Species composition and molecular phylogeny of the Indo-Pacific teatfish (Echinodermata : Holothuroidea) bêche-de-mer fishery

Sven Uthicke[A,D], *Tim D. O'Hara*[B] *and Maria Byrne*[C]

[A]Australian Institute of Marine Science, PMB No 3, Townsville, Qld 4810, Australia.
[B]Museum Victoria, GPO Box 666E, Melbourne, Vic. 3001, Australia.
[C]Department of Anatomy and Histology, F13, University of Sydney, NSW 2006, Australia.
[D]Corresponding author. Email: s.uthicke@aims.gov.au

Abstract.   Using mtDNA sequences we found that the Indo-Pacific teatfish fishery comprises at least three species, clarifying confusion on the taxonomic status of these commercially important holothurians. Traditional taxonomic characters, including the morphology of skeletal structures, could not be used to differentiate the species. Sequences of the *COI* gene (529 bp) distinguished three haplotype clusters, corresponding to distinct colour forms and, to some extent, previously described species. The white teatfish, *Holothuria fuscogilva*, comprises a range of colour morphs and has a wide distribution over the tropical Indo-Pacific region. The large sequence divergence indicates potential for the presence of several cryptic species in the white teatfish complex. In contrast to current taxonomy, we identified two species of black teatfish that appear to be allopatric: *H. whitmaei* is entirely black and has a Pacific distribution; whereas *H. nobilis* has white ventro-lateral dots and only occurs in the Indian Ocean. There is evidence for allopatric speciation between the black teatfish species, possibly driven by separation of the oceans and altered current patterns during the Pliocene, resulting in relatively young species with low intraspecific sequence divergence. These results provide insight into speciation in these tropical holothurians and are crucial for their conservation management.

Extra keywords: Aspidochirotida, diversity and conservation, Holothuriidae, invertebrate fishing, sea cucumber, taxonomy.

Introduction

The bêche-de-mer fishery in the tropical Indo-Pacific includes ~20 species of aspidochirotid sea cucumbers (Conand 1990). Many reefs have been serially depleted over the past 25 years in areas as disparate as Australia, Papua New Guinea, Fiji and Madagascar, with fishers continually moving on to more remote locations, deeper water and/or less valuable species (Conand 1990; Conand and Byrne 1993; D'Silva 2001; Buckner *et al*. 2003; Uthicke 2004). Stocks of the most valuable species are now severely depleted throughout the tropical Indo-Pacific region (Conand 1998*a*, 2001; Lovatelli *et al*. 2004). Holothurians are particularly susceptible to over-exploitation because of their limited mobility, late maturity, density-dependent reproduction and low rates of recruitment (Uthicke and Benzie 2000*a*; Uthicke 2004; Uthicke *et al*. 2004). Despite having a relatively-long lived pelagic larvae (Martinez and Richmond 1998; Ramofafia *et al*. 2003), there has been little or no recruitment over several years to heavily fished reefs on the Great Barrier Reef despite the presence of unfished stocks in nearby No-Take zones (Uthicke and Benzie 2000*a*; Byrne *et al*. in press *a*; Uthicke 2004; Uthicke *et al*. 2004).

Teatfish are among the most valuable species in the bêche-de-mer fishery. These holothurians are distinguished by a row of prominent processes or teats along the ventrolateral margin of the body. They are assigned to the genus *Holothuria* subgenus *Microthele*, which besides the teatfish also includes the elephant trunkfish (*H. (M.) fuscopunctata*). Despite their large size, ecological importance in nutrient recycling and bioturbation (e.g. Uthicke 2001), economic importance, and concerns for their conservation, there is considerable confusion among scientists, traders and fishermen as to the composition of the teatfish fishery. Fishers have long recognised two forms, the black and white mottled colour form (= white teatfish) and the black form (= black teatfish), but taxonomists and ecologists have variously treated them as separate species or as colour morphs of a single species.

At least seven scientific names have been applied to the Indo-Pacific teatfish. Cherbonnier (1952) believed that *Holothuria guamensis* (Quoy & Gaimard, 1833) was likely to be the first scientific description of a teatfish. However, this species has recently been shown to be an unusual colour form of the wide-ranging *Actinopyga mauritiana* (see Paulay 2003). The next name, *Holothuria maculata* Brandt, 1835, faired little better because it had been already used for a different species, *H. maculata* de Chamisso & Eysenhardt, 1821. The first available name is *Mülleria* (= *Holothuria*) *nobilis* (Selenka, 1867). Unfortunately, Selenka included

both a black-and-white specimen from Zanzibar and several completely black specimens from Hawaii within his description. He also described a specimen from Tahiti as *Mülleria hadra* on the basis of its apparently distinct calcareous ring. Bell (1887) added to the confusion by describing a black specimen from Samoa as *Holothuria whitmaei* without comparing it to previously known species. Saville-Kent (1890) described a black teatfish from the Great Barrier Reef as *H. mammifera*, which Rowe and Gates (1995) referred to *H. whitmaei*. Important taxonomic reviews by Clark (1921), Panning (1929), Rowe (1969) and Clark and Rowe (1971) treated all teatfish as a single species.

Scientists working in New Caledonia recognised that sympatric black and black/white mottled forms were distinct species. Cherbonnier (1980) listed the black specimens as *H. nobilis* and described a new species, *H. fuscogilva*, for the mottled black-and-white form. These species were also distinguished by differences in their body-wall skeleton (Cherbonnier 1980). The presence of two species in New Caledonia was confirmed by differences in their reproductive cycles and habitat preferences (Conand 1981). White teatfish generally inhabit deeper areas than the black form (Conand 1989, 1990; Reichenbach 1999; Lincoln Smith *et al.* 2000; Byrne *et al.* in press *b*). The black teatfish in the Pacific is a winter spawner and the white teatfish spawns in summer, although there is some regional variation in reproduction, apparently influenced by latitude and seasonal monsoons (Conand 1981, 1993; Reichenbach 1999; Ramofafia *et al.* 2000).

In a recent review, Rowe and Gates (1995) pointed out that Selenka's material of *H. nobilis* contained two colour forms, and that the original description and illustrations primarily referred to the black and white specimen that they designated as the lectotype. Consequently, according to the rules of zoological nomenclature, the name *H. nobilis* (with synonyms *M. hadra* and *H. fuscogilva*) was assigned to white teatfish (Rowe and Gates 1995). These authors did not recognise that the lateral white-spotted colour pattern of Selenka's *H. nobilis* and the mottled black and white colour pattern of Cherbonnier's *H. fuscogilva* differed. Rowe and Gates (1995) assigned *H. whitmaei* to the entirely black teatfish. These name changes have caused considerable confusion, with authors variously using *H. nobilis* for black (e.g. Uthicke and Benzie 2000*a*, 2003) or mottled black-and-white (e.g. Massin 1999) species. Uthicke and Benzie (2003), using molecular methods, further complicated the situation by suggesting the presence of a second species of black teatfish.

