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Natural hybridization does not dissolve species boundaries in commercially important sea cucumbers

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The study of species boundaries in areas of sympatry provides important insight into speciation processes. We investigated whether (i) two sympatric holothurians, *Holothuria scabra* and *H. s. var. versicolor* constituted species, and (ii) specimens of intermediate phenotype hybrids. Results from allozyme and 16S mtDNA sequence analyses indicated these two sea cucumbers to be distinct but young biological and phylogenetic species. Several private allozyme alleles existed and a Bayesian analysis grouped varieties into separate clusters. MtDNA sequences hardly varied within each taxon, and nine single bp changes were diagnostic between these two taxa. Allozyme allele frequencies in individuals of intermediate phenotype were intermediate to those of *H. scabra* and *H. s.* var. *versicolor*, most private alleles were present and heterozygote frequencies were higher than in either species. Ancestry coefficients modelled for these individuals were close to 0.5, indicating that the two taxa contributed equally to their genome. MtDNA sequences were identical to those of either species. We conclude that individuals of intermediate phenotype represent F1 hybrids. The presence of hybrids demonstrates that the opportunity for introgression exists, but is not realized, as backcrossing and introgression were not supported by the data. Thus, the genetic integrity of either holothurian species remains intact through an unknown postzygotic mechanism, possibly hybrid sterility.

ADDITIONAL KEYWORDS: echinoderm – holothurians – hybrids – introgression – speciation.

INTRODUCTION

Despite advances in the understanding of molecular mechanisms of inheritance and novel tools being available to evolutionary biologists, species concepts and modes of speciation are still hotly debated (reviewed in Mallet, 1995; Avise & Wollenberg, 1997). Although marine habitats cover most of the planet's surface, and diversity is as high as it is in terrestrial ecosystems, there have been few attempts to investigate speciation in the sea (Palumbi, 1994).

The study of natural hybridization and hybrid zones can be an important tool with which to understand these processes (Palumbi, 1994). Hybridization has been studied rarely in marine animals (but see examples given in Gardner, 1997). Despite the importance of echinoderms in the ecology of coral reefs and in other ecosystems (Birkeland, 1989), little work on speciation and hybridization has been conducted in this phylum. Echinoderms are usually broadcast spawners. Closely related species of echinoids (sea urchins, Strathmann, 1981; Palumbi & Metz, 1991; Rahman, Uehara & Pearse, 2001, 2004; Levitan, 2002;) and of asteroids (sea stars, Byrne & Anderson, 1994) can cross-fertilize in vitro. In some of these, F_1 hybrids have been raised in the laboratory, but the only echinoderms shown unambiguously to produce hybrids in the field were from the classes Asteroidea (Schopf & Murphy, 1973; Kwast, Foltz & Stickle, 1990) and Echinoidea (Lessios & Pearse, 1996; Pearse, 1998).

Holothuria scabra (sea cucumber, class Holothuroidea) is one of the most important holothurian species for the tropical bêche-de-mer fishery (Hamel *et al*., 2001). In most areas in the Indian and Pacific Ocean this species shows distinct signs of over-fishing. We previously studied the population genetics of this species

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using allozyme analyses (Uthicke & Benzie, 2001; Uthicke & Purcell, 2004), and inferred a surprisingly high degree of population separation for a marine invertebrate with a planktonic larval duration of 10–14 days. This species shows clear patterns of isolation by distance over large geographical scales, and less predictable and mosaic-like patterns over smaller scales.

Conand (1990) found individuals similar to *H. scabra*, but with slight differences in morphology and small-scale distribution patterns, in New Caledonia. However, based on skeletal features, Conand could not describe a new species, and suggested classifying these animals as a variety, *H. s.* var. *versicolor,* reflecting their variation in colour patterns. During collections of *H. scabra* and *H. s.* var. *versicolor* in New Caledonia (Uthicke & Purcell, 2004), we observed these variants in sympatry at only three out of nine sites. In addition, we detected individuals of intermediate phenotype at two sites, which had high densities because they have been closed to fishing. The presence of both putative species and intermediate forms in sympatry prompted this study into species boundaries in holothurians. Firstly, we tested the hypothesis that *H. scabra* and *H. s.* var. *versicolor* constitute separate species. Secondly, we investigated whether intermediate forms constitute hybrids between the putative species. It was expected that by investigating these intermediate forms we would gain further information on the biological mechanisms leading to or preventing speciation in these species, for example, providing information of postzygotic isolation mechanisms, or the potential for introgression. This was achieved by investigating eight nuclear allozyme loci and one mitochondrial locus (16S rDNA) and applying both traditional statistics and model-based Bayesian approaches to analyse the data.

