorata	Vanuatu	UF5572	S046	JN543461	
B. marmorata	Vanuatu	UF5575	S045	JN543460	
B. ocellata	Australia	QM SBD507784	FLMNH057A08	JX683881	
B. ocellata	Guam	UF7143	S471	JN543483	JN543404

Table 1. *Continued*

Species	Sample locality	Voucher	Taxa ID	GenBank accession numbers	
				COI	16S
B. ocellata	Kiribati	UF5766	A090	JN543472	JN543400
B. ocellata	Kiribati	UF5767	A092	JN543473	
B. ocellata	Kosrae	UF7085	S451	JN543482	
B. ocellata	Lizard Island		EU848270	(EU848270)	
B. ocellata	Lizard Island		EU848271	(EU848271)	
B. ocellata	Majuro	UF7141	S367	JN543478	
B. ocellata	Majuro	UF7135	S369	JN543479	JN543406
B. ocellata	Majuro	UF7137	S370	JN543480	
B. ocellata	Majuro	UF6796	S389	JN543481	JN543420
B. ocellata	Malaysia	UF4467	G196	JN543476	
B. ocellata	Okinawa	UF11157	N747	JX838804	
B. ocellata	Philippines	UF5638	N368	JN543477	JN543414
B. ocellata	PNG		EU848248	(EU848248)	
B. paradoxa	French Frigate Shoals	UF6244	A085	JN543465	
B. paradoxa	French Frigate Shoals	UF6076	A109	JN543466	JN543409
B. paradoxa	French Frigate Shoals	UF6245	A112	JN543467	
B. paradoxa	Hawaii	UF6158	FLMNH023D05	JX683897	
B. paradoxa	Hawaii	UF6242	FLMNH023F04	JX683882	
B. paradoxa	Hawaii	UF6073	FLMNH038E11	JX683896	
B. sp. 1	Hawaii	UF4901	N082	(EU220816)	(EU220795)
B. sp. 1	Australia	QM G212567	FLMMH057C04	JX683886	
B. sp. 1	Okinawa	UF11022	N517	JX838805	
B. sp. 2	Egypt	No voucher	FLMNH084A05	JX683890	
B. subrubra	Madagascar	UF6876	FLMNH043H08	JX683889	
B. subrubra	Mayotte	UF6889	FLMNH001F06	JX683883	
B. subrubra	Mayotte	UF6899	FLMNH001G04	JX683895	
B. subrubra	South Africa	MRAC2001	C077	JN543484	
B. subrubra	Réunion	UF6330	S243	JN543485	
B. vitiensis	Guam	UF4706	G172	JN543496	
B. vitiensis	Kenya	MRAC2032	C074	JN543494	
B. vitiensis	Kenya	MRAC2036	C081	JN543495	
B. vitiensis	Kosrae	UF6913	S429	JN543492	JN543422
B. vitiensis	Kosrae	UF6925	S456	JN543493	JN543407
B. vitiensis	Kosrae	UF6945	S424	JN543448	
B. vitiensis	Kosrae	UF6912	S431	JN543449	JN543424
B. vitiensis	Lizard Island		EU848267	(EU848267)	
B. vitiensis	Madagascar	UF7561	A088	JN543489	JN543399
B. vitiensis	Madagascar	UF7304	A101	JN543490	
B. vitiensis	Mayotte	UF6888	FLMNH001E12	JX683891	
B. vitiensis	Mayotte	UF7022	FLMNH001F02	JX683888	
B. vitiensis	New Caledonia	UF8674	FLMNH080H04	JX683885	
B. vitiensis	Philippines	UF5646	N309	JN543497	JN543410
B. vitiensis	Philippines	UF5639	N330	JN543498	
B. vitiensis	Philippines	UF5640	N347	JN543499	JN543412
B. vitiensis	Philippines	UF5642	N374	JN543500	JN543415
B. vitiensis	Philippines	No Voucher	N622	JX838806	
B. vitiensis	Réunion	No Voucher	N474	JX838807	
B. vitiensis	Réunion	UF6561	S174	JN543501	
B. vitiensis	Réunion	UF6354	S227	JN543502	
B. vitiensis	Réunion		EU848255	(EU848255)	
B. vitiensis	Yap		AY574879	(AY574879)	
B. vitiensis	Yap		AY574880	(AY574880)	
P. graeffei	Gulf of Aqaba	GZG MB385c	G302	JN543503	
P. graeffei	Philippines		EU848285	(EU848285)	

Voucher numbers and taxa IDs match the Florida Museum of Natural History (FLMNH), Royal Museum of Central Africa (MRAC), Queensland Museum (QM) and University of Göttingen (GZG) collections database, and the abbreviation in the phylogenetic trees, respectively. GenBank accession numbers acquired from previous studies are in parentheses.

were used to determine the best trees with random starting trees, 10^4 random-taxon-addition sequences, and TBR branch-swapping setting. For ML analyses, nucleotide substitution models and parameters suggested by AIC were used with a genetic algorithm implemented in Garli (Zwickl, 2006). For BI analyses, four independent Markov chain Monte Carlo (MCMC) runs were conducted with four Markov chains for 10^7 generations and phylogenetic trees were sampled every 100 generations. After 10^7 generations, the standard deviations of split frequencies were 0.0055 for COI and 0.0027 for 16S. Both standard deviations were well below 0.01, meaning that the number of generations run was sufficient (Ronquist, Huelsenbeck & van der Mark, 2005). The first 25 000 trees were disregarded as burn-ins. Robustness of the inferred trees was assessed using 100 bootstrap pseudoreplicates for the MP and ML analyses, while posterior probability was calculated for the BI analysis.

Character analyses

We examined ossicle morphology, internal anatomy and external morphology, and several ecological characters for their potential value as taxonomic characters. The evolution of morphological and ecological characters was analysed using maximum-parsimony mapping with a delayed transformation on the molecular phylogeny. Ossicles were isolated from small portions of mid-dorsal body wall, mid-ventral body wall, tentacles, and internal organs. Differences in the number of bifurcations, length of main branch length and maximum ossicle length of 20 rosette ossicles, and length and width of 20 grain ossicles were measured from the dorsum and ventrum of two preserved adult specimens per species ranging from 15 to 40 cm in length for a total of five characters scored (Fig. 1). The concatenated data set of all ossicle measurements was analysed by discriminant analysis using SPSS v.19.0 software. The classification results were cross-validated using a jack-knife method to produce a more reliable function.

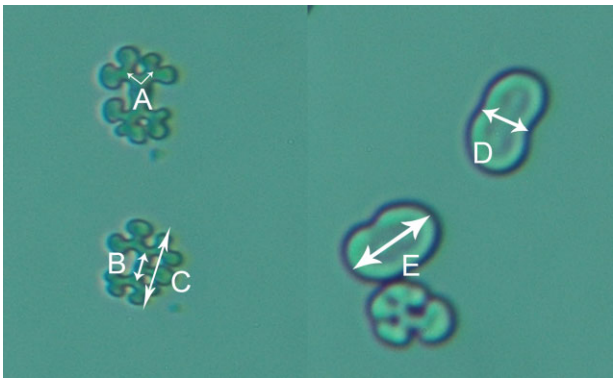

Figure 1. Measured ossicle characters (of *B. marmorata*). A, bifurcation of rosettes; B, rosette main branch length; C, rosette total length; D, grain width; E, grain length.

One to three specimens per species were examined (Appendix 1) for internal anatomy and external morphology. Internal organs, including Polian vesicle, stone canal, and madreporite, as well as external morphological characters, such as colour patterns, were evaluated for each specimen.

Ecological characteristics, including circadian activity pattern, burrowing behaviour and habitats, were investigated in the field.

RESULTS

Colour forms and species based on field observations

We distinguished 12 forms of *Bohadschia* based on field observations, where a 'form' indicates a general patterning and palette of colours. Two of these forms were genetically indistinguishable from other forms and appear to represent (1) a colour polymorph, and (2) a hybrid taxon. An additional genetically distinct species was encountered from a specimen that was available as a tissue sample only; this is listed as a 13th 'form' even though its appearance remains unknown, for a total of 13 major forms, representing 11 species (Figs 2, 3).

