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REPORT

Hybridization of reef fishes at the Indo-Pacific biogeographic barrier: a case study

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Abstract Hybridization is recognized as an important source of genetic variation. In some reef fishes, including the Acanthuridae, hybridization has been detected due to intermediate colouration. This study used a molecular genetic approach to investigate hybridization in two Acanthurid species: *Acanthurus leucosternon* and *Acanthurus nigricans*, which have Indian and Pacific Ocean distributions respectively and are sympatric in the eastern Indian Ocean. In this area a putatitve hybrid, *Acanthurus* cf. *leucosternon* has been recognized based on intermediate colouration and restriction to the sympatric region of otherwise allopatric putative parental species. This study aimed to test this hypothesis using genetic tools. The three species were sampled from Cocos (Keeling) and Christmas Islands, the biogeographic boundary where many Indian and Pacific Ocean biota meet. Representatives from allopatric populations of both parental species and outgroups were also sampled. Mitochondrial COI and intron 1 of the nuclear ribosomal protein S7 were sequenced from 13 and 30 specimens respectively. Although sample sizes in this study are relatively small and more genetic data, including an extended phylogeographic sampling, is required to further evaluate these findings, the COI results support hybrid origins of *Acanthurus* cf. *leucosternon*, but S7 data are inconclusive due to the possibility of incomplete lineage sorting. The fourfold more abundant *Acanthurus nigricans* is most often the maternal parent. Inter-fertile hybrids apparently backcross with rare *Acanthurus leucosternon* males, transferring *Acanthurus nigricans* mitochondria to this species. These results suggest that *Acanthurus leucosternon* may eventually be lost from these islands, due to their relative rarity and introgressive hybridization.

Keywords *Acanthuridae* · Hybridization · Introgression · COI and S7 intron 1 · Biogeography · Mating behaviour

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Introduction

Coral reefs support a great diversity of fishes, which are equally diverse in colouration. The responsible mechanisms for this diversity are poorly understood, particularly as there are few barriers to dispersal in the marine environment (McMillan et al. 1999; Bernardi et al. 2002). The identification of many closely related coral reef fishes relies on colour differences (Lieske and Meyers 1999) as they are often morphologically and meristically indistinguishable (Jansson et al. 1991; Wilkins et al. 1994). However, many species have multiple colour morphs (Taylor and Hellberg 2003; Messmer et al. 2005). The relationship between colour pattern and genetic divergence has been identified in several studies (for example McMillan et al. 1999;

Messmer et al. 2005). Moreover, there are cryptic species, where no colour or meristic differences are apparent, but populations are genetically differentiated (e.g. *Dascyllus*; Bernardi et al. 2002). This is further complicated by the fact that genetic differentiation has been identified between some colour morphs, but not others, both within and between populations. For example, the spiny damselfish, *Acanthochromis polyacanthus*, has different colour morphs on the same reef but these cannot be distinguished genetically (Planes and Doherty 1997; van Herwerden and Doherty 2006). The contrary was found for the same species on other reefs (van Herwerden and Doherty 2006). In addition, it appears that same colour morphs exhibit genetic differences between isolated locations (van Herwerden and Doherty 2006). Contrary to the spiny damselfish, at a range of locations in the Caribbean, the hamlets of the genus *Hypoplectrus* consists of many colour morphs, but these are genetically indistinguishable (Ramon et al. 2003; Garcia-Machado et al. 2004).

It is therefore important to use genetic approaches to identify whether there is gene flow between populations and/or colour morphs (Knowlton 2000). The genetic identification of species can however, be confounded by gene flow between them due to hybridization or incomplete lineage sorting. Hybrids are individuals that are the offspring of dissimilar parents, which are distinguishable by one or more heritable characters (Harrison 1993). Hybrids generally occur in hybrid zones which are geographical regions in which previously isolated populations/species have come into contact and inter-bred (Harrison 1993). Such interbreeding is favoured by factors such as abundance differences between the participant species (Arnold 1997). There are many examples in the terrestrial environment of diverged species hybridizing when they come into secondary contact at biogeographic borders (see review by Mallet 2005). Biogeographic borders also exist in the marine environment (Williams and Benzie 1998; Lessios et al. 1999; Bernardi et al. 2001), however, due to a lack of studies it is unclear whether secondary contact at biogeographic boundaries is a typical cause of hybridization in coral reef fishes.