The aim of the present study was to provide molecular and morphological evidence to resolve the confusion surrounding the identity of teatfish from the western Indian and south-western Pacific Oceans. We examined Selenka's original material and revisited the morphological characters used to separate the species. A molecular phylogeny using mtDNA sequences presents the relationships between the teatfish species. Recent discussions have highlighted the need to list the teatfish, and other heavily fished holothurian species, on the Convention on International Trade in Endangered Species of Wild Fauna and Flora ('CITES', Buckner *et al.* 2003). Establishing the correct identity of the species comprising the teatfish fishery is essential to progress the conservation and fishery management of these holothurians.

Materials and methods

Specimens of teatfish were sampled from the Great Barrier Reef (GBR) at Raine Island (11°36′S 144°02′E), Wharton Reef (14°8′S 144°00′E) and One Tree Island (23°30′S 152°04′E); Western Australia (WA) (Tantibbiddi, 21°55′S 113°56′E); Fiji (Great Astrolabe Reef, 18°55′S 178°30′E); Papua New Guinea (PNG) (New Ireland 18°55′S 178°30′E); and La Réunion (21°09.8′S 55°16.9′E) in the Western Indian Ocean (Table 1). Some of the black teatfish sequences have been previously published (Uthicke and Benzie 2003). Sequences derived from specimens from Madagascar were provided by Igor Eeckhaut (University of Mons-Hainaut, Belgium). Outgroup samples of other aspidochirotid holothurians were obtained from the GBR at Wharton Reef, Linnet Reef (14°47′S 145°21′E), Davie Reef (13°58′S 144°29′E), Reef 19-156 (19°40′S 149°46′E) and One Tree Island. Tissue samples were obtained from cleaned gut lining or ventral body wall, preserved in 100% ethanol or, in some instances, snap frozen and stored at −80°C. Specimens from Raine Island, Lizard Island (14°40′S 145°27′E) and One Tree Island were preserved in 70% ethanol and deposited in Museum Victoria and the Australian Museum.

Morphological characters

We examined the lectotype of *H. nobilis* (MCZ 819) and holotype of *H. hadra* (MCZ 801) from the Museum of Comparative Zoology, Harvard University. Sections of skin from these specimens, other museum specimens, and freshly collected teatfish were dissolved in bleach to isolate the ossicles for scanning electron microscopy (SEM). The ossicles were washed with water, transferred to ethanol, mounted on a stub topped with a glass coverslip, and spluttered with gold. They were examined with a Philips XL20 scanning electron microscope using secondary electron imaging. The shape of the calcareous ring and the presence of Cuvierian tubules were also noted. Photographs of teatfish from various localities throughout the Indian and Pacific Oceans were sourced from the literature, and from scientists who have worked in the region, to determine the distribution of the various colour forms.

DNA extraction, PCR amplification and sequencing

Holothurian tissue samples were extracted with a DNeasy tissue extraction kit (Qiagen, Valencia, CA) following the instructions of the supplier with modifications as described in Uthicke and Benzie (2002). A region of the mitochondrial cytochrome oxidase I (*COI*) gene was amplified using primers described by Arndt *et al.* (1996) (COIe-F: 5′ATAATGAT AGGAGGRTTTGG3′, COIe-R: 5′GCTCGTGTRTCTACRTCCAT3′). These primers amplify 674 nucleotides of the echinoderm *COI* gene, corresponding to positions 6001–6674 of the mitochondrial genome of *Strongylocentrotus purpuratus* (Jacobs *et al.* 1988). PCR amplification was conducted as described in Uthicke and Benzie (2003). PCR products were cleaned using QIAquick PCR purification kit (Qiagen), diluted to final concentrations between 10 and 25 µg mL^{-1} and sequencing reactions conducted using Amersham (Dyenamic; Uppsala, Sweden) sequencing reagents. DNA from each sample was sequenced in both directions, and sequencing products were cleaned using Autoseq 50 (Amersham) clean-up columns. These samples were sequenced on an ABI 377 sequencer (Applied Biosystems, Foster City, CA). All

Table 1. Classification, sample locations and sample abbreviations used in this study

Species	Location/No.	Collection date	Reference	GenBank No.
Holothuriidae				
Holothuria (Microthele) fuscogilva	Raine Island 1	Nov. 2002	This study	AY700767
H. fuscogilva	Raine Island 2	Nov. 2001	This study	AY700765
H. fuscogilva	Raine Island 3	Nov. 2002	This study	AY700766
H. fuscogilva	Wharton Reef 1	Sep. 1998	This study	AY700763
H. fuscogilva	Wharton Reef 2	Sep. 1998	This study	AY700764
H. fuscogilva	Fiji 1	July 2001	This study	AY700768
H. fuscogilva	Fiji 2	July 2001	This study	AY700769
H. fuscogilva	Madagascar 1	–	3	AY700231
Holothuria (Microthele) whitmaei	Raine Is 4	Nov. 2002	This study	AY700762
H. whitmaei	Raine Island 5	Nov. 2002	This study	AY700761
H. whitmaei	One Tree Island 1	Jan. 2002	This study	AY700760
H. whitmaei	Fiji 3	July 2001	2	AY176777
H. whitmaei	Fiji 5	July 2001	2	AY176778
H. whitmaei	WA 1		2	AY176810.1
H. whitmaei	WA 2		2	AY176811.1
H. whitmaei	PNG 1		2	AY176780.1
H. whitmaei	PNG 2		2	AY176781.1
Holothuria (Microthele) nobilis	La Réunion 1	Dec. 1999	2	AY176775
H. nobilis	La Réunion 2	Dec. 1999	2	AY176776
H. nobilis	Madagascar 2	–	3	AY700232
Holothuria (Halodeima) atra	Reef 19-156	May 1999	This study	AY700770
Bohadschia marmorata	One Tree Island	March 2003	This study	AY700772
Actinopyga miliaris	Linnet Reef	Sep. 1998	This study	AY700773
Stichopodidae				
Parastichopus californicus	Pacific USA		1	U32198
Thelenota ananas	Davie Reef	Oct. 1998	This study	AY700771

References: 1 = Arndt *et al*. (1996), 2 = Uthicke and Benzie (2003), 3 = Igor Eeckhaut (unpublished). PNG, Papua New Guinea; WA, Western Australia.

sequences obtained have been submitted to GenBank (see Table 1 for Accession numbers).