1. *Bohadschia argus* (Fig. 3A, B). This is a well-known species subject to little controversy. It has two colour morphs: light grey vs. dark brown background, overlain by a characteristic ocellation. The two forms intergrade and were not genetically distinguishable with the markers used.
2. *Bohadschia atra* (Fig. 3C). This is a recently recognized form, quite close to *B. argus*, but always very dark, blackish brown, with inconspicuous, dark red ocellation. It is an allopatric sister species of *B. argus*, currently known only from the south-western Indian Ocean.
3. *Bohadschia cousteaui* (Fig. 3D). A uniformly dark brown species with relatively elongate papillae known from the Red Sea and western Indian Ocean only. No other species with that colour pattern are known from the western Indian Ocean. The only other solid dark brown species is a form of *B. ocellata*, which can be distinguished by the lighter coloured dots encircling each papilla.
4. *Bohadschia koellikeri* (Fig. 3E, F). The overall colour pattern is light, creamy-tan, with distinctive blotches of light brown that are not strongly demarcated. The gross colour pattern itself is recognizable from a distance once one

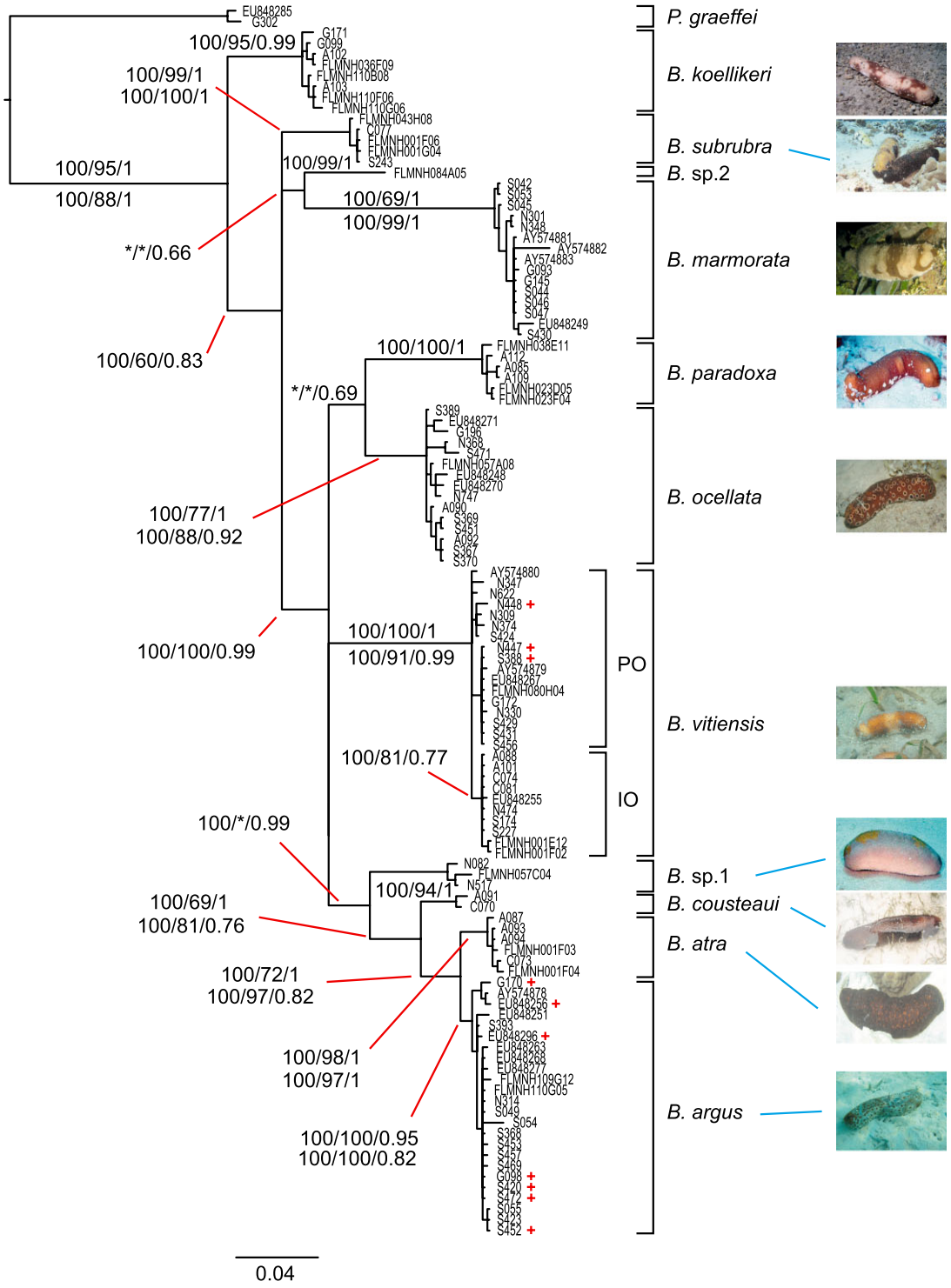

Figure 2. Fifty per cent majority-rule consensus tree from COI Bayesian analysis. Both mtDNA markers and all phylogenetic reconstruction methods produced virtually identical phylogenetic tree topologies with the COI Bayesian tree having the highest resolution. Numbers on branches indicate bootstrap support values and posterior probabilities (PP) from MP/ML/BI analyses, respectively. Values above branches are from COI sequence data and values below are from 16S sequence data. Branches with one set of values indicate data are only from COI sequence analyses. Potential hybrids between *B. argus* and *B. vitiensis* are marked with red crosses. Asterisks represent branch support less than 50% (bootstrap) and 0.5 (PP). *Pearsonothuria graeffei* served as an outgroup. Scale indicates expected substitutions per site.