The use of molecular genetic analyses is particularly helpful in the study of hybridization and has been applied to a number of marine species (e.g. Brown 1995; Gardner 1996), especially corals (see references in van Oppen and Gates 2006). The Atlantic cod, *Gadus morhua*, which has been the subject of population genetic studies for several decades (Ruzzante et al. 2000; Hutchinson et al. 2001) provides evidence of mixing between differentiated populations of Atlantic cod in a temperate marine hybrid zone (e.g. Ruzzante et al. 2000). An increasing number of studies on coral reef fishes have further improved our understanding of hybridization in the marine environment, for example in *Siganus* (Lacson and Nelson 1993), several damselfish genera (Lacson 1994; Lacson and Clark 1995, van Herwerden and Doherty 2006), *Chaetodon* (McMillan et al. 1999), serranids (van Herwerden et al. 2006) and wrasses (Yaakub et al. 2006, 2007), but none of these studies have examined hybridization at a significant marine biogeographic boundary.

In this study, three morphologically similar fish "species" that have distinct colours were characterised genetically: *Acanthurus nigricans* (Linnaeus 1758), the whitecheek surgeonfish, *Acanthurus leucosternon* (Bennett 1833), the powderblue surgeonfish and *Acanthurus* cf. *leucosternon*, a putative hybrid of the afore-mentioned species. These species are Perciformes in the class Actinopterygii. *Acanthurus nigricans* and *Acanthurus leucosternon* are widespread in the Pacific and Indian Oceans respectively (Fig. 1). These allopatric species are separated by the east Indian Ocean biogeographic barrier and their ranges overlap at Christmas and Cocos (Keeling) Islands (Fig. 1) where they are sympatric. These sympatric populations are in contact, presumably due to range expansions by each species from their respective locations. Thus, gene flow between these species is theoretically possible and hybridization may therefore occur.

In order to test the hypothesis that hybridization has produced the *Acanthurus cf leucosternon* type, genetic analyses were performed using both mitochondrial and nuclear DNA markers. The mtDNA will permit identification of the maternal contributor and the nuclear marker will identify both parental contributions in first generation (F1) hybrids, if complete lineage sorting has been achieved. Thus, the aims of this study were (1) to determine the status of the hybrid; (2) to identify if there is a single or multiple maternal parent(s) and (3) to examine the ecological factors that

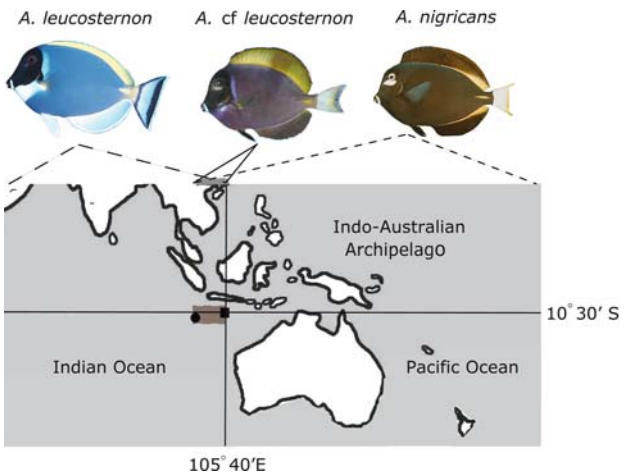

Fig. 1 Christmas (indicated by a *closed square*) and Cocos (Keeling) (indicated by a *closed circle*) Islands in the east Indian Ocean are situated 900 km apart. The ocean basin within which each species occurs is shown on the plate by *dashed*, *dotted* and *solid lines*, respectively. The zone of sympatry sampled in this study is indicated by an *opaque rectangle*

may promote hybridization between these two species that come into contact in the vicinity of a known marine biogeographic boundary.

Materials and methods

Sampling procedures

Cocos (Keeling) and Christmas Island are approximately 900 km apart, situated at 12°10′S, 96°52′E and 10°30′S, 105°40′E respectively (Fig. 1). These islands are situated at the biogeographic break between Indian and Pacific Ocean species (Williams and Benzie 1998). Individuals whose colour patterns were intermediate to the putative parent species were labelled as "putative hybrids" (also known as *Acanthurus* cf. *leucosternon*) (see Table 1). There was an abundance of additional possible putative hybrids that appeared similar in colour to one parent, but contained some minor colouration from the other putative parent. Numbers of specimens of the three species sampled at Christmas and Cocos (Keeling) Islands are given in Table 2. Additional specimens from allopatric populations of the putative parental species were obtained, three from west Australian Scott Reef (*Acanthurus nigricans*) in the east Indian Ocean and one from the Seychelles in the west Indian Ocean (*Acanthurus leucosternon*). Two specimens each of *Naso lituratus* (from the Marquesas, Pacific Ocean and from west Australian Scott Reef) and *N. elegans* (Seychelles) were also obtained for outgroup rooting purposes (see Klanten et al. 2004), as per Table 2. Fish were collected using spearfishing equipment in May 2004 and June 2006. Tissues (fin clips) were preserved in 80% ethanol.