Analyses of molecular data

We chose to analyse a slightly shortened fragment of the *COI* gene (529 bp between 6072 and 6600 of the mitochondrial genome of the echinoid *Strongylocentrotus purpuratus*) because of ambiguities in the beginning or end of some sequences. Sequences were aligned using SequEd software (version 1.03, Applied Biosystems, Foster City, CA). Previous analyses of a large dataset of black teatfish (Uthicke and Benzie 2003) showed highest likelihood scores using a model with a transition to transversion ratio of 7.8 and a gamma shape parameter of 0.749. Therefore, we also used the Kimura two parameter (Kimura 1980) substitution model with those parameters for genetic distance calculations conducted in Arlequin 2.000 (Schneider *et al*. 2000). Sequences were analysed using MEGA2 (version 2.1; available online at http://www.megasoftware.net/text/downloads.sht, verified 10 November 2004) and MrBayes (version 3.0B4, Huelsenbeck and Ronquist 2001) software that were used to create neighbour-joining (using Kimura two-parameter distances)/maximum-parsimony and Bayesian consensus trees respectively (burnin value = 100). Minimum spanning haplotype networks were created using TCS (version 1.13, Clement *et al*. 2000).

Results

Colour and habitat

The holotype of *Holothuria* (*M.*) *hadra* appears whitish (Fig. 1*a*) and superficially similar to some white teatfish morphs (see below). Most of the white teatfish from Australia and the south-west Pacific had off-white to beige background body-wall colour with irregular brown/black markings dorsally (Fig. 1*b–e*). These dark markings took two forms, small circles of black pigment around some of the tube feet, and larger more irregular black/brown markings spread across the dorsal surface (Fig. 1*c*). The ventral surfaces of white teatfish are white, yellow or grey. In some individuals from Australia and the south-west Pacific, the dark markings were not well developed. Two specimens found in very shallow water (2 m) on the reef flat at Raine Island were a lighter colour form (Fig. 1*b,c*). The specimen from Madagascar sequenced for this study was consistent with the darker colour pattern (I. Eeckhaut, personal communication). Photographs of putative white teatfish from various western and northern Indian Ocean localities (Table 2) revealed other colour variations, including grey, beige (Fig. 1*f,g*) and reticulate forms (not shown). White teatfish from Australia and the south-west Pacific were generally (but not always) present in deeper water (>10 m) than black ones (0–10 m).

Black teatfish from Australia and the south-west Pacific were uniform black in colour as adults (Fig. 1*h*). Juvenile black teatfish may have beige or white markings on the dorsal surface (Fig. 1*i*), but their sole is usually black (Fig. 1*j*). Black
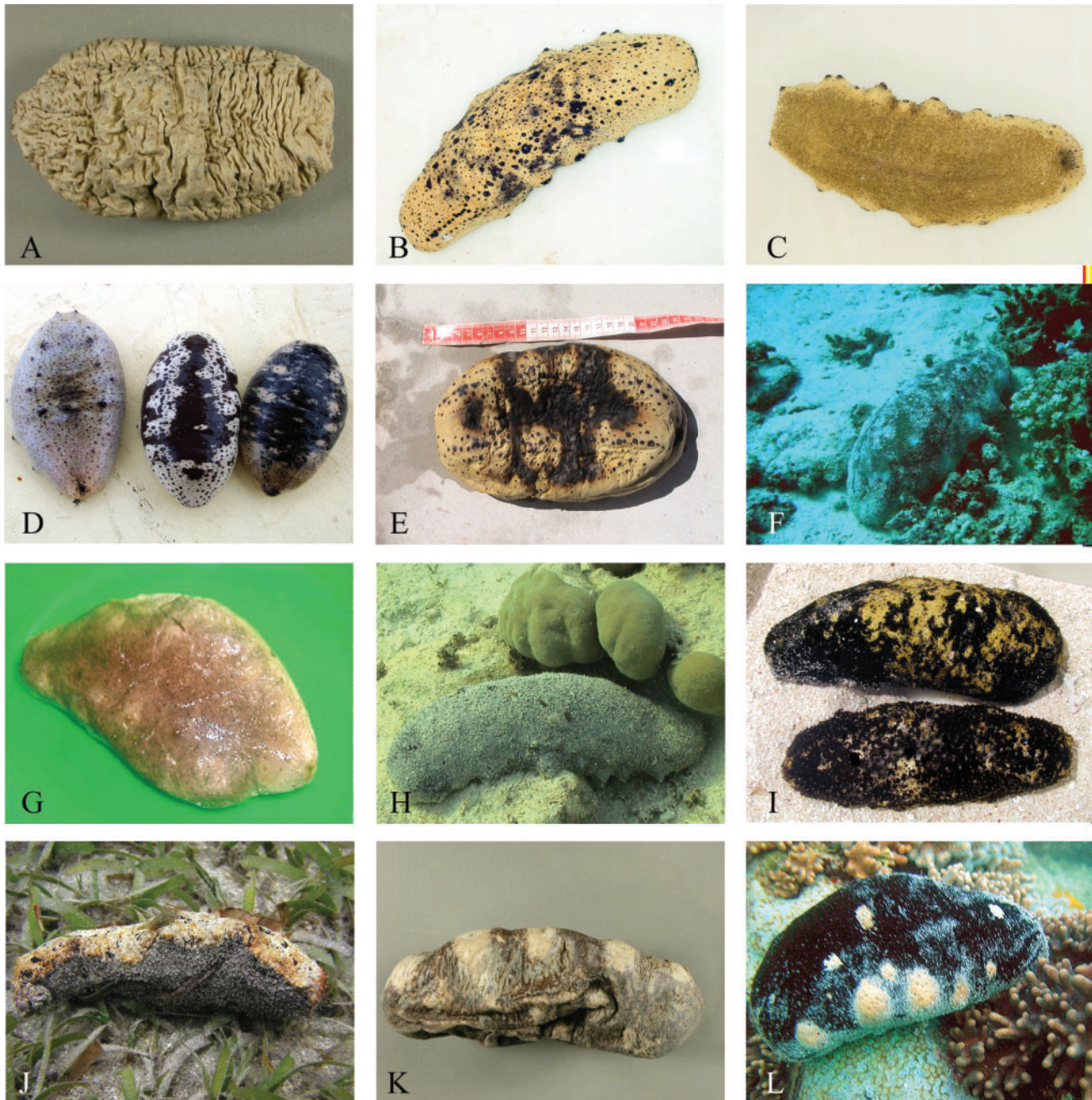
Fig. 1. *Holothuria* (*Microthele*) spp.: colour variation in the *H. hadra* (*A*), white teatfish (*H. fuscogilva*, *B–G*) and black teatfish (*H. whitmaei*, *H–J*; *H. nobilis*, *K–L*). *A*, *H. hadra* holotype (Selenka 1867) (MCZ 801, 14 cm) from Tahiti. This species was previously synonymised with *H. nobilis* (Cherbonnier 1980). The type investigated shows more similarities to *H. fuscogilva*. However, the outer epidermis is heavily damaged and its taxonomic status is unresolved. *B–G*, *H. fuscogilva*: *B*, small and rather white individual as occasionally found on the reef top (Raine Island, 16 cm); *C*, same individual as *B*, ventral view (the ventral side is uniformly white/beige for all colour variants of *H. fuscogilva*); *D*, colour variation in *H. fuscogilva* as seen in Papua New Guinea (animals 30–35 cm in length); *E*, most common variant in deeper areas of the GBR (Ethanol preserved specimen 25 cm, *c*. 35 cm before preservation); *F/G*, more greyish brown individuals as found in the Red Sea, but also generally in the Indian Ocean (both *c*. 40 cm). *H–J*, *H. whitmaei*: *H*, typical entirely black individual (*c*. 30 cm), the ventral side may also be dark brown or grey (*in situ* these animals appear often grey because of a cover with fine sediment); *I/J*, the dorsal side of juveniles can be yellowish or with black and yellow patches, somewhat similar to *H. fuscogilva* (however, the ventral side is always entirely dark); *I*, specimen from a seagrass bed in Raine Island (both individuals *c*. 16 cm); *J*, specimen from Vanuatu (*c*. 12 cm). *K*, *L*, *H. nobilis*: *K*, lectotype from Zanzibar (MCZ 819, Selenka 1867, 35 cm); *L*, *in situ* in the Red Sea (35 cm). Credits for pictures not taken by the authors: *D*, Tim Skewes, CSIRO, Brisbane Australia; *F/G/L*, Mohammed I Ahmed, University of Hull, UK; *J*, Kim Friedman, South Pacific Commission, Noumea, New Caledonia.