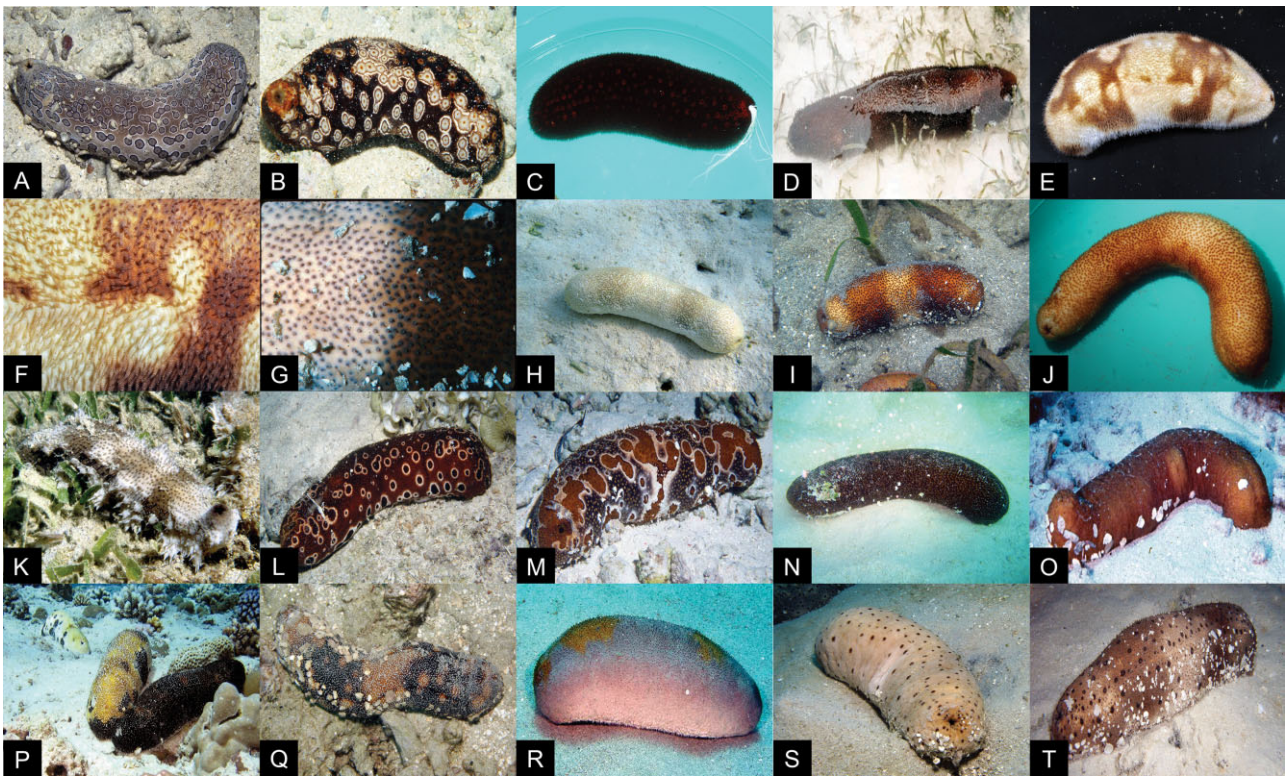

Figure 3. Colour patterns of *Bohadschia* species. A, *B. argus* with grey background and grey ocellar spots. B, *B. argus* with dark brown background and light beige ocellar spots. C, *B. atra* with black background and red ocellar spots. D, *B. cousteaui* with uniform dark brown body. E, *B. koellikeri* with cream body and brown blotches. F, Close-up of *B. koellikeri*. Note numerous fine grooves giving lineated/reticulated pattern. G, Close-up of *B. vitiensis*. Note absence of reticulations. H, *B. vitiensis* with beige background and two light brown transverse bands. I, *B. vitiensis* with brown background and two thick darker brown transverse bands. J, *B. vitiensis* with uniform light brown body. K, *B. marmorata* with beige background and light brown, well-delineated, dorsolateral blotches. L, *B. ocellata* with maroon background and moderate number of blotches. The blotches are encircled by cleanly delineated white rings. M, *B. ocellata* with dark chestnut background and brown blotches. N, *B. ocellata* with uniform brown body, lacking characteristic blotches. O, *B. paradoxa* with uniform brown body. P, *B. subrubra* with variably background coloration and blotch patterns. Q, *B. subrubra* with rusty, light maroon background and dark chestnut blotches. R, *B.* sp. 1 with lavender background and yellow blotches. S, putative *argus–vitiensis* hybrid with *vitiensis*, background colour and ocellar dots similar to *argus*. T, putative *argus–vitiensis* hybrid with *vitiensis* background, including transverse bands, and ocellar spots of *argus*. Photo credits: A, C, F, G, K, O, Q: G. Paulay; B: Lisa Kirkendale; D, P: Yves Samyn; E, J, L, M: François Michonneau; H, I, N: A. Kerr; R: John Hoover; S: David Burdick; T: S. Kim.

is familiar with it, but is difficult to describe. Fine lineation (Fig. 3F) covers the dorsum, which unequivocally identifies this species and clearly distinguishes it from the morphologically similar *B. vitiensis*. This species shares the same habitat with the widely confused *B. vitiensis* at locations where both occur, such as Guam. We have not seen any intermediates or evidence of hybridization between these species. The recently illustrated (http://www.echinodermata.be/index.php?option=com_content&view=article&id=355:holothuria-koellikeri-semper-1868–holotype&catid=39:hamburg-museum&Itemid=57) type specimen of *Holothuria koellikeri* Semper, 1868 (ZMH E.2637) from Samoa clearly matches this form. Our records show this species ranges through the West Pacific, from Australia to Micronesia and eastern Polynesia.

5. *Bohadschia marmorata* (Fig. 3K). Readily distinguished from other species in the complex but consistent colour pattern, smaller size, and mammillate ventrolateral papillae. It has a cream background, with paired, dorsolateral series of crisply delineated, light brown blotches of variable shape and extent. *Bohadschia marmorata* appears to be restricted to the West Pacific, and tends to live more

inshore than *B. vitiensis* or *B. koellikeri*. Like those species, it burrows in the sediment during the day.

6 & 7. *Bohadschia ocellata* (Fig. 3L, M, N). A distinctive form that somewhat resembles *B. argus*, but has large, blotchy spots on the dorsum, and has been photographed and discussed by naturalists for some time. It has a rich brown dorsum, with colour fading out toward the cleanly delineated blotches; the blotches vary in shape and size substantially (Fig. 3L, M). Dark brown animals lacking blotches were genetically indistinguishable and appear to form a second colour morph of this species (Fig. 3N). This type can be distinguished from other brown *Bohadschia* by the lighter dots encircling papillae.

8. *Bohadschia paradoxa* (Fig. 3O). This is a well-known Hawaiian endemic that is uniformly golden to yellow brown, and often slightly wrinkled. This species lives exposed on deeper fore-reef and sand, and has distinctly larger ossicles (Fig. 3O).

9. *Bohadschia subrubra* (Fig. 3P, Q). This striking, colourful species, known only from the south-west Indian Ocean, was redescribed and illustrated by Massin *et al*. (1999) and Samyn, Vanden Spiegel & Massin (2005). It has a variable colour pattern with yellowish, rusty to black-brown blotches. It is found exposed most of the time, but will also burrow in sand.

10. *Bohadschia vitiensis* (Fig. 3G, H, I, J). This is most similar to *B. koellikeri* and *B. marmorata*, but can be distinguished from those species by the characters listed. Like those species it buries itself in sediment diurnally, and prefers quiet, sandy habitats. It ranges in colour from cream to light brown (always lighter in colour than *B. cousteaui* and *B. ocellata*), frequently with two, broad, loosely demarcated transverse bands across the dorsum. The podia tend to lie in a small, darker dot.

11. *Bohadschia* '*maculisparsa*' = *B. argus-vitiensis* hybrids (Fig. 3S, T). The putative hybrid between *B. argus* and *B. vitiensis* possessed a colour pattern mix of both parent species; the spotting is *B. argus*-like but less demarcated, while the frequent occurrence of ill-defined, broad, transverse banding and the dark dotting around podia matches *B. vitiensis*. *Bohadschia maculisparsa* described from New Caledonia may represent this hybrid form. Our attempts to secure fresh specimens from the type locality of this putative species have yet been successful.

12. *Bohadschia* sp. 1 (Fig. 3R). This is a distinctive, rare *Bohadschia* known to us from Bali, Okinawa, Philippines, and Hawaii, with grey and off-yellow blotches over a grey or lavender to cream background. It is genetically distinct and does not match types of any of the described species we have been able to check.

13. *Bohadschia* sp. 2. A unique sequence indicated as *Bohadschia* sp. 2 (Fig. 2), obtained from a tissue sample (without voucher or photograph) from the Red Sea may represent *Bohadschia steinitzi*, a species only known from the holotype taken in the northern Red Sea. We have not yet secured a specimen of *B. steinitzi* to compare it against.

Phylogenetic analyses

In a trimmed alignment of 656 characters, the COI gene fragment had 424 invariant sites (65%), 42 parsimony-uninformative variable sites (6%), and 190 parsimony-informative sites (29%). In a trimmed alignment of 543 characters, the 16S gene had 341 invariant sites (63%), 39 parsimony-uninformative variable sites (7%), and 163 parsimony-informative sites (30%). The two markers produced essentially identical phylogenetic tree topologies, with the COI tree having a higher resolution. The *Bohadschia* species formed a monophyletic group with strong support (Fig. 2). The 13 forms distinguished above fell into 11 clusters, each representing a putative species, recovered with strong branch support regardless of the phylogenetic reconstruction method or marker used (Fig. 2). *Bohadschia koellikeri* was sister to a group consisting of all other congeners in the COI gene analyses. *Bohadschia atra*, *B. argus*, and *B. cousteaui* formed a clade in both COI and 16S analyses regardless of phylogenetic estimation method. Specimens referable to *B. maculisparsa* fell within the clades otherwise consisting only of *B. argus* or of *B. vitiensis*. The various colour morphs of *B. vitiensis* (e.g. as represented by *B. bivittata* and *B. similis*) fell within a single tight clade.