MATERIAL AND METHODS

Study animals

The two morphologically similar sea cucumbers *H. scabra* and *H. s.* var. *versicolor* are distinguished by features listed by Conand (1990) and Hamel *et al*. (2001). In this study, we distinguished between *H. scabra* and three colour morphs of *H. s.* var. *versicolor* (Conand, 1990; blotchy morph, black morph and beige morph), which can be distinguished in the field (Fig. 1). Apart from colour patterns, the main external distinguishing features are deeper body wrinkles in *H. scabra*, and longer dorsal papillae in *H. s.* var. *versicolor* (Conand, 1990). In specimens of intermediate phenotype, in situ measurements showed that dorsal papillae (2.1 mm) and body wrinkles (1.7 mm) were intermediate to those in *H. scabra* (dorsal papillae, 1.5 mm; body wrinkles, 3.1 mm) and *H. s.* var. *versicolor* (dorsal papillae, 3.1 mm; body wrinkles, 0.5 mm). Type specimens of all colour variants were lodged in the Australian Museum, Sydney (registration numbers: J24079–J24085).

Sampling

A location with approximate size of 4 ha and high abundance of *H. s.* var. *versicolor* (blotchy morph, 63/ha; black morph, 119/ha; beige morph, 119/ha; all densities: S. Purcell, B. Blockmans & S. Uthicke, unpubl. data), *H. scabra* (7/ha) and individuals of intermediate phenotype (19/ha) were found in sympatry (nearest-neighbour distances between types averaged between 2.6 and 3.6 m) at a reef flat near Ilot Maître (22°20.4′S; 166°24.8′E), New Caledonia. Although there is extensive fishing in nearly all available habitats in New Caledonia, this reef was declared a marine reserve in 1990.

For allozyme analysis we collected gut tissue from 31 individuals of *H. s.* var. *versicolor* (blotchy morph = 11, black morph = 11, beige morph = 9) and 19 individuals of intermediate phenotype; samples were stored and processed as described in Uthicke & Purcell (2004). Gut tissue samples from the 14 individuals of *H. scabra* used for allozyme electrophoresis were the same as those used in the previous study on population genetics. For mtDNA analyses, we obtained five additional samples of *H. s.* var. *versicolour*, five of the intermediate phenotype and two additional *H. scabra* samples from Ilot Maître. In addition, we obtained three samples of *H. scabra* from Moreton Bay, Australia (27°4.8′S; 153°21.0′E) and two samples from Magnetic Island, Australia (19°10.2′S; 146°48.6′E). Tissues for DNA extractions were either gonad or respiratory tree, and samples were preserved in 20% DMSO (saturated NaCl, 250 mM EDTA, pH 7.5).

Allozyme and mtDNA analysis

Electrophoresis of seven enzyme loci that are polymorphic in *H. scabra* was conducted as described previously (Uthicke & Benzie, 1999, 2001) on 12% horizontal starch gels using two buffer systems (TEC7.9, TG8.4). These loci were glucose-6-phosphate isomerase (E.C. 5.3.1.9, *Gpi**), hexokinase (E.C. 2.7.1.1, *Hk**), malate dehydrogenase (E.C. 1.1.1.37, *Mdh**), phosphoglucomutase (E.C. 5.4.2.2, *Pgm**), and peptidases (E.C. 3.4.11/13), which were reported as peptidase locus 1 (*Pep-1**), *Pep-2** and *Pep-3** (Uthicke & Benzie, 2001). In addition, we screened for variation in 6-phosphogluconate dehydrogenase (E.C. 1.1.1.44, *Pgd**) on TEC7.9 gels. Alleles not previously detected were labelled relative to the most common allele of the previous analysis.