Table 2. Distribution of teatfish species based on reported molecular evidence or colour pattern

Locality	*H. whitmaei*	*H. fuscogilva*	*H. nobilis*	Reference
Easter Island		X[A]		Massin 1996
Hawaii	X[A]			Selenka 1867
Tahiti		X		Selenka 1867
Samoa	X			Bell 1887
Northern Mariana Island	X			Trianni 2002
Guam	X[A]	X		Rowe and Doty 1977; Kerr *et al.* 1992
Fiji	X	X		This study
New Caledonia	X[A]	X		Cherbonnier 1980; Conand 1981, 1990, 1998*b*
Vanuatu	X	X		K. Friedman, personal communication
Solomon Island	X[A]	X		Ramofafia *et al.* 2000; Lincoln Smith *et al.* 2000
Papua New Guinea	X	X		This study; Gosliner *et al.* 1996
Coral Sea	X	X		S. Uthicke (unpublished data)
Great Barrier Reef	X	X		This study
Western Australia	X	X		This study; Russell and Vail 1988
Indonesia (Sulawesi)		X[A]		Massin 1999
Malaysia	X[A]	X		Forbes and Baine 1998
Philippines	X[A]	X[A]		Anon. 1984; Schoppe 2000
Taiwan	X[A]			Chao and Chang 1989
India		X[B]	X[C]	James 2001
Maldives		X[B]	X[C]	Reichenbach 1999
Red Sea (Egypt)		X[B]	X	M. I. Ahmed, personal communication
Seychelles		X[B]	X	R. Aumeeruddy, personal communication
East Africa		X[B]	X	Samyn 2003
La Réunion			X	This study
Madagascar		X[B]	X	This study; Cherbonnier 1988; Conand 1999

[A]Recorded as *H. nobilis*. [B]It is still unclear whether there are one or more white teatfish from the Western and northern Indian Oceans (see text). [C]The species status of black teatfish from India and the Maldives requires confirmation (see text).

teatfish specimens from La Réunion and Madagascar were mainly black with large white patches around some dorsal tube feet and along the teats on the ventrolateral margin of the animal (Fig. 1*l*). Photographs of putative black teatfish from off India (James 2001) and the Maldives (N. Reichenbach, personal communication) differed in having lateral white patches similar to those of east African *H. nobilis* except that they are larger and sometimes fused.

Morphology and nomenclature

The colour pattern on the body wall of the lectotype of *H. nobilis* (MCZ 819, Fig. 1*k*) from Zanzibar (Selenka 1867) exactly matches the black form with lateral white dots of specimens from Madagascar and La Réunion sequenced for this study (Fig. 1*l*). The Indian Ocean morph is thus consistent with the original *H. nobilis*. The holotypes of *H. whitmaei* (BMNH 1875.10.2.6) and presumably *H. mammifera* (locality of types not known) are completely black (Rowe and Gates 1995). Consequently, the appropriate name for the black teatfish from Australia and the Western Pacific sequenced for this study is *H. whitmaei*, as proposed by Rowe and Gates (1995). The remaining two names, *H. hadra* and *H. fuscogilva*, are available for the white teatfish from the East Indian and Pacific Oceans, with *H. hadra* having date priority. The holotype of *H. hadra* (MCZ 801, Fig. 1*a*) is small (14.5 cm in length) and coloured off-white with a few faint spots of dark

pigment on the dorsal surface, faint dark markings near the anus and a light brown colour ventrally. The epidermal pigment layer of this specimen may have been damaged before preservation (Selenka 1867), so its identity is not clear. There is no other morphological character that allowed us to decide whether this is really a white teatfish, a black teatfish or a further species. An attempt to extract DNA from the type was unsuccessful. Consequently, we have retained the name *H. fuscogilva* for the white teatfish.

Scanning electron microscopy revealed fenestrated ellipsoids and rudimentary tables (Fig. 2*a*) in the tentacles of *H. nobilis*, *H. whitmaei* and *H. fuscogilva*. Further examination of body wall and tentacle ossicles in specimens of the three species revealed little difference in the overall shape or size of tables and buttons from the body wall (Fig. 2*b,e,h*) or rods from the tentacles (Fig. 2*c,f,i*). In most cases, the body-wall buttons were complex fenestrated ellipsoids (e.g. Fig. 2*b* lower left). However, relatively simple buttons with central or rim-like projections were common in the ventral body wall of the smaller *H. fuscogilva* specimens from Australia and the south-west Pacific (Fig. 2*d*) and, more rarely, in specimens of *H. whitmaei* (Fig. 2*g*). They were present in the type specimen of *H. hadra*, but not *H. nobilis*.