Laboratory procedures

DNA extraction procedures followed Sambrook and Russell (2001). A PCR cocktail was made to a final concentration

Table 1 Relative abundance, ecological and morphological aspects of *Acanthurus nigricans*, the putative hybrid and *Acanthurus leucosternon* as per Kuiter and Debelius (2001)

Relative abundance	*A. nigricans*	Putative hybrid	*A. leucosternon*
	16-fold	Onefold F1, eightfold (F1 and post-F1)[a]	Twofold
Ecological strategies			
Habitat	Hard substrate areas and seaward reefs from the lower surge zone	Around rocky reefs subjected to strong currents	Reef flats and along upper seaward slopes
Mating strategy	Monogamous	No data	Monogamous
Clustering strategy	Solitary or in group	Solitary or in small group	Solitary or in a large feeding aggregation
Diet	Filamentous algae	No data	Benthic algae
Morphological aspects			
Maximal size	21 cm	20 cm	23 cm
Number of dorsal spines	9–9	No data	9–9
Number of dorsal soft rays	28–31	No data	28–30
Number of anal spines	3–3	No data	3–3
Number of anal soft rays	26–28	No data	23–26
Number of gill rakers on the anterior row	17–19	No data	17–19
Number of gill rakers on the posterior row	18–20	No data	18–20
Colouration			
Below the eye	A horizontally elongated white spot	A white spot	No distinct white spot
Pectoral fin	Black and blue	Blue with yellow margins	Yellow
Dorsal fin	Black with blue margins and yellow base	Yellow with white margins and black submarginal	Yellow with white margins and black submarginal
Ventral and anal fin	Blue with yellow base and white margins	Blue with yellow base and white margins	Blue with white margins
Caudal peduncle	Yellow surrounded by blue	Yellow surrounded by blue	Yellow surrounded by yellow
Caudal fin	Sub-marginal yellow band	Sub-marginal yellow band	Sub-marginal black band

[a] Putative hybrids sampled were all F1s, based on intermediate colouration, however a range of putative post-F1 hybrids, not sampled here, were also observed at much greater abundance than the F1s and *A. leucosternon* (J. P. Hobbs, personal observation)

Table 2 Material examined with collection locations, number of samples (*n*) from each location and marker used for each species from each location

Genus	Species	Location	*n*	Primer used
Acanthurus	*leucosternon*	Christmas Island	4	COI
Acanthurus	*leucosternon*	Cocos Island	1	COI
Acanthurus	cf. *leucosternon*	Christmas Island	3	COI
Acanthurus	*nigricans*	Christmas Island	4	COI
Acanthurus	*nigricans*	Cocos Island	1	COI
Naso	*elegans*	Seychelles	2	COI
Naso	*lituratus*	Marquesas	2	COI
Naso	*lituratus*	West Australia	2	COI
Acanthurus	*leucosternon*	Christmas Island	7	S7
Acanthurus	*leucosternon*	Cocos Island	4	S7
Acanthurus	*leucosternon*	Seychelles	1	S7
Acanthurus	cf. *leucosternon*	Christmas Island	7	S7
Acanthurus	*nigricans*	Christmas Island	7	S7
Acanthurus	*nigricans*	Cocos Island	1	S7
Acanthurus	*nigricans*	West Australia	3	S7
Naso	*elegans*	Seychelles	2	S7
Naso	*lituratus*	Marquesas	2	S7
Naso	*lituratus*	West Australia	2	S7