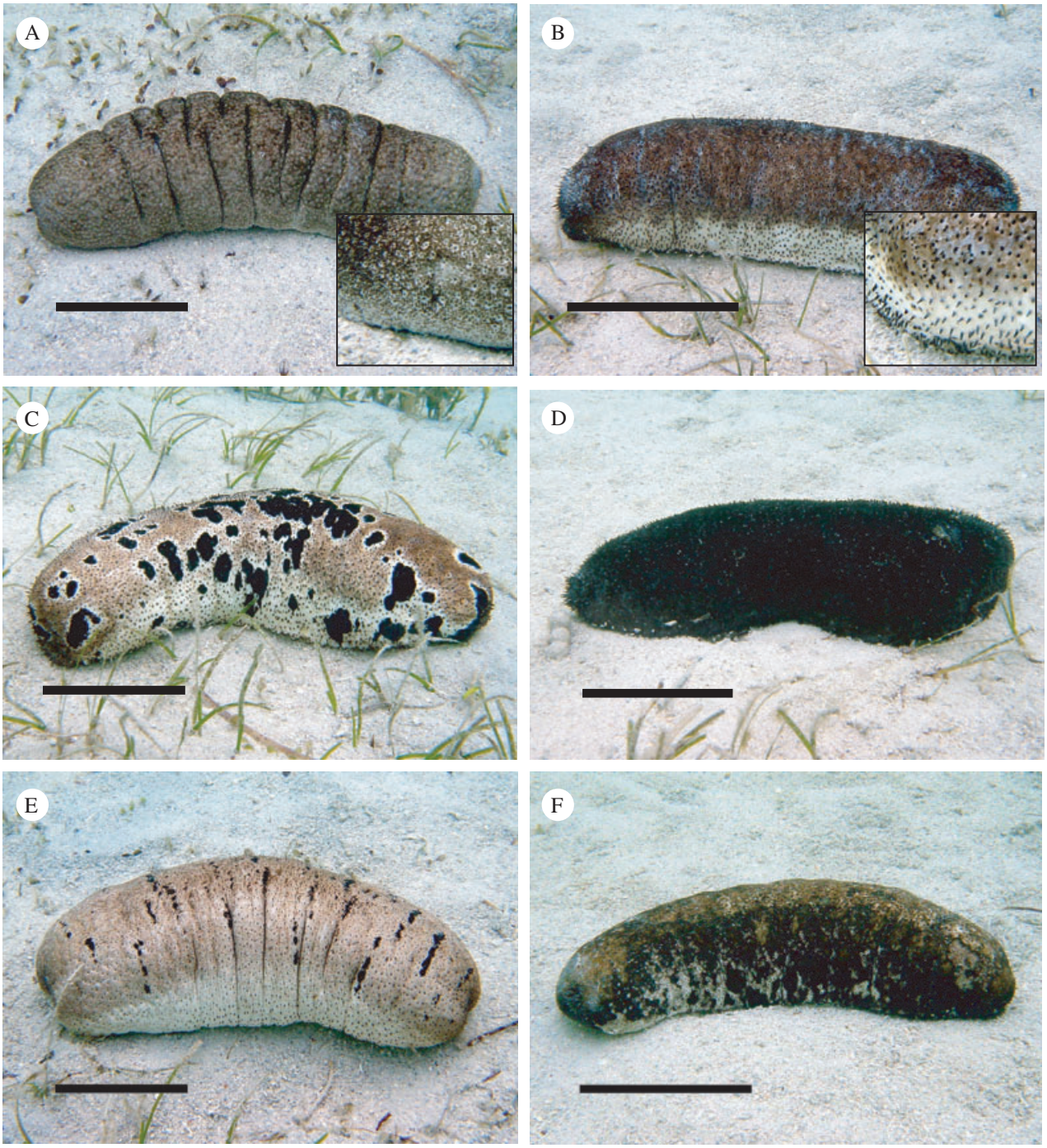

Figure 1. Phenotypic variation in *Holothuria scabra* (A), *H. s.* var. *versicolor* (B, beige; C, blotchy; D, black) and varieties of intermediate phenotypes (E, light; F, dark) from New Caledonia. Inserts show the longer papillae in *H. s.* var. *versicolor* (B) compared with *H. scabra* (A). All scale bars represent 10 cm; inserts are 2× magnification.

DNA from preserved tissues was extracted using Qiagen DNeasy extraction kits as described in Uthicke & Benzie (2003). A region of the mitochondrial large subunit ribosomal DNA (16S rDNA) gene was amplified with primers 16SA-R and 16SB-R (Palumbi *et al*., 1991), using PCR conditions as described in Uthicke & Benzie (2003).

PCR products were screened for the presence of bands of the expected size on 1% agarose gels. For sequencing, we cleaned the PCR products with a

QIAquick PCR purification kit (Qiagen). Amersham DYEnamic ET Terminator sequencing kits were used, with 20–60 ng purified PCR-product, and sequencing was performed in both directions. Sequencing products were cleaned with Amersham Autoseq G-50 columns and run on an ABI 377 sequencer.