The calcareous ring is variable in all teatfish colour forms examined. The radial plate is larger than the interradial plate, but the relative height of each plate can differ, with the radial
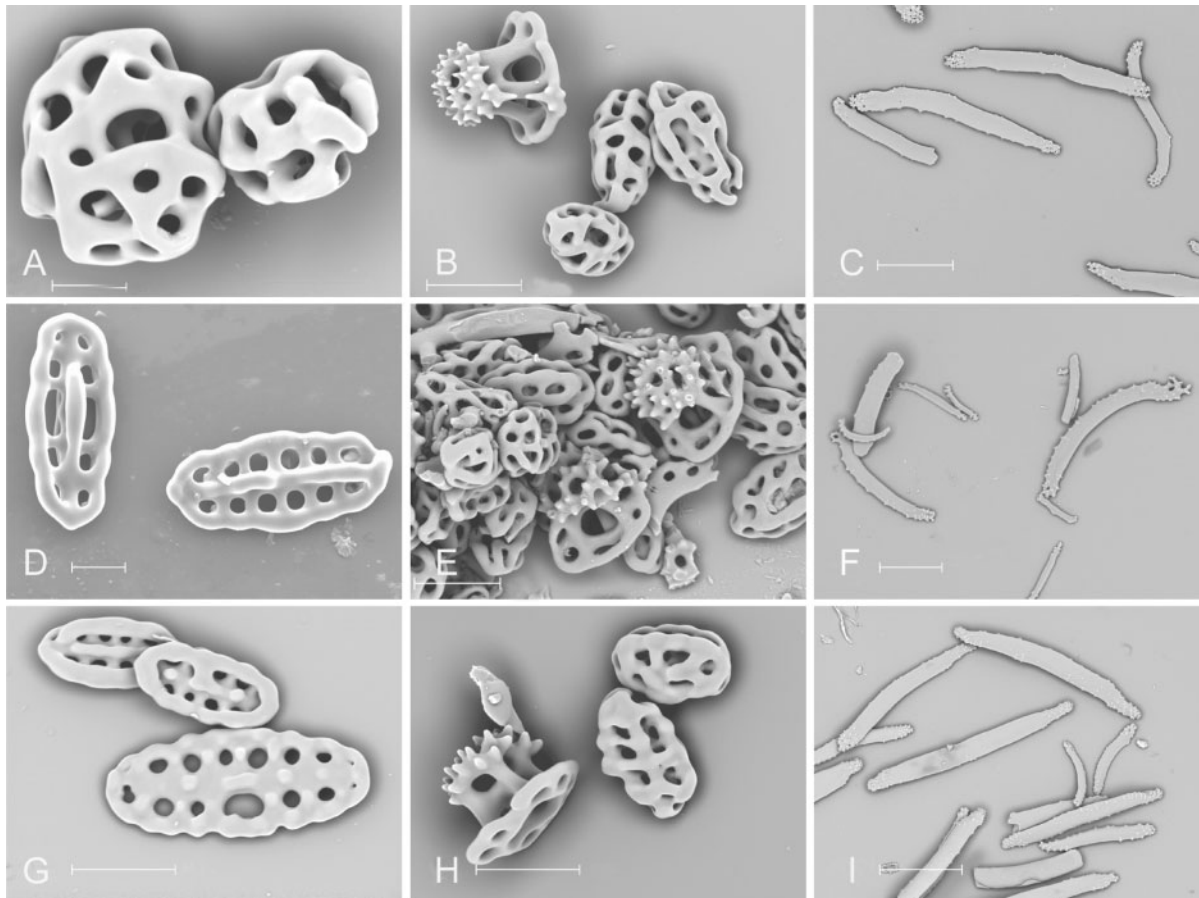
Fig. 2. *Holothuria* (*Microthele*) spp. SEM photographs of ossicles of the three species. *A–D, H. fuscogilva* (Wharton Reef, MoV F101794): *A*, tentacle ellipsoids (scale bar: 20 µm); *B*, body-wall tables and fenestrated ellipsoid buttons (50 µm); *C*, tentacle rods (200 µm); *D*, ventral body-wall simple buttons (20 µm). *E, F, H. nobilis* (Zanzibar, Lectotype, MCZ 819): *E*, body-wall tables and fenestrated ellipsoid buttons (50 µm); *F*, tentacle rods (100 µm). *G–I, H. whitmaei* (One Tree Island, MoV F101795): *G*, ventral body-wall simple buttons (200 µm); *H*, body-wall towers and fenestrated ellipsoid buttons (50 µm); *I*, tentacle rods (200 µm).

plate ranging from 1.2 to 2.5 times as high as the radial one. Some of this variation is related to size, with smaller specimens having relatively larger radial plates. The anterior projections on the radial plate consist of a central notched one and a lateral one on each side, but the shape and size of these projections can vary from large and scalloped to small and narrow. The posterior margin of the radial plate is also notched. Selenka (1867) distinguished his species *H. hadra* from *H. nobilis* on the basis that the radial plates of the calcareous ring were relatively much larger than the interradial plates in *H. hadra*. However, re-examination of the types of these species showed that Selenka's (1867) illustration of the ring of the *H. nobilis* lectotype is not accurate. The radial plates were 7.5 mm high, 2.5 times the height of the interradial ones, similar to that from *H. hadra*.

The specimens of *H. whitmaei* examined here and in previous studies (*n* >1000; Uthicke and Benzie 2000*b*, 2003) from Australia and the south-west Pacific, had thick white Cuvierian tubules (5–10 cm long). When present, the tubules are rarely discharged after disturbance in the field and are not

sticky. Cuvierian tubules observed in *H. nobilis* in Madagascar are similar to those in *H. whitmaei* (C. Conand, personal communication). In the white teatfish examined (*n* = 10), the Cuvierian tubules are rudimentary or absent. If present, they were 2–3 cm long and 1–2 mm in diameter and were not sticky.