Phylogenetic analyses show evidence of allopatric divergence within two clades. The morphologically similar *B. argus* and *B. atra* are closely related, apparently allopatric, sister species. The range of *B. argus* is challenging to ascertain as older literature records (e.g. Cherbonnier, 1988) probably misidentified *B. atra* under the same name. Recent fieldwork, by us and others, has encountered only *B. atra* in the south-west Indian Ocean (Massin *et al*., 1999; Samyn, 2003; Samyn *et al*., 2005). *Bohadschia argus* ranges at least from the north-east Indian Ocean to the central Pacific. In addition, *B. vitiensis* included a strongly

supported Indian Ocean clade in an unresolved polytomy of otherwise Pacific Ocean exemplars (Fig. 2).

Sequence divergence

Intra-specific variability ranged from 0.2 to 1% (mean ± SD = 0.6 ± 0.3%) for COI and from 0.2 to 0.5% (mean = 0.3 ± 0.1%) for 16S rRNA (Table 2). Inter-specific divergence among *Bohadschia* species varied from 1.8 to 15.6% (mean = 11.1 ± 2.8%) for COI and from 1.7% to 9.5% (mean = 5.5 ± 2.0%) for 16S rRNA (Table 2). The lowest divergence was between the morphologically similar, allopatric sister species *B. argus* and *B. atra*.

Character analyses

There were significant differences among species in the concatenated data set of ossicle measurements, largely stemming from *B. paradoxa*'s distinct ossicles (Fig. 4). The classification statistics for *Bohadschia* species showed that only *B. paradoxa* specimens were correctly classified with its species (Appendix 2). When *B. paradoxa* was eliminated from the discriminant analysis, only 34.4% of originally grouped specimens and 25.6% of cross-validated specimens were correctly classified (Appendix 3).

There were no differences among species in the presence or absence of ossicles or of ossicle types in internal organs. Longitudinal muscles, cloacal muscles and the intestine lacked ossicles, while respiratory trees and gonads possessed rosettes in all species investigated. Major features of the internal anatomy also did not differ among species. All specimens investigated possessed 20 tentacle ampullae, a single Polian vesicle, a stone canal with a small stubby madreporite embedded in the dorsal mesentery, and the two respiratory trees joined before inserting at the cloaca via a common duct. All specimens displayed evidence of numerous Cuvierian tubules.

Phylogenetic relationships among *Bohadschia* species showed weak correlation with ecological characters (Fig. 5A). A few *Bohadschia* species were nocturnally active and buried in sediment during the day: *B. koellikeri*, *B. marmorata*, and *B. vitiensis*. On the other hand, several *Bohadschia* species were diurnally active and always epibenthic: *B. argus*, *B. atra*, *B. cousteaui*, *B. paradoxa*, and *B.* sp. 1. Other species were observed mostly epibenthic during the day, but also buried: *B. ocellata* and *B. subrubra*. Most *Bohadschia* species inhabited sandy to silty areas of shallow, tropical coral reefs. A Hawaiian endemic, *B. paradoxa*, and a rare Pacific species, *B.* sp. 1, were found on deeper fore reef. *Bohdachia ocellata* and *B. argus* are also frequently found on hard substrata.

Table 2. Intra-specific and inter-specific maximum likelihood distances for *Bohadschia*

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
(1) *B. argus*	**0.002/0.002**	0.017	0.023	0.063	0.077	0.043	0.040	–	0.065	0.041	–
(2) *B. atra*	0.018	**0.004/0.004**	0.029	0.068	0.082	0.050	0.043	–	0.067	0.043	–
(3) *B. cousteaui*	0.045	0.05	**0.008/0.007**	0.054	0.071	0.037	0.037	–	0.063	0.035	–
(4) *B. koellikeri*	0.135	0.145	0.121	**0.004/–**	0.091	0.065	0.066	–	0.080	0.060	–
(5) *B. marmorata*	0.148	0.155	0.128	0.13	**0.005/0.005**	0.085	0.083	–	0.095	0.076	–
(6) *B. ocellata*	0.105	0.105	0.087	0.094	0.128	**0.01/0.002**	0.044	–	0.061	0.034	–
(7) *B. paradoxa*	0.132	0.132	0.114	0.111	0.133	0.082	**0.004/–**	–	0.065	0.037	–
(8) *B. subrubra*	0.112	0.126	0.104	0.091	0.151	0.095	0.112	**0.009/–**	–	–	–
(9) *B. vitiensis*	0.13	0.136	0.114	0.111	0.138	0.123	0.141	0.156	**0.008/0.005**	0.060	–
(10) *B.* sp.1	0.091	0.09	0.063	0.117	0.151	0.078	0.101	0.09	0.123	**0.005/–**	–
(11) *B.* sp.2	0.118	0.123	0.097	0.098	0.143	0.092	0.138	0.094	0.112	0.1	**–/–**

Numbers in bold are intra-specific divergence (COI/16S), above diagonal are 16S distances and below diagonal are COI distances. Dashes indicate distances missing due to lack of samples.

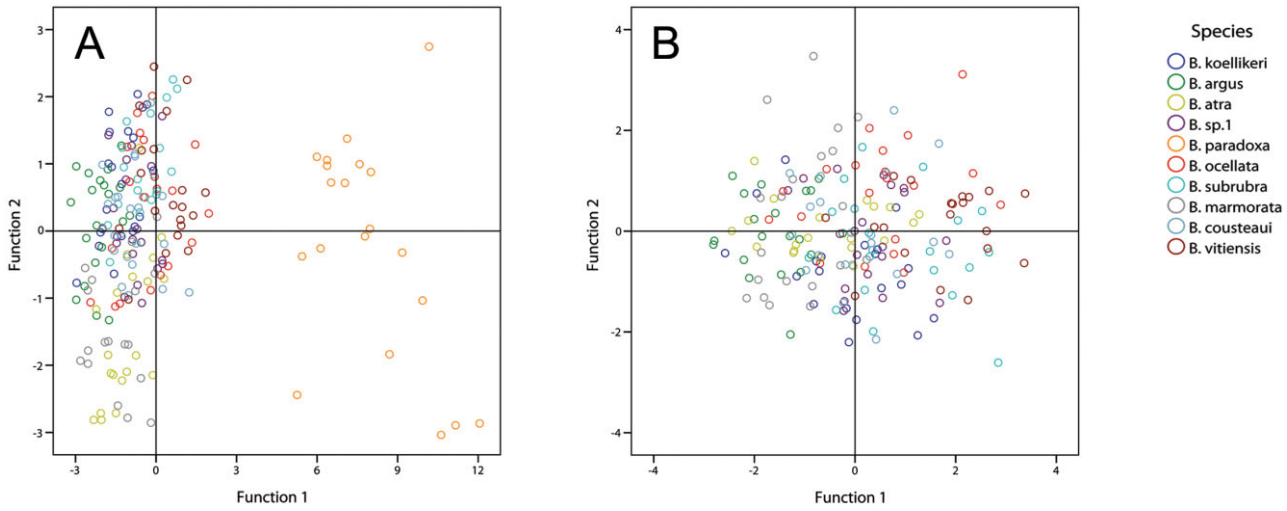

Figure 4. Discriminant analysis result for: A, all *Bohadschia* species; B, excluding *B. paradoxa*. The *x* and *y* axes display discriminant scores from canonical discriminant functions 1 and 2, respectively. Navy: *B. koellikeri*. Green: *B. argus*. Gold: *B. atra*. Purple: *B.* sp.1. Orange: *B. paradoxa*. Red: *B. ocellata*. Teal: *B. subrubra*. Grey: *B. marmorata*. Light blue: *B. cousteaui*. Burgundy: *B. vitiensis*.