Statistical analyses

Estimation of genetic variation and exact tests (1000 dememorization steps, ten batches with 2000 permutations) were performed using the 'Tools for Population Genetic Analyses' package (Miller, 1997). We additionally analysed the allozyme data with a model-based approach (Pritchard, Stephens & Donelly, 2000). These authors have developed a program (STRUCTURE, available at http://www.stats.ox.ac.uk/~pritch/home.html) to cluster genetic data adopting a Bayesian approach and applying Markov Chain Monte Carlo methods to approximate posterior distributions. Individual samples are assigned to K clusters, based on their multilocus genotypes, and membership coefficients to each cluster (q_{ik} = ancestry probabilities of individual i in cluster k) calculated. We tested various lengths of burn-in period and simulation run under different numbers of clusters (K) using the admixture model in STRUCTURE. A burn-in length of 30.000 and a length of the simulation and data collection of 10^6, as suggested by Pritchard *et al.* (2000), gave highly consistent results, and these parameters were used in simulations. Initially, we used the admixture model without prior population information. We conducted five replicate runs each for a K between 1 and 5. The decision on the number of clusters best fitting the data followed the ad-hoc procedure suggested by Pritchard *et al.* (2000), using the average ln Pr(X | K) of five runs to calculate the posterior probability of K. These calculations only showed high probability ($P = 1$) for a model with two clusters, which was therefore used for analyses.

In a second step of the analysis, we used the admixture model including prior population information, by defining animals of the *H. scabra* and *H. s. var. versicolor* phenotypes as belonging to separate clusters. No information was given for intermediate forms. This model facilitates the detection of misclassifications or introgression between taxa and improves the ancestry estimates for potential hybrids (Pritchard *et al.*, 2000).

MtDNA sequences were aligned with SEQUENCHER (Gene Codes Corporation, Miami). Due to some ambiguities at the 5′ or 3′ end of some sequences, we reduced sequences to 489 bp. We used programs from Phylip 3.5 (Felsenstein, 1993) to obtain 100 bootstrap replicates of the dataset, and to calculate Kimura 2-parameter distances (transition–transversion ratio = 2) for each replicate. These were subjected to neighbour-joining analysis with a sequence from *H. whitmaei* (collected in the northern Great Barrier Reef) as an outgroup. We removed some deletions and insertions from that sample to align it with the other sequences. Thus, the genetic distances from the outgroup to the ingroup were minimum estimates and indicative only.

RESULTS

Allozyme analyses

In the samples of *H. scabra* from Ilot Maître, we detected most alleles commonly found in previous allozyme studies of this species (Uthicke & Benzie, 2001; Uthicke & Purcell, 2004; Table 1). The three colour variants of *H. s.* var. *versicolor* had similar allele frequencies in each locus, and exact tests for individual loci or a pooled analysis did not detect differences ($P > 0.3$ in each case) between any combination of these morphs. Thus, the three colour morphs were accepted as being the same species, and samples were combined. Although most alleles appeared both in *H. scabra* and in *H. s.* var. *versicolor* (Table 1), the combined sample of *H. s.* var. *versicolor* had eight alleles not detected in *H. scabra*. Four of these alleles (Pgm^{74}, Hk^{90}, and $Pep\text{-}3^{91,106}$) occurred at relatively high frequencies in *H. s.* var. *versicolor*. Similarly, three alleles common in *H. scabra* ($Pgm^{80,86,90}$) were absent from *H. versicolor*, and a fourth allele (Hk^{100}) very frequent in *H. scabra* was found in very low frequencies in *H. s.* var. *versicolor*.

Nearly all alleles detected in both putative species were found in the individuals of intermediate phenotype (Table 1). Each allele occurring in frequencies of > 0.2 in *H. scabra* and *H. s.* var. *versicolor* occurred at intermediate frequencies in the intermediate forms. The lowest number of alleles occurred in *H. scabra*, and the highest in the intermediate forms (Table 1). The observed heterozygosity was highest in the intermediate phenotype sample for most polymorphic loci, with the average heterozygosity being nearly twice as high compared with *H. scabra* and *H. s.* var. *versicolor* (Table 2). The heterozygosity observed in the intermediate phenotypes was similar to expected values for F_1 hybrids between *H. scabra* and *H. s.* var. *versicolor*. (Table 2; calculation based on allele frequencies for *H. scabra* and *H. s.* var. *versicolor* given in Table 2). The *Pep-2* locus was most instructive in exemplifying the effects on heterozygosity. This locus was nearly fixed (frequencies of > 0.92, Table 2) for different alleles in *H. scabra* and *H. s.* var. *versicolor*, resulting in very low numbers of heterozygotes in either variety (two out of 14 samples and two out of 31 samples, respectively). In contrast, 13 out of 19 samples of the