Molecular analyses

The sequences were readily aligned, with no insertion and/or deletion events detected. A total of 201 nucleotides were variable in the total dataset, including outgroups. As expected, most of these (160) were on the third codon position and thus many were silent substitutions. However, translation of the sequences to amino-acid sequences showed that 20 amino acids differed between the species. When only compared among the ingroup, 119 base pairs were polymorphic and only 9 amino-acid substitutions occurred. The Bayesian consensus tree produced from 9900 maximum-likelihood analyses (Fig. 3) had a similar topology to trees generated by neighbour-joining and maximum parsimony (both not
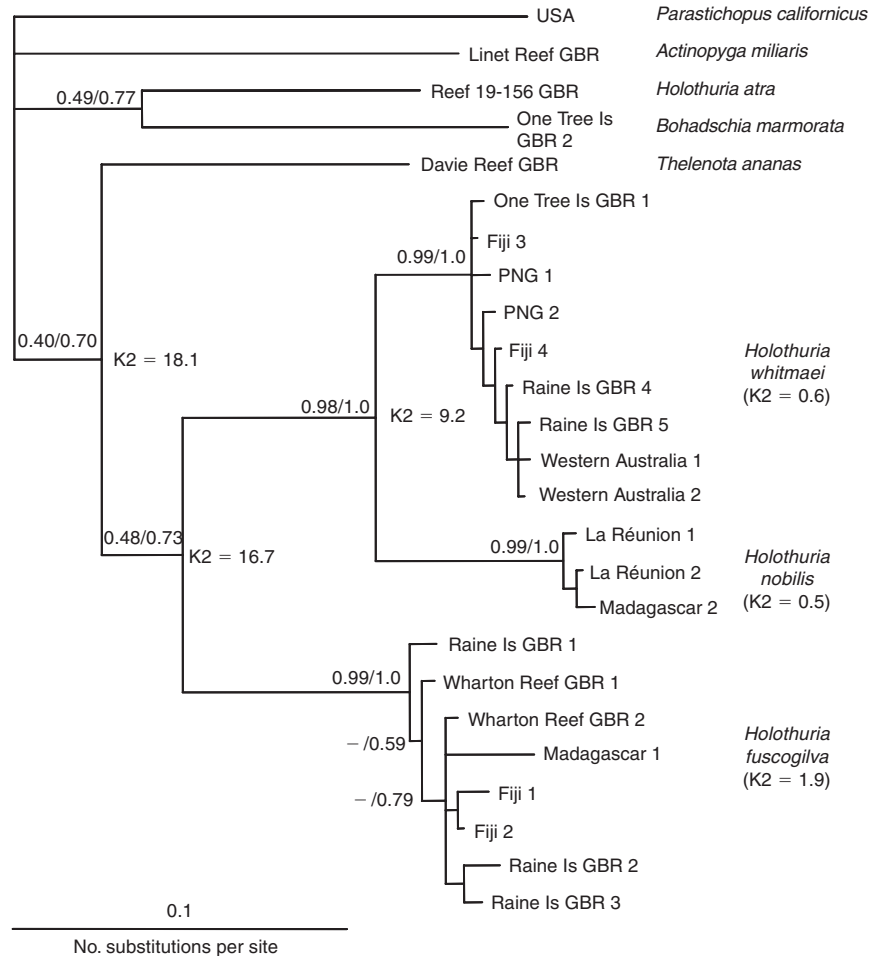
Fig. 3. *Holothuria* (*Microthele*) spp. Bayesian consensus tree for teatfish and outgroups based on 529 bp of the mtDNA gene *COI* and 9900 maximum-likelihood trees (burnin value of 100). Numbers on the left side of the nodes identify maximum parsimony (MP) bootstrap values/Bayesian maximum-likelihood (BML) posterior probabilities. Maximum parsimony boot-strap values are not given for the two internal *H. fuscogilva* clades because the MP tree had a slightly different configuration with the Madagascar specimen being a sister branch to all other *H. fuscogilva* specimens. Numbers on the right side of the nodes are average pairwise Kimura-2 distances between samples in each branch. The numbers in brackets under the species names are average Kimura-2 parameter distances within each species. GBR, Great Barrier Reef; PNG, Papua New Guinea.

shown), differing only in the topology of the terminal clus-ters. All teatfish haplotypes were monophyletic with respect to the outgroup. Within this major cluster, all specimens that were coloured white with dark patches formed a single clus-ter (labelled on Fig. 3 as *Holothuria fuscogilva*). This cluster included the unusual light-coloured specimens from Raine Island (see above). The black teatfish from Australia and the south-west Pacific also clustered together (*H. whitmaei*). A third cluster was formed from black and white specimens from La Réunion and Madagascar (*H. nobilis*).

The variation within these three clusters was far less than variation between clusters (Fig. 3). Average Kimura genetic distances within species were 1.9, 0.6 and 0.5% for *H. fuscogilva*, *H. whitmaei* and *H. nobilis* respectively. In

contrast, average genetic distance between clusters was 9.2% between *H. nobilis* and *H. whitmaei* and 16.7% between *H. nobilis–H. whitmaei* and *H. fuscogilva*.

The relationship between the teatfish and other aspidochi-rotid holothurian (outgroup) species was unresolved, and the few patterns emerging are not consistent with current under-standing of their phylogeny. The average Kimura genetic distances between the teatfish and the other holothurian sam-pled exceeded 18%. The relationship between number of transitions and Kimura distance ceased to be linear after distances exceeding 10%, indicating substitution saturation (data not shown). Consequently, the anomalous result of *The-lenota ananas* (in the family Stichopodidae) appearing to be more closely related to the *Holothuria* (*Microthele*) cluster
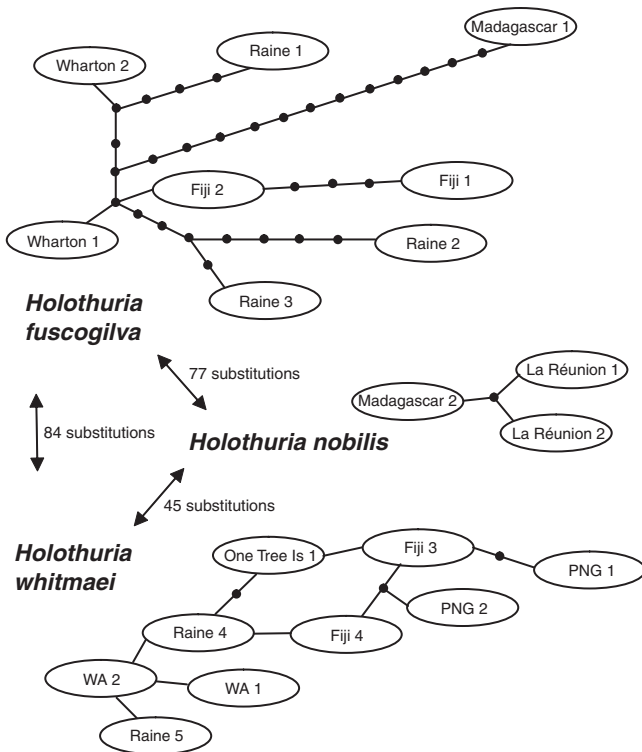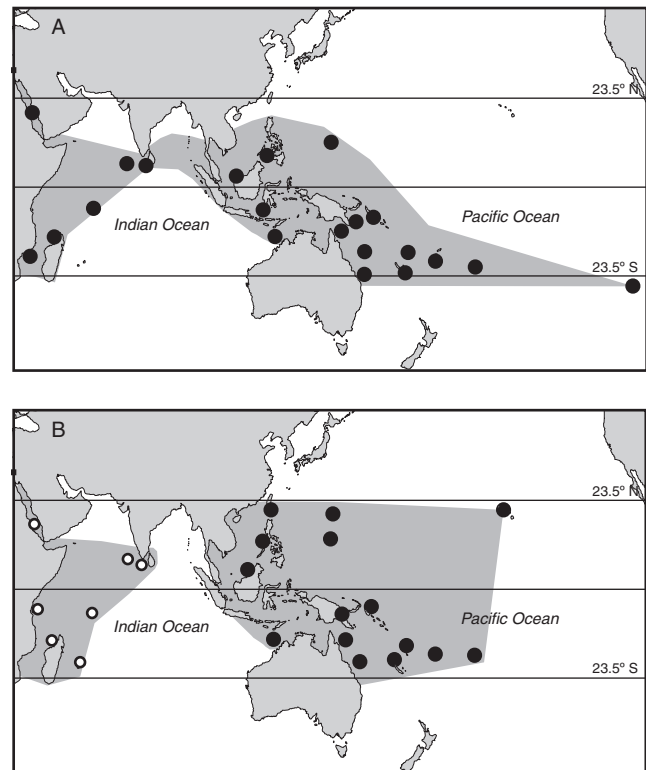
Fig. 4. *Holothuria* (*Microthele*) spp. Minimum spanning trees for 529 bp *COI* sequences of *Holothuria fuscogilva*, *H. nobilis* and *H. whitmaei*. Black dots represent intermediate un-sampled haplotypes. Numbers next to the double-headed arrows are the mean number of substitutions between haplotypes of each species. PNG, Papua New Guinea; WA, Western Australia.