DISCUSSION

SYSTEMATICS OF *BOHADSCHIA*

We delimited 11 *Bohadschia* species, including one potentially new taxon and two that have not been correctly recognized since their original description [*B. ocellata* Jaeger, 1833, and *B. koellikeri* (Semper, 1868)]. These 11 species may represent all currently known *Bohadschia*, with the following reservations: (1) the potential identification of the tissue sample from the Red Sea as representing *B. steinitzi* is based solely on biogeographical grounds and needs further evaluation, ideally based on new material; (2) the status of *B. mitsioensis* described from two specimens off north-west Madagascar needs evaluation; and (3) specimens from the type locality of *B. maculisparsa* are needed to verify that this corresponds to the *B. argus-vitiensis* hybrid recognized here. Other nomina within *Bohadschia* appear to fall within these 11 species, although further work is needed to unequivocally demonstrate this. A taxonomic revision of the genus is in preparation.

We confirmed the species status of several widely recognized *Bohadschia*. The species status of *B. argus* has never been challenged despite wide variation in colour, and was strongly supported in our analyses. Recently described *B. atra*, once thought to be a dark form of *B. argus* in the Indian Ocean, formed a well-supported, though recently diverged clade. Samples from the central Indian Ocean need to be examined to establish which of the two forms is/are present there, if they co-occur, and whether they hybridize. Two other Indian Ocean endemics, *B. cousteaui*, with an invariant dark brown colour, and *B. subrubra*, with a striking, characteristic, though variable colour pattern were also confirmed genetically as distinct species. Finally, the uniformly yellow brown Hawaiian endemic *B. paradoxa*, long distinguished by its divergent ossicles, was also genetically confirmed as distinct.

We distinguished four species in the *B. marmorata*-complex, a group with a confusing taxonomic history as noted above. We confirmed the distinction between two forms that have frequently been recognized (e.g. Féral & Cherbonnier, 1986; Clouse *et al*., 2005), although under varied names (see Introduction): *B. marmorata* and *B. vitiensis*. While *B. marmorata* is unmistakable with its small size, mammillate ventrolateral papillae, and strongly delineated, paired dorsolateral blotches, *B. vitiensis* is less distinct and more variable. The latter has a loaf-like, large body as for all *Bohadschia* except *B. marmorata*, and varies in colour from largely cream (*B.* '*similis*') to brown (*B. vitiensis*), with many individuals possessing two diffuse, broad, transverse bands across the dorsum (*B.* '*bivittata*'). The frequent misidentification of *B. marmorata* as *B. similis* in some of the literature stems from Féral & Cherbonnier's (1986) treatment and excellent colour figures; *B. similis* and *B. bivittata* are in fact synonyms of *B. vitiensis*. In contrast, Clouse *et al*.'s (2005) use of *marmorata* follows our concept. We also recognized two additional species that differ in field appearance, and showed these to be genetically distinct as well. One is characterized by a cream-tan body with diffuse brownish blotches that are not organized into well-formed bands, but vary in

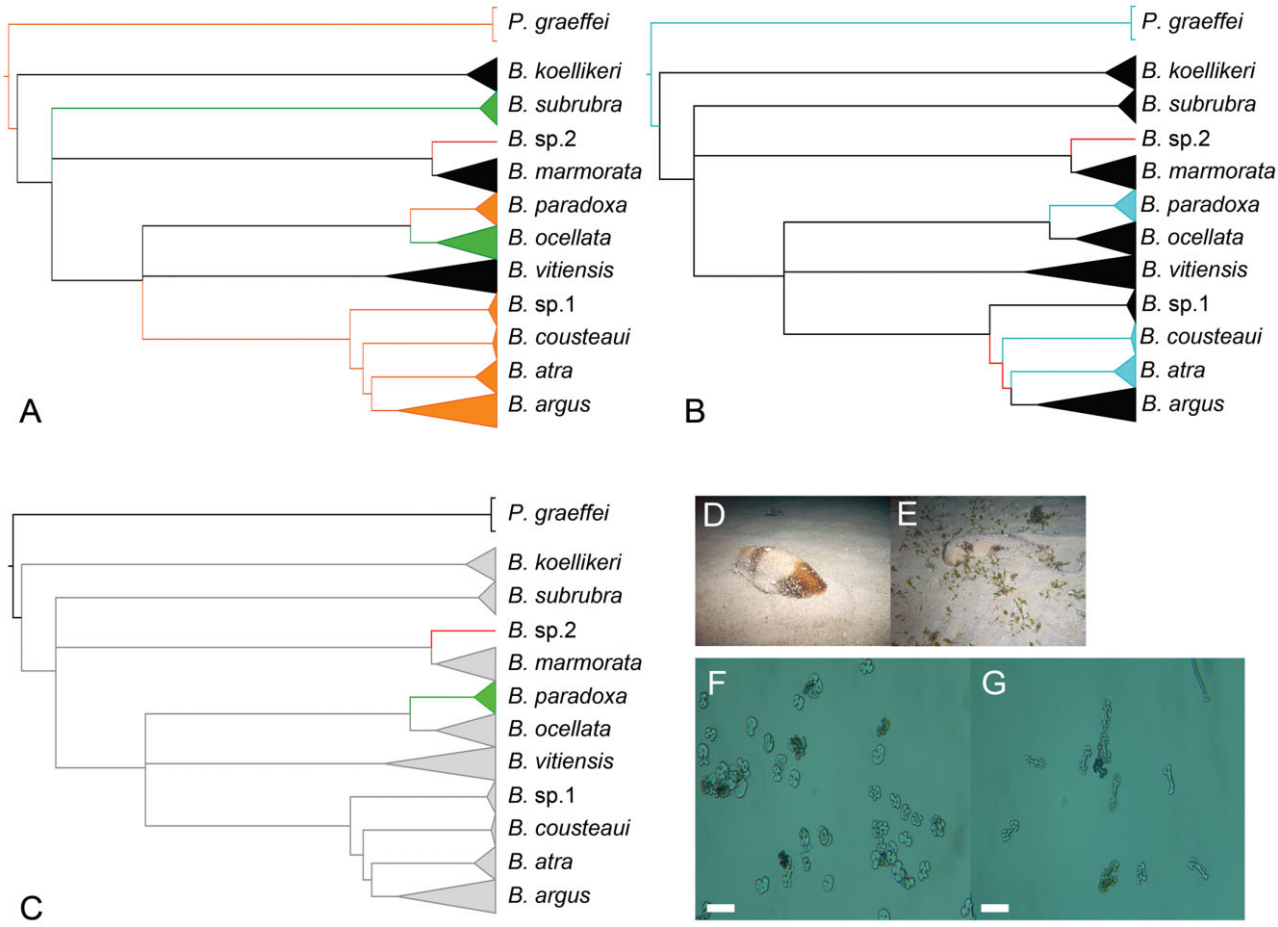

Figure 5. Ancestral reconstruction of: A, burrowing behaviour and circadian activity pattern. Branches in black indicate nocturnally epibenthic species. Branches in orange indicate diurnal species. The branch in green indicates nocturnally active, yet often diurnally epibenthic species, *B. ocellata* and *B. subrubra*. The branch in red indicates an unresolved character-state reconstruction. B, colour variability. Branches in blue indicate species with uniform colour patterns. Branches in black indicate species with intra-specific colour variability. Branches in red indicate unresolved character-state reconstructions. C, ossicle evolution. Branches in grey indicate *Bohadschia*-specific ossicles. Green branch is the evolution of *B. paradoxa*-specific ossicle. The branch in red indicates an unresolved character-state reconstruction. D, *B. vitiensis*'s burrowing behaviour. E, *B. koellikeri*'s burrowing behavior. F, typical ossicles of *Bohadschia* species. Shown are from *B. argus*. G, ossicles of *B. paradoxa*. Scale in F indicates 20 μm and scale in G indicates 40 μm.

distribution over the body. A diagnostic fine pattern of somewhat reticulate brown lines is overlain on this pattern, visible close-up only (Fig. 3F). The same colour pattern is still evident in the type of *Holothuria koellikeri*, indicating that this name applies to this species. The last member of the complex has two colour morphs that show similar COI sequences. One is striking, with strongly demarcated blotches (Fig. 3L, M); it has surprisingly been overlooked in the taxonomic literature partly perhaps because it is uncommon, although it is commonly featured now on the internet, often identified as *B. argus* (Fig. 3A, B); the other is solid dark brown, with slightly lighter dots encircling the papillae (Fig. 3N). The recently relocated type of Jaeger's *B. ocellata*, photographs kindly provided by Carsten Lueter (ZMB1133), matches the blotchy form. This name has generally been misinterpreted as representing a species of *Holothuria* (*H. ocellata* – see, for example, Liao & Clark, 1995; Teo & Ng, 2009) that belongs in the *Holothuria* (*Theelothuria*) *kurti* Ludwig, 1891 complex.