of 2.5 mM $MgCl_2$, 1 × Buffer (10 mM Tris–HCL, 5 mM KCL, pH 8.3), 0.2 mM deoxynucleotide triphosphates, 10 μM each primer and 0.75 units Taq polymerase. Two markers, both of which differentiate between congeneric species (e.g. Read et al. 2006; Bernardi et al. 2004) and both of which have been previously used to identify hybridization in coral reef fish (Yaakub et al. 2006) were used in this study. The cytochrome oxidase I (COI) region of the mitochondrial genome was amplified using universal primers LCO 1490, 5′-GGTCAACAAATCATAAAGAT ATTGG-3′ and HCO 2198, 5′-TAAACTTCAGGGTGA CCAAAAAATCA-3′ (Folmer et al. 1994). The first intron of the nuclear ribosomal S7 protein was also amplified using universal primers S7RPEX1F, 5′-TGGCCTCTTCC TTGGCCGTC-3′ and S7RPEX2R, 5′-AACTCGTCTGG CTTTTCGCC-3′(Chow and Hazama 1998). For both markers, PCR conditions varied among samples, as different annealing temperatures and $MgCl_2$ concentrations were required. COI amplifications were performed at an annealing temperature of 48°C for 35 cycles or at 50°C for 5 cycles, followed by 30 cycles at 48°C. S7 amplifications were either performed at an annealing temperature of 50°C or using the same touchdown procedure described for the COI amplification. All PCR conditions included an initial denaturation step at 94°C for 2 min, followed by 35 cycles of PCR at 94°C for 30 s, annealing at the specified temperature for 30 s and an extension at 72°C for 1 min and 30 s. A final extension was done for 10 min to complete the PCR procedure. PCR products were purified using either isopropanol precipitation (as per Sambrook and Russell 2001) or using a QIAquick gel extraction procedure in the event of multiple PCR products being produced, by following manufacturer's instructions (Qiagen).

The samples were sent to Macrogen (Seoul, Republic of Korea) for sequencing on an ABI 377 sequencer in both directions. DNA sequences were inspected individually for quality and then spliced together using the computer program BioEdit Sequence Alignment Editor (Hall 1999). Sequences were aligned using ClustalW from within BioEdit and alignments were checked and modified by hand where necessary. Aligned data was exported as a NEXUS file, and used for phylogenetic analyses to determine the relationships between individuals.

Analytical procedures

Sequences have been submitted to Genbank with accession numbers EF648221 to EF648275. To identify the evolutionary relationships between samples, neighbour joining, (NJ) and maximum parsimony (MP) methods were used. Phylogenetic analyses were conducted using MEGA version 3.1 (Molecular Evolutionary Genetics Analysis) (Kumar et al. 2004) and PAUP* version 4 (Swofford 1999). A likelihood approach was used in Modeltest version 3.06 (Posada and Crandall 1998) to find the best substitution model for NJ analyses. One thousand bootstrap replicates were performed during the NJ analysis for evaluation of the level of support for the tree topology. Both complete and pairwise deletions of gaps and missing data were used for S7 sequences. Only lineages supported by bootstrap values greater than 50% were retained when majority rule bootstrap consensus trees were constructed.

The haplotype diversity, *h*, was calculated (as per Nei 1987) for COI sequences and nucleotide diversities, π, were calculated for both S7 and COI data as implemented in the software program Arlequin, version 3.1 (Excoffier et al. 2005). A minimum spanning tree was constructed for COI haplotypes using Arlequin version 3.1 (Excoffier et al. 2005) in order to identify the relationships between haplotypes.

Results

COI

Sequence data was obtained from five *Acanthurus leucosternon*, five *Acanthurus nigricans* and three putative hybrids. The nucleotide frequency was: $A = 26.22\%$, $C = 25.17\%$, $G = 17.07\%$ and $T = 31.54\%$. There were 14

transversions and 2 transitions. There were no gaps in the partial COI sequence and when sequences were translated, functional partial COI protein was obtained, as verified by a Blast search of the GenBank sequence database. Of 686 bases, only 16 were variable, eight of which (1.17%) were parsimony informative. The remaining eight variable sites were singletons.

The best substitution model identified by the Akaike Information Criterion was HKY + I + G, with $I = 0.5418$ and $G = 0.3050$. The MP and NJ trees had very similar topologies, therefore MP bootstrap support values are indicated on the NJ tree (Fig. 2). The only difference between the two is in the placement of L4CI, which is part of the *Acanthurus nigricans* clade in the MP, but distinct in the NJ analysis. There were 190 equally likely MP trees, each with a length of 16 steps. The consistency index (CI) and retention index (RI) were both one (Fig. 2). Samples separated into two strongly supported clades, one for each species (Figs. 2, 4). All eight parsimony informative sites contributed to the split between these two species clades. The *Acanthurus nigricans* clade contained all *Acanthurus nigricans* samples for which data was obtained as well as one or two (analysis dependent) of the five *Acanthurus leucosternon* and two of the three putative hybrids for which COI sequences were obtained. There were six substitutions in this clade, all of which were transitions. Only one of the six haplotypes in this clade was shared (Fig. 3). Four individuals, two of which were *Acanthurus nigricans* (N1 and N4), one *Acanthurus leucosternon* (L1) and one hybrid (H2) shared this haplotype, suggesting that these four individuals are all descendants of an *Acanthurus nigricans* mother. This clade also includes five other specimens, three of