Fig. 5. *Holothuria* (*Microthele*) spp. Geographic distribution of *H.* (*M.*) *fuscogilva* (*A*) and *H.* (*M.*) *nobilis* (*B*, white dots) and *H.* (*M.*) *whitmaei* (*B*, black dots). Dots represent areas of confirmed observations explained in more detail in Table 2.

than *Holothuria* (*Halodeima*) *atra* (Fig. 3) is likely to be an artefact of the analysis.

The genetic variability within the *H. fuscogilva* clade is much higher than for *H. whitmaei*. This can be demonstrated with minimum-spanning networks of haplotypes (Fig. 4). In particular, the Madagascar 1 specimen differs from other *H. fuscogilva* haplotypes by 15 substitutions or more (3% of the sequence). Its placement on the various trees is variable. On the Bayesian consensus tree, it is nested well within the *H. fuscogilva* cluster, whereas on the neighbour-joining and maximum-parsimony trees, it is sister to all other *H. fuscogilva* specimens (not shown). More samples from east Africa are required to resolve whether specimens from that area should be regarded as a separate species.

Distribution

Current data on the distributions of *H. nobilis*, *H. whitmaei* and *H. fuscogilva*, where identities are matched with figures and photographs, is given in Table 2 and illustrated in Fig. 5. *Holothuria fuscogilva* (including all its colour variants) occurs from Madagascar across to Easter Island and from southern China south to Lord Howe Island. This species

(or species complex) covers roughly the range of the combined black teatfish species. *Holothuria nobilis* occurs from the east coast of South Africa to the Red and Arabian Seas, including Madagascar and La Réunion. Its presence in India and the Maldives is uncertain (see above). *Holothuria whitmaei* ranges from Western Australia east to Hawaii/Tahiti and southern China south to Elizabeth Reef in the Tasman Sea.

Discussion

Molecular and morphological analyses have indicated that there are more species of teatfish than currently assumed by taxonomists and for fisheries management. Although there are two recognised species of teatfish (Rowe and Gates 1995), our molecular analysis resolved three haplotype clusters. These corresponded with distinct colour forms: black/dark brown specimens from Australia and south-west Pacific (*H. whitmaei*), white/beige specimens with dark markings from Australia and the south-west Pacific (*H. fuscogilva*), and black specimens with white ventro-lateral patches from the western Indian Ocean (*H. nobilis*). Previously, *H. fuscogilva* had been confused with *H. nobilis*. However, genetic analyses indicated that *H. nobilis* is a sister species to *H. whitmaei* and more distantly related to *H. fuscogilva*.

The most useful taxonomic characters for distinguishing holothurian species are thought to be the distribution and morphology of the calcareous ossicles present in the body wall, tentacles and tube feet, and the structure of the calcareous ring. White teatfish have been distinguished from black teatfish by having more complex table ossicles, the presence of simple button ossicles (as well as tables and ellipsoids) in the ventral body wall, larger tentacle rod ossicles, the presence of spiny rods in the tube feet (Cherbonnier 1980) and the shape of the calcareous ring (Selenka 1867). We found, however, that most types of ossicles and calcareous rings have a similar range of morphological variability in each of the three species and cannot be relied on to distinguish them. The exception is the presence of simple button ossicles in the ventral body wall, which appear from our material to be common in *H. fuscogilva*, rare in *H. whitmaei* and absent from *H. nobilis*. However, both Massin (1999) and Samyn (2003) reported some white teatfish specimens without these buttons, and the presence of these buttons cannot be considered a definitive taxonomic character at this time.

Although the presence of Cuvierian tubules in teatfish species has been considered to be useful for taxonomy (Cherbonnier 1980), these structures have been shown to be highly variable within species (Massin 1999; Samyn 2003). We found that rudimentary Cuvierian tubules were present in some *H. fuscogilva* specimens, but not others, an observation also reported in previous studies (Massin 1999; Samyn 2003). On the other hand, all specimens of *H. whitmaei* examined here and in previous studies (Uthicke and Benzie 2000b, 2003) possessed Cuvierian tubules. In contrast to the well developed sticky tubules with a defence function of other holothuroids (Vanden Spiegel *et al.* 2000), the Cuvierian tubules of teatfish are small or absent, and non-adhesive. Teatfish have a characteristic ring of anal teeth that may serve a defensive role, for instance to deter parasitic pearl fishes that inhabit the cloaca of tropical aspidochirotids. Possession of anal teeth in teatfish may be associated with reduced selection to maintain a Cuvierian tubule system as a major line of defence, resulting in variable development of these structures in some species.

The presence of three species explains why previous authors have had difficulties assigning the correct names to teatfish species. Much of the confusion stems from Selenka's inclusion of two different colour forms in his original description (Selenka 1867). Based on Selenka's sample locations and our comparative data, we can confirm that the black form with one or two lines of lateral white dots from east Africa, as illustrated by Conand (1999) and Samyn (2003) is *H. nobilis*, and the entirely black specimens from Hawaii are *H. whitmaei*. *Holothuria whitmaei* and *H. nobilis* appear strictly allopatric, roughly exhibiting Pacific and Indian Ocean distributions, respectively, whereas *H. fuscogilva* occurs in both oceans. Many authors may have only been aware of two species within their main region of work. For example,

Samyn (2003; in Kenya) conducted a detailed analysis using Cherbonnier's (1980; in New Caledonia) criteria to distinguish *H. whitmaei* (as *H. nobilis*) and *H. fuscogilva*. Similarly, authors from the Pacific (including ourselves) were not aware that a black teatfish with white areas (*H. nobilis*) occurs in the western Indian Ocean. Photographic evidence from India and the Maldives (James 2001; N. Reichenbach, personal communication) suggests that yet another colour form exists. Unfortunately, we could not obtain material, and thus cannot decide if this is a third black teatfish species, or a colour variant of *H. nobilis* or *H. whitmaei*.