One *Bohadschia* species in our study has not matched any of the types studied so far and may be undescribed. *Bohadschia* sp. 1 has a lavender grey background with yellow blotches. It is known from a few specimens from the Hawaiian Islands, Australia, Okinawa, Philippines, and a photo taken in Bali

(Fig. 3R). The large gap in sample localities suggests that this species may be widespread in the Pacific.

Ossicle morphology is of limited value for differentiation of *Bohadschia* species because they possess a few ossicle types and these show substantial intra-specific variability and little inter-specific differentiation (Fig. 4). *Bohadschia* have only two types of ossicles in their body wall: rosettes and grains. Both vary substantially in form and completely intergrade (Fig. 5F); we consider them to be end-members of a single ossicle type. These ossicles are scattered in the outer part of the body wall like buttons, rosettes, and rods do in *Holothuria*, and do not form a mono-layer like tables, the second major type of body wall ossicle in the family. Ossicles are absent in several other body parts (muscles and gut), further limiting their utility.

We were not able to distinguish most species by meristic analysis of body wall ossicles with the exception of *B. paradoxa*. Intra-specific variation in ossicle shape and size was substantially greater than inter-specific differences, except for *B. paradoxa* (Fig. 4). A recent study by Clouse *et al*. (2005) also found that ossicle features varied more geographically within species than between *B. marmorata* and *B.* '*bivittata*' (= *vitiensis*). In addition, juvenile and adult holothuroids often have ossicles of different forms (Cutress, 1996), and several studies have pointed out substantial ontogenetic variation in *Bohadschia* ossicles (Panning, 1944; Rowe & Doty, 1977; Massin *et al*., 1999). Together, ossicle morphology, while traditionally the primary criterion for species-level classification in Holothuroidea, appears to be of limited value in differentiating *Bohadschia* species. This partly accounts for the complex taxonomic history of the genus, as has been previously noted by many workers (Panning, 1944; Samyn, 2003; Clouse *et al*., 2005).

Conversely, each species displayed a unique set of colour patterns. The conspicuous colour patterns are diagnostic of species despite considerable intra-specific variation; this intra-specific variability is based on regular patterns with limited combinations of colour forms. As a result, virtually all specimens can now be identified in the field based on colour pattern alone.

Intra-specific variation in COI (0.6%, Table 2) was similar to other echinoderms (0.6%, Ward, Holmes & O'Hara, 2008), whereas average congeneric divergence (COI 11.2%, Table 2) was slightly lower than that reported by Ward *et al*. (2008) for echinoderms (15.3%) and Uthicke *et al*. (2010) for aspidochirotids (16.9%). These differences at least partly represent the more thorough sampling of species diversity in this study.

Hybridization

Sequence and morphological data suggest frequent reciprocal hybridization between *B. argus* and *B. vitiensis*. Unusual colour variants mixing the colour pattern of these two forms were occasionally encountered in Micronesia, and contributed to the taxonomic confusion surrounding the *B. marmorata* complex. These variants have ocellated spots surrounding single podia, similar to but less substantial and defined than in *B. argus* (Fig. 3S, T). They have a background colour pattern of tan to brown, often with ill-defined double transverse bands as in *B.* '*bivittata*', and papillae are surrounded with dark dots as in *B. vitiensis* (Fig. 3S, T). All animals with this colour pattern had mtDNA sequences that matched either *B. argus* or *B. vitiensis* (marked as hybrids in Fig. 2). Uthicke *et al*. (2010) also encountered two specimens of this morphotype with *B. argus* mtDNA sequenced, and noted that they may represent very rapid mtDNA evolution or hybridization. The mixed morphology and alternative mtDNA genotypes suggests these animals are hybrids of these species, and that hybridization occurs in both directions. At some locations, such as Guam, these parental species and putative hybrids were all commonly encountered in the same habitat: sandy reef flats and shallow lagoons. *Bohadschia maculisparsa*, described from New Caledonia from a single specimen, is similar to these *argus–vitiensis* hybrids and may be based on one. Fresh specimens from the type locality are needed to test this hypothesis.

Bohadschia argus and *B. vitiensis* are not sister taxa, but separated by several (at least three) speciation events (Fig. 2). The potential hybridization between these species is surprising as distantly related species usually hybridize less frequently than closely related species because prezygotic barriers are more likely to have evolved (Foltz, 1997; Mendelson, 2003; Bolnick & Near, 2005). Further molecular analyses using nuclear DNA markers are needed to confirm hybridization between *B. argus* and *B. vitiensis*.

Biogeography

Bohadschia is endemic to the IWP region and ranges through almost its entire extent, from the Red Sea to South Africa, east to Hawaii and south-eastern Polynesia, although not reaching isolated Easter Island in the east (Massin, 1996). However, none of the 11 species shows an IWP-wide range. *Bohadschia vitiensis* is the most widespread, extending from East Africa to western Polynesia. Four species are known only from the Red Sea and/or western Indian Ocean, where they co-occur in various combinations: *B. atra*, *B. cousteaui*, *B. steinitzi*, and *B. subrubra*. Five range across the western to central Pacific, and again frequently co-occur: *B. argus*, *B. koellikeri*, *B. marmorata*, *B.*

ocellata, and *B.* sp. 1, with the first species extending at least to the eastern and possibly to the central Indian Ocean as well. The last species, *B. paradoxa*, is known only from the Hawaiian Islands, where it co-occurs with *B.* sp. 1. The easternmost species in the central and south Pacific are *B. argus*, *B. koellikeri*, and *B. ocellata*; we collected all three in the Line Islands and French Polynesia.

Present phylogenetic resolution limits analysis of speciation pattern across the genus. Only the relationship of (*B.* sp. 1 (*B. cousteaui* (*B. argus* – *B. atra*))) is resolved consistently regardless of phylogenetic estimation methods. The terminal sister species show allopatric divergence across the central Indian Ocean, a speciation pattern frequently observed in IWP taxa. The restriction of the other two species to the Red Sea – western Indian Ocean (*B. cousteaui*) and western Pacific (*B.* sp. 1) suggests that repeated allopatric differentiation may have been the cause of diversification in the group.

Bohadschia vitiensis specimens from the Indian Ocean were found in the inclusive polytomy of otherwise Pacific Ocean specimens (Table 1, Fig. 2). The divergence in COI gene trees between the two geographically separated populations occurred more recently compared with that between *B. argus* and *B. atra* (Fig. 2). The Indian Ocean specimens were uniformly light brown whereas specimens from the Pacific Ocean showed wide intra-specific plasticity in colour pattern – illustrating a similar pattern to morphologically consistent Indian Ocean endemic *B. atra* and variable Pacific Ocean *B. argus*.