Table 1. Allele frequencies at seven enzyme loci of *Holothuria scabra, H. s.* var. *versicolor* and individuals of intermediate phenotype

Locus	*H. scabra*	*H. s.* var. *versicolor*				Intermediate phenotype
		Blotchy	Black	Beige	Total	
*Pgm**	(14)	(11)	(11)	(9)	(31)	(19)
100	0.214	0.454	0.818	0.722	0.661	0.500
94	0.000	0.182	0.046	0.111	0.113	0.132
90	0.357	0.000	0.000	0.000	0.000	0.132
86	0.215	0.000	0.000	0.000	0.000	0.158
80	0.214	0.000	0.000	0.000	0.000	0.053
74	0.000	0.364	0.136	0.167	0.226	0.026
*Hk**	(14)	(11)	(11)	(9)	(31)	(19)
100	0.821	0.000	0.000	0.111	0.032	0.526
94	0.179	0.682	0.909	0.833	0.807	0.395
90	0.000	0.318	0.091	0.056	0.161	0.158
*Gpi**	(14)	(11)	(11)	(9)	(31)	(19)
113	0.000	0.000	0.045	0.055	0.032	0.000
100	1.000	0.909	0.909	0.889	0.903	0.974
90	0.000	0.091	0.045	0.055	0.065	0.026
*Mdh**	(14)	(11)	(11)	(9)	(31)	(19)
100	1.000	1.000	1.000	1.000	1.000	1.000
91	0.000	0.000	0.000	0.000	0.000	0.000
*Pgd**	(14)	(11)	(11)	(9)	(31)	(19)
115	0.000	0.046	0.000	0.000	0.016	0.000
100	1.000	0.954	1.000	1.000	0.984	1.000
*Pep-1**	(14)	(11)	(11)	(7)	(29)	(19)
106	0.357	0.136	0.136	0.000	0.103	0.184
100	0.643	0.864	0.864	1.000	0.897	0.816
*Pep-2**	(14)	(11)	(11)	(9)	(31)	(18)
100	0.929	0.000	0.046	0.056	0.032	0.472
94	0.071	1.000	0.954	0.944	0.968	0.528
*Pep-3**	(14)	(11)	(11)	(8)	(30)	(19)
106	0.000	0.318	0.455	0.313	0.367	0.201
100	0.893	0.455	0.227	0.250	0.317	0.579
95	0.107	0.091	0.045	0.062	0.067	0.184
91	0.000	0.136	0.273	0.375	0.250	0.026

Numbers in parentheses indicate the sample size at each locus.

intermediate phenotypes were heterozygous at this locus.

H. scabra and *H. s.* var. *versicolor* grouped into two separate clusters (Fig. 2A) using the admixture model without prior population information and K = 2. Each individual had only small membership coefficients (q_{ik}) to the cluster dominated by the other taxon. The individuals of intermediate phenotype had varying membership coefficients to the two clusters. Including population information in the model further clarified the results (Fig. 2B). No outliers (misidentifications or migrants) were observed within the *H. scabra* or *H. s.* var. *versicolor* clusters; most individuals had ancestry coefficients of nearly 1 for their cluster. The membership coefficients of the individuals of intermediate phenotype were in a narrow band around 0.5, with an average ancestry coefficient to the *H. scabra* cluster of 0.51.

mtDNA (16SrDNA) analysis

We obtained 489 bp of sequence for seven specimens of *H. scabra*, five specimens of *H. s.* var. *versicolor* and five individuals of intermediate phenotype (GenBank Accession No. AY509130–AY50947, the latter being the outgroup sequence). Hardly any variation existed among *H. scabra* samples; only one sequence differed from all the others by 1 bp (0.20%). No sequence variation was observed between samples of *H. s.* var. *versicolor* (Fig. 3), including all three of the

Table 2. Summary statistics and parameters describing genetic variability at eight allozyme loci of *Holothuria*