In contrast to the relatively consistent colour morphs of the two black teatfish species, *H. fuscogilva* specimens in the south-west Pacific were variable, ranging from almost all white to white with large dark patches on the dorsal surface. However, our genetic analyses did not separate out light and dark specimens as separate clusters. The two small *H. fuscogilva* specimens found atypically in very shallow water (2 m) on the reef flat at Raine Island were a lighter colour form, similar to those illustrated by Massin (1999). In the Maldives, shallow water *H. fuscogilva* specimens are smaller and appear to migrate to deeper areas as they grow (Reichenbach 1999).

The identity of white teatfish from the Indian Ocean is unclear. The sequence of a specimen from Madagascar differed, on average, by 3% from specimens of *H. fuscogilva* from Australia and the south-west Pacific. Some white teatfish from the Maldives and Seychelles are white with reticulate brown markings dorsally (R. Aumeeruddy, N. Reichenbach, personal communication), these specimen are referred to as 'pentard' or 'tiger bum' by local fishers. Other colour variations include a greyish brown form from east Africa and a brown form from India and the Red Sea (Conand 1999; James 2001). More material is required to resolve whether these animals are part of one variable species or represent distinct species. For the present, all the white teatfish from the Indo-Pacific are referred to as *H. fuscogilva*.

Evolutionary history

The molecular tree (Fig. 3) indicates that the primary division within the teatfish is between the mainly black (*H. whitmaei* and *H. nobilis*) and mainly white (*H. fuscogilva*) species, with an average genetic distance between these lineages of 16.7%. Estimates of evolutionary rates within the *COI* gene for echinoderms have ranged from 2 to 5% per million years (Benzie 1999; Lessios *et al.* 2001), based on divergence after the closure of the Isthmus of Panama. This evolutionary rate would date the divergence at 3.3–8.3 million years. It is unclear what event would have prompted the speciation. The late Miocene (6–10 million years) was a period of global cooling, lowered sea-levels and exposed continental shelves (McGowan *et al.* 2000), which would have increased the potential for allopatric speciation. It is unclear where

this speciation event would have occurred because current distributions are largely sympatric.

The black teatfish are divided into two allopatric species: *H. nobilis* in the western Indian Ocean and *H. whitmaei* in Australia and the Pacific. A genetic distance of 9.2% between these implies a divergence during the Pliocene of ~1.8–4.6 million years. Phylogeographic breaks across the Indian Ocean have been recorded for other echinoderm species. The western and northern Indian Ocean populations of the sea-stars *Linckia laevigata* and *Acanthaster planci* differed from those in Australia and the Western Pacific (Williams and Benzie 1998; Benzie 1999). The situation in the south-east Indian Ocean was complex. For both seastars, populations from Western Australia clustered with the Pacific clade. In both cases, the difference between the Indian and Pacific clades was interpreted as intraspecific based on relatively low levels of sequence divergence.

Reduced gene flow through altered current patterns and closures of sea straits such as the Torres Strait between Papua New Guinea and Australia during low water levels of cool geological periods have been evoked to explain population genetic differences between Indian Ocean and Pacific marine invertebrate populations (Benzie 1999). Similar events earlier in the Pliocene may have separated populations of the two black teatfish species, and subsequently allopatric speciation has occurred and prevented mixing after a change of conditions.

Holothuria whitmaei has a flat genetic structure ('star phylogeny') that might imply a recent population bottleneck or rapid population expansion (Uthicke and Benzie 2003). Although only three sequences are available, the sequences of *H. nobilis* also only differ by one or two base pairs. In contrast, haplotypes of the *H. fuscogilva* complex are quite diverse, even if only samples within the west Pacific regions are compared (Fig. 4). Sequence distances between samples range from 0.4 to 4.1% and in our sample every haplotype was unique. This implies either a stable population with a high long-term effective population size, or a recent mixing of haplotypes from previously allopatric populations (Grant and Bowen 1998). It is interesting to note that any events that lead to speciation among the other two species, did not appear to lead to distinct speciation in the white forms, unless later studies suggest that the unusual colour forms from the Indian Ocean constitute other species. One reason for this might be the deeper habitat of white teatfish. Generally, the shallow coral reef habitat of black teatfish can be quite reduced during glacial low water levels. Thus, in addition to physical barriers and changes in current regimes (see above), residual populations might be quite distant from each other, reducing gene flow and potentially promoting allopatric speciation. This might be less consequential for species that also occur in deeper habitats, which have access to more refugia during lower sea levels. Thus, populations might undergo fewer bottlenecks, leading to more diverse haplotypes. However,

this hypothesis needs further testing with a larger sample size using animals of the *H. fuscogilva* complex from a wide variety of locations.

Conservation significance

Morphological and molecular data presented here indicate that diversity in the teatfish is higher than assumed: three species can now clearly be delineated, with the potential for several more cryptic species. The description of three teatfish species has significant implications for conservation and fisheries management. Although rarely practised for holothurian fisheries, a responsible management plan should be based on biological information such as growth rates, reproductive time and output, juvenile recruitment and habitat of each species. Scientific work on these parameters is only possible after the species are clearly defined. Indeed, the few ecological studies on *H. whitmaei* (referred to mainly as *H. nobilis*) and *H. fuscogilva* indicated large differences, for example, in reproductive time and habitat preferences. It is now apparent that there is hardly any information on the ecology of the 'real' *H. nobilis*. With the number of fished species greater than previously assumed, individual species would be more vulnerable to extinction, because species regionally overfished cannot be replenished from regions where only sibling species occur.

The lack of morphological markers (apart from colour) that distinguish the teatfish species carries implications for our understanding of holothurian biodiversity and for their conservation. Currently, holothurian taxonomy extensively utilises the shape of ossicles in the body wall to delineate species. Many of these morphological species might prove to be species complexes and our current assessment of holothurian diversity might be a considerable underestimate. The lack of morphological markers also increases the difficulty of identifying species that have been processed for human consumption. Processing holothurians for bêche-de-mer typically removes the epidermal pigment layer and so colour cannot be used for identification. It thus becomes important to know the origin of processed material to assist with species identification. The difficulty in identifying processed material is one of the main obstacles for listing holothurians under Appendix 2 of the CITES convention on trade in endangered species. Further studies are required to resolve the identity of teatfish from the Indian Ocean. Until such a study is completed, it will be difficult to manage teatfish fisheries in this region.

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