Character evolution

Bohadschia are reef-associated, but several species spend part of the time buried in sediment but emerge to feed, usually at night. Nocturnal activity plus diurnal crypsis (e.g. under rubble or in crevices) is a lifestyle widespread in the Holothuriidae. Parsimony-based ancestral-state reconstruction suggests that circadian activity pattern and burrowing behaviour evolved once at the base of the *Bohadschia* lineage (Fig. 5A). The character-state has reverted once at the base of the clade comprising diurnal species, *B. atra*, *B. argus*, *B. cousteaui* and *B.* sp. 1, and once in *B. paradoxa*. *Bohadschia ocellata* and *B. subrubra* have been observed exposed during the day, but they are also capable of burrowing and have been seen buried in sand during the day; more field observations are required to determine diurnal activity pattern of these species.

The evolution of colour patterns in *Bohadschia* is striking. Nearly every species possesses a diagnostic and conspicuous pattern or sets of pattern that are different from its sister species. Several species display substantial intra-specific variation in colour pattern. This intra-specific colour variation appears to have evolved at the base of *Bohadschia* (Fig. 5B) because most holothuriids, including the proximate outgroup in this study, show little to no variation in colour patterns within species. A few *Bohadschia* (*B. paradoxa*, *B. atra*, and *B. cousteaui*) likewise show a uniform coloration. These uniform colorations within *Bohadschia* appear to be secondary acquisitions from ancestral intra-specific colour variability. The only instance of sister taxa with similar colour patterns is *B. atra*, an Indian Ocean endemic, and *B. argus* from the Pacific. *Bohadschia atra* possesses a single colour pattern across its geographical range that is modified from a subset of the variably patterned *B. argus*.

In contrast to other characters, ossicle form appears to have evolved little in *Bohadschia*. Character reconstruction revealed that grains in the ventrum evolved once and at the base of *Bohadschia* (Fig. 5C). On the other hand, rosettes in both dorsum and ventrum are found in the closely related genus *Actinopyga*. *Bohadschia* may have evolved rosettes independently or acquired them from a common ancestor of these two genera. Regardless, size and complexity of rosettes have increased dramatically only in *B. paradoxa* (Figs 4, 5C, G).

In summary, the complex taxonomic history of *Bohadschia* is partly due to the high intra-specific variability and little inter-specific differentiation in the traditional criterion, ossicle morphology. The high intra-specific variability in colour pattern also contributed to the vexing taxonomic history of the genus. In this study, we delimited boundaries of *Bohadschia* species based on molecular analyses and morphology. Most importantly, we clarified that the variable colour patterns are based on limited combinations of regular patterns, which can be used as a diagnostic tool to confidently identify *Bohadschia* species.

ACKNOWLEDGEMENTS

This study was funded by NSF PEET (DEB 0529724) to G.P. and A.M.K., the Marine Resources Pacific Consortium fund (MAREPAC) to A.M.K., and a Lerner-Gray grant from American Museum of Natural History to S.W.K.. Sequencing of some specimens was funded as part of the Marine Barcode of Life (MarBOL) by the Sloan Foundation. Korea Institute of Ocean Science and Technology fund PE99161 and PM57122 supported S.W.K. when writing. We thank Arthur Anker, John Hoover, David Lane, François Michonneau, Mike Reich, Yves Samyn, John Starmer, Didier Vandenspiegel, and two reviewers for providing many samples and/or discussion. We thank Sarah McPherson, François Michonneau, Chris Meyer and his lab for sequencing some samples.

REFERENCES

Akaike H. 1974. A new look at the statistical model identification. *IEEE Tran Automatic Control* **19:** 716–723.

Arndt A, Marquez C, Lambert P, Smith MJ. 1996. Molecular phylogeny of eastern pacific sea cucumbers (Echinodermata: Holothuroidea) based on mitochondrial DNA sequence. *Molecular Phylogenetics and Evolution* **6:** 425–437.

Bolnick DI, Near TJ. 2005. Tempo of hybrid inviability in centrarchid fishes (Teleostei: Centrarchidae). *Evolution* **59:** 1754–1767.

Borrero-Pérez GH, Pérez-Ruzafa A, Marcos C, González-Wangüemert M. 2009. The taxonomic status of some Atlanto-Mediterranean species in the subgenus *Holothuria* (Echinodermata: Holothuroidea: Holothuriidae) based on molecular evidence. *Zoological Journal of the Linnean Society* **157:** 51–69.

Cherbonnier G. 1952. Les Holothuries de Quoy et Gaimard. Mémoires de l'Institut Royal des Sciences Naturelles de Belgique.

Cherbonnier G. 1954. Note préliminarie sur les holothuries de la Mer Rouge. *Bulletin Muséum National Histoire Naturelle Paris. 2 série* **26:** 252–260.

Cherbonnier G. 1963. Les Holothuries de la Mer Rouge de l'Université Hébraïque de Jerusalem. *Bulletin Sea Fishery Research Station Haifa* **34:** 5–10.

Cherbonnier G. 1988. Echinoidermes: Holothurides. Faune de Madagascar. Publicé sous les auspices du Gouvernment de la République Malgache, 70, Editions de l'ORSTOM, Paris.

Cherbonnier G, Féral JP. 1984. Les Holothuries de Nouvelle-Calédonie. Deuxième contribution. (Première partie: Synallactidae et Holothuriidae). *Bulletin du Muséum National d'Histoire Naturelle, Paris 4 série* **6:** 659–700.

Chomszynski P, Mackey K, Drews R, Wilfinger W. 1997. DNAzol®: a reagent for the rapid isolation of genomic DNA. *BioTechniques* **22:** 550–553.

Clouse R, Janies D, Kerr AM. 2005. Resurrection of *Bohadschia bivittata* from *B. marmorata* (Holothuroidea: Holothuriidae) based on behavioral, morphological, and mitochondrial DNA evidence. *Zoology* **108:** 27–39.

Cutress BM. 1996. Changes in dermal ossicles during somatic growth in Caribbean sea cucumbers (Echinodermata: Holothuroidea: Aspidochirotida). *Bulletin of Marine Science* **58:** 44–116.

Féral JP, Cherbonnier G. 1986. Les holothurides. In: Guille A, Laboute P, Menou JL, eds. *Guide des Étoiles de Mer, Oursins et autres Échinodermes du Lagon de Nouvelle-Calédonie*. Faune Tropicale, 25. Paris: Editions de l'ORSTOM, 57–107.

Foltz DW. 1997. Hybridization frequency is negatively correlated with divergence time of mitochondrial DNA haplotypes in a sea star (*Leptasterias* spp.) species complex. *Evolution* **51:** 283–288.

Haacke W. 1880. Holothurien. In: Möbius K, Richter F, eds. *Beiträge zur Meeresfauna der Insel Mauritius und der Seychellen*. Berlin: Gustav Fischer, 46–48.

Jaeger GF. 1833. *De Holothuriis*. Turici: Dissertatio Inauguralis.

Kerr AM. 1994. Shallow-water holothurioids (Echinodermata) of Kosrae, Eastern Caroline Islands. *Pacific Science.* **48:** 161–174.

Knowlton N. 1993. Sibling species in the sea. *Annual Review of Ecology and Systematics* **24:** 189–216.

Koehler R, Vaney C. 1908. *Holothuries recuielles par l'INVESTIGATOR dans l'Ocean Indien. II. Les Holothuries Littorales*. Calcutta: Trustees Indian Museum.

Lane DJW. 2004. Sea cucumber diversity and resources in Brunei, Borneo Island. In: Heinzeller T, Nebelsick JH, eds. *Echinoderms: Muenchen. Proceedings of the 11th International Echinoderm Conference*. Munich, Germany, 6-10 October 2003. London: Taylor & Francis, 231–237.

Liao Y, Clark AM. 1995. *The echinoderms of southern China*. Beijing: Science Press.

Ludwig H. 1875. Beitraege zur Kenntnis der Holothurien. *Arbeiten Zoology Institut Wuerzburg* **2:** 77–120.

Ludwig H. 1881. Revision der Mertens-Brandts'chen Holothurien. *Zeitschrift Wissenschaftliche Zoologie* **35:** 575–599.

Ludwig H. 1883. Verzeichniss der Holothurien des Kieler Museums. *Bericht der Oberhessischen Gesellschaft für Natur-und Heilkunde* **22:** 155–176.

Massin C. 1994. Ossicle variation in antarctic dendrochirote holothurians (Echinodermata). *Bulletin de l'Institut Royal des Sciences Naturelles de Belgique. Biologie* **64:** 129–146.

Massin C. 1996. The holothurians of Easter Island. *Bulletin Institut Royal Sciences Naturelles Belgique, Biologie* **66:** 151–178.

Massin C. 1999. Reef-dwelling Holothuroidea (Echinodermata) of the Spermonde Archipelago (South-West Sulawesi, Indonesia). *Zoologische Verhandelingen* **329:** 1–144.

Massin C, Rasolofonirina R, Conand C, Samyn Y. 1999. A new species of *Bohadschia* (Echinodermata, Holothuroidea) from the Western Indian Ocean with a redescription of *Bohadschia subrubra* (Quoy & Gaimard, 1833). *Biologie* **69:** 151–160.

Mendelson TC. 2003. Sexual isolation evolves faster than hybrid inviability in a diverse and sexually dimorphic genus of fish (Percidae: Etheodstoma). *Evolution* **57:** 317–327.

Meyer CP. 2003. Molecular systematics of cowries (Gastropoda: Cypraeidae) and diversification patterns in the tropics. *Biological Journal of the Linnean Society* **79:** 401–459.

Mitsukuri K. 1912. Studies on Actinopodous Holothurioidea. *Journal College Science, Tokyo Imperial University* **29:** 1–284.

Nylander JAA. 2004. *MrModeltest v2*. Program distributed by the author. Uppsala University: Evolutionary Biology Centre.

Palumbi SR, Martin A, Romano S, McMillan WO, Stice L, Grabowski G. 1991. A simple fools guide to PCR, vers. 2.0. Special publication of the University of Hawaii Department of Zoology and Kewalo Marine Laboratory. Department of Zoology and Kewalo Marine Laboratory, Univ. Hawaii, Honolulu.

Panning A. 1929. Die gattung *Holothuria*. *Mitteilungen aus dem Zoologischen Museum und Institut, Hamburg* **44:** 91–138.

Panning A. 1940. Holothurien von den Kanaren und von Dakar. *Videnskabelige Meddelelser Dansk Naturhistorik Forening, København.* **103:** 523–546.

Panning A. 1944. Die Trepangfischerei. *Mitteilungen aus dem Hamburgischen Zoologischen Museum und Institut, Hamburg* **49:** 1–76.

Pearson J. 1903. Holothurioidea. In: Herdman WA, ed. *Report to the Government of Ceylon on the Pearl Oyster Fisheries of the Gulf of Manar*. London: Royal Society, Supplementary Report 5, 181–208.

Pearson J. 1913. Notes on the Holothuroidea of the Indian Ocean I. The genus *Holothuria*. *Spolia Zeylanica* **9:** 49–101.

Pearson J. 1914. Proposed re-classification of the genera *Mülleria* and *Holothuria*. *Spolia Zeylandica* **9:** 163–172 pl. 26.

Reyes-Leonardo LD. 1984. A taxonomic report of shallow water holothurians of Calatagan, Batangas. *Philippine Journal of Science.* **113:** 137–172.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19:** 1572–1574.

Ronquist F, Huelsenbeck JP, van der Mark P. 2005. MrBayes 3.1 Manual. Available at: http://mrbayes.csit.fsu.edu/manual.php.

Rowe FWE, Doty JE. 1977. The shallow-water holothurians of Guam. *Micronesica* **13:** 217–250.

Rowe FWE, Gates J. 1995. Echinodermata. In: Wells A, ed. *Zoological catalogue of Australia*. Vol. 33. Melbourne: CSIRO Australia, 1–578.

Samyn Y. 2000. Conservation of aspidochirotid holothurians in the littoral waters of Kenya. *SPC Bêche-de-mer Information Bulletin* **13:** 12–17.

Samyn Y. 2003. Shallow-water Holothuroidea (Echinodermata) from Kenya and Pemba Island (Tanzania). *Studies Afrotropical Zoology* **292:** 1–158.

Samyn Y, Vanden Spiegel D, Massin C. 2005. Sea cucumbers of the Comoros Archipelago. *SPC Bêche-de-mer Information Bulletin* **22:** 14–18.

Semper C. 1868. Holothurien. Reisen im Archipel der Philippinen.

Swofford DL. 2003. *PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4*. Sunderland, MA: Sinauer Associates.

Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA 4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **25:** 1596–1599.

Tan Tiu AS. 1981. The intertidal holothurian fauna (Echinodermata: Holothuroidea) of Mactan and the neighboring islands, Central Philippines. *Philippine Scientist* **18:** 45–119.

Tavaré S. 1986. Some probabilistic and statistical problems in the analysis of DNA sequences. In: Miura RM, ed. *Some mathematical questions in biology-DNA sequence analysis*. Providence: American Mathematical Society, 57–86.

Teo S, Ng CSL. 2009. New record of a sea cucumber, *Holothuria ocellata* (Jaeger, 1833) (Holothuroidea: Aspidochirotida: Holothuriidae) in Singapore. *Nature in Singapore* **2:** 411–414.

Théel H. 1886. Report on the Holothuroidea dredged by H.M.S. Challenger during the years 1873–76. Part 2. *Report on the Scientific Results of the Voyage of the 'Challenger' (Zoology)* **39:** 1–290.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignments through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22:** 4673–4680.

Uthicke S, Byrne M, Conand C. 2010. Genetic barcoding of commercial Bêche-de-mer species (Echinodermata: Holothuroidea). *Molecular Ecology Resources* **10:** 634–646.

Ward R, Holmes B, O'Hara T. 2008. DNA barcoding discriminates echinoderm species. *Molecular Ecology Resources* **8:** 1202–1211.

Wiedemeyer WL. 1994. Biology of small juveniles of the tropical holothurian *Actinopyga echinites*: growth, mortality, and habitat preference. *Marine Biology* **120:** 81–93.

Zwickl DJ. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. PhD dissertation, The University of Texas at Austin.

APPENDIX 1

List of voucher specimens at the FLMNH investigated for morphological analyses.

Genus	Species	FLMNH voucher number
Bohadschia	*argus*	UF 6966
Bohadschia	*argus*	UF 7087
Bohadschia	*argus*	UF 5565
Bohadschia	*atra*	UF 7258
Bohadschia	*atra*	UF 7353
Bohadschia	*atra*	UF 7574
Bohadschia	*cousteaui*	UF 7328
Bohadschia	*koellikeri*	UF 4705
Bohadschia	*koellikeri*	UF 5768
Bohadschia	*koellikeri*	UF 4744
Bohadschia	*marmorata*	UF 956
Bohadschia	*marmorata*	UF 5558
Bohadschia	*marmorata*	UF 4748
Bohadschia	*ocellata*	UF 7805
Bohadschia	*ocellata*	UF 6796
Bohadschia	*ocellata*	UF 1638
Bohadschia	*paradoxa*	UF 6276
Bohadschia	*paradoxa*	UF 6076
Bohadschia	*paradoxa*	UF 6158
Bohadschia	sp. 1	UF 4901
Bohadschia	*subrubra*	UF 6330
Bohadschia	*vitiensis*	UF 4716
Bohadschia	*vitiensis*	UF 5640
Bohadschia	*vitiensis*	UF 6912

APPENDIX 2

Classification statistics for all *Bohadschia* species.

(a) Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.
(b) 39.5% of original grouped cases correctly classified.
(c) 31.5% of cross-validated grouped cases correctly classified.

APPENDIX 3

Classification statistics for *Bohadschia* species excluding *B. paradoxa*.

(a) Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.
(b) 34.4% of original grouped cases correctly classified.
(c) 25.6% of cross-validated grouped cases correctly classified.