	H. scabra		*H. s.* var. *versicolor*		Intermediate phenotype		
Sample site/location	No. of alleles	Observed heterozygosity	No. of alleles	Observed heterozygosity	No. of alleles	Observed heterozygosity	Expected (F_1) heterozygosity
*Pgm**	4	0.714	3	0.355	6	**0.737**	0.859
*Hk**	2	0.357	3	0.387	3	**0.684**	0.831
*Gpi**	1	0.000	3	0.194	2	0.053	0.097
*Mdh**	1	0.000	1	0.000	1	0.000	0.000
*Pgd**	1	0.000	2	0.032	1	0.000	0.016
Pep-1	2	0.427	2	0.207	2	0.368	0.386
Pep-2	2	0.143	2	0.065	2	**0.722**	0.901
Pep-3	2	0.214	4	0.633	4	**0.790**	0.711
Total/average	15	0.232	20	0.234	21	**0.412**	0.475

Heterozygosity = direct count heterozygosity. Bold print indicates heterozygosities in the intermediate phenotypes that are higher than found in both *H. scabra* and *H. s.* var. *versicolor*.

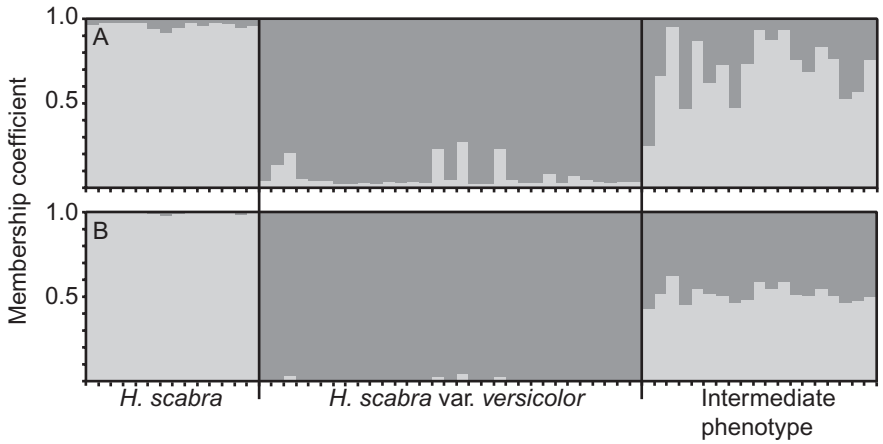

Figure 2. Estimated structure of samples of *Holothuria scabra*, *H. s.* var. *versicolor* and animals of intermediate phenotype (K = 2). Vertical lines on the *x*-axis represent single individuals. The light grey contribution to each line represents the membership fraction to the first cluster, the darker grey to the second cluster. The upper graph (A) is derived from an admixture analysis without population information; (B) includes prior population information.

colour morphs. Neighbour-joining analysis placed sequences of the *H. scabra* and *H. s.* var. *versicolor* into two separate clusters, supported by high (97%) bootstrap values (Fig. 3). The sequences of *H. scabra* and *H. s.* var. *versicolor* all differed by the same 9 single bp (1.84%). Sequences from individuals of intermediate phenotype fell into either of the two clusters. Thus, individuals from intermediate phenotypes had sequences typical of either *H. scabra* or *H. s.* var. *versicolor* haplotypes.

DISCUSSION

Understanding speciation and species concepts in marine animals requires studies on hybridization between species, specifically in hybrid zones (Palumbi, 1994). Results recently emerging from studies on potential introgressive hybridization in scleractinian corals (van Oppen *et al*., 2000; Marquez *et al*., 2002; Vollmer & Palumbi, 2002) demonstrate the insight into evolutionary processes that can be gained through such studies. Among echinoderms, hybridization in the field has been shown only for two asteroid genera (*Asterias*: Schopf & Murphy, 1973; *Leptasterias*: Kwast *et al.*, 1990) and within one echinoid genus (*Diadema*: Lessios & Pearse, 1996). However, the fact that simultaneous spawning of different species also occurs in other echinoderms (McEuen, 1988) and that several species of echinoids and asteroids can be cross-fertilized (Strathmann, 1981; Byrne & Anderson, 1994; Rahman *et al.*, 2001) indicates that the potential for introgressive hybridization may also exist in these

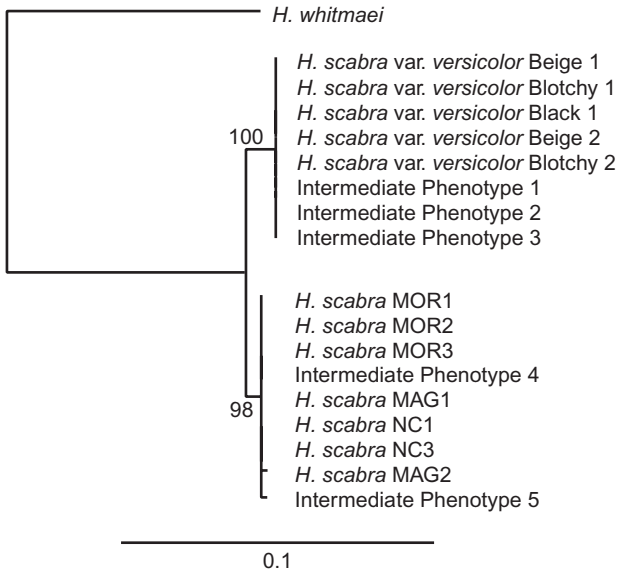

Figure 3. Neighbour-joining tree of 16S mtDNA sequences obtained from individuals of *Holothuria scabra*, *H. s.* var. *versicolor* and animals of intermediate phenotype. Numbers on branches represent the percentage of bootstrap replicates which gave the same result. MOR, MAG and NC denote samples from Moreton Bay, Magnetic Island and New Caledonia, respectively.

groups. The detection of individuals of phenotype intermediate between *H. scabra* and *H. s.* var. *versicolor* in an area of sympatry allowed us to study species boundaries and potential hybridization in holothurians, another ecologically important group (Uthicke, 2001).

ARE *H. SCABRA* AND *H. S.* VAR. *VERSICOLOR* DIFFERENT SPECIES?

Whether two groups of animals constitute two species may depend on the species concept that one follows: a classical biological species concept (Mayr, 1942), a more phylogeny-based concept (e.g. Rosen, 1979), or a modern synthesis species definition (Mallet, 1995). We argue that the present molecular data obtained on *H. scabra* and *H. s.* var. *versicolor* are consistent with the two taxa being separate species under most concepts. Genetic data support the finding that *H. scabra* and *H. s.* var. *versicolor* form reproductively isolated units, albeit producing F_1 hybrids (see below). Despite substantial ancestral polymorphisms at allozyme loci, and the sharing of 12 out of a total of 23 alleles, there were several fixed allele differences in these two sympatric varieties. Seven alleles were private to *H. s.* var. *versicolor*. Although our sample size of *H. scabra* was relatively small, we could compare these results with a dataset of nearly 900 individuals collected at sites from New Caledonia to Bali (Uthicke & Benzie, 2001; Uthicke & Purcell, 2004). Four of these alleles (Pgm^{94}, Hk^{90}, $Gpi^{90,113}$) were found in very low numbers in the total dataset of *H. scabra* (frequencies: 0.077, 0.002, 0.018 and 0.027, respectively), but the remaining ones were never observed in *H. scabra*. Fewer alleles were unique to *H. scabra* than to *H. s.* var. *versicolor*. Whether this indicates that the latter is a phylogenetically older species requires further investigation.

The model-based admixture analysis strongly supports the hypothesis that the two species are reproductively isolated. For individuals of each of the two groups, we estimated hardly any contribution to the genome from the other group. The fact that animals fell into two distinct clusters also supports the suggestion that the two taxa are phylogenetically distinct units, thereby satisfying more phylogeny-based species concepts. This is also supported by the mtDNA analysis; the haplotypes of the two species fell into two clades, and the species appeared to be separated by nine single diagnostic bp changes. Variation within taxa was low to non-existent, the 1 bp change observed in *H. scabra* was in the range, which may be caused by PCR errors. Given that samples from *H. scabra* were collected over a large geographical region (separated by *c.* 1200–2000 km), this low amount of variation was unexpected. The samples were from three regions, with significantly different allozyme–allele frequencies, and large restrictions in gene flow (Uthicke & Purcell, 2004).

The 9 bp fixed difference between sequences of *H. scabra* and *H. s.* var. *versicolor* is small (1.84% sequence divergence), suggesting that the two species have not been separated long; this was also implied by the incomplete lineage sorting that was shown by the allozyme analysis. In comparison, the congeneric outgroup (*H. whitmaei*) has sequence differences from the other two species of over 16%, and has several additional insertions and deletions. Inter-generic differences in 16S sequences among other aspidochirotid holothurians are generally larger than 9% (Kerr *et al.* cited from Samyn, 2003). The mitochondrial COI gene shows differences of up to 2.4% within species, and differences between species of the genus *Holothuria* within the Aspidochirotida can be in excess of 25% (Uthicke & Benzie, 2003; Uthicke *et al*., 2004).

No statistical differences in allele frequencies occurred between the different colour morphs of *H. s.* var. *versicolor,* implying that these constitute one species, as suggested by Conand (1990). This finds further support in the fact that these colour varieties showed no differences in 16S mtDNA haplotypes.

What are the individuals of intermediate phenotype?

The frequencies for most alleles in the intermediate phenotypes were between those of *H. scabra* and *H. s.* var. *versicolor*, and nearly all alleles unique to one species occurred in these forms. This is consistent with the intermediate specimens being hybrids between the two species, and the high number of heterozygotes observed in the intermediate forms substantiates this hypothesis. The Bayesian ancestry analysis (Pritchard *et al.*, 2000) further suggested that individuals with intermediate phenotype receive equal amounts of their genome from both *H. scabra* and *H. s.* var. *versicolor*. In addition, mtDNA haplotypes found in these intermediates belonged either to the *H. scabra* or *H. s.* var. *versicolor* group. Assuming that mtDNA is inherited only maternally, as in most other animals, this indicates that hybridization can be reciprocal, with oocytes originating from either species. Thus, we accept that the animals of intermediate phenotype are hybrids between *H. scabra* and *H. s.* var. *versicolor*.

There were no indications of back-crossing between these F_1 hybrids and the two parent species. The occurrence of some unique alleles in high frequencies in either parent species (especially considering the large dataset available for *H. scabra*, see above) strongly supports this. Backcrossing with parental species would result in introgression of alleles into the other species. Also, the ancestry coefficient derived from the Bayesian modelling was close to 0.5 for each hybrid. There were no indications of introgressive hybridization as individuals from the two species clearly belonged to separate clusters, with ancestry coefficients to that respective cluster being close to one. Thus, despite the existence of hybrids, we could not detect indications of backcrossing and therefore it seems that introgression of these alleles does not occur or is very rare in these species.

Amongst echinoderms, introgressive hybridization has been observed in some asteroid species, but only in geographically restricted hybrid zones (Schopf & Murphy, 1973; Kwast *et al.*, 1990). *H. scabra* and *H. s.* var. *versicolor* co-occur over a large geographical range, both in the tropical Indian and West Pacific Oceans (both species occur from Madagascar to the Solomon Islands and New Caledonia; *H. scabra* also occurs slightly further east in Fiji and Tonga, as summarized in Conand, 1998; Hamel *et al.*, 2001). However, they often occupy different microhabitats, with *H. s.* var. *versicolor* often being found deeper (Conand, 1990). Therefore, under a strict definition they are not sympatric in many areas. The spawning season of the two putative species overlaps in New Caledonia (Conand, 1993). It appears that in some locations, where the microhabitat distributions overlap, spawning times are sufficiently similar to allow hybridization. The only hybrids we observed were in areas of high population density because they were in areas protected from fishing, particularly at the marine reserve investigated here, Ilot Maître. Higher densities provide more opportunity for interfertilization in broadcast spawners. The finding of hybrids only in areas of species overlap provides some further evidence for our previous suggestion that there is a high incidence of self seeding despite the 10–14-day planktonic larval phase (Uthicke & Purcell, 2004). Whether hybridization between these two species would be more prevalent if stocks were better protected from over-fishing is an interesting topic for further studies investigating interactions between no-take zones, gene flow and genetic diversity. This is now possible, because our study has shown that two species exist and hybridize, and that these species and hybrids can be distinguished reliably in the field.

CONCLUSION

For speciation to occur and for species boundaries to be maintained, either prezygotic or postzygotic isolation to gene exchange must exist (Dobzhansky *et al.*, 1977). The fact that the two species hybridize in situ, and hybrids are more common in the population than is one of the parent species (see Material and methods), indicates that it is not gamete incompatibility or other prezygotic mechanisms that are keeping gene pools from merging. However, hybrids are also viable and we have observed both male and female individuals with gonads in various stages of maturity. Thus, if hybrid sterility is assumed as a postzygotic mechanism, this must be independent of gonad development.

Most species concepts rely to a large extent on species definitions. One problem is to make predictions about the species integrity after contact of closely related species. Paradoxically, the existence of hybrids between species may assist in this prediction. The existence of hybrids can be considered as proof that two species are in close contact and have the opportunity to interbreed. If it can be shown that despite this opportunity the genetic integrity of the two groups remains intact, these two species fulfil the requirements for all generally accepted species concepts. This was the case in the two species candidates discussed here and we suggest that the variety *versicolor* should be elevated to species rank.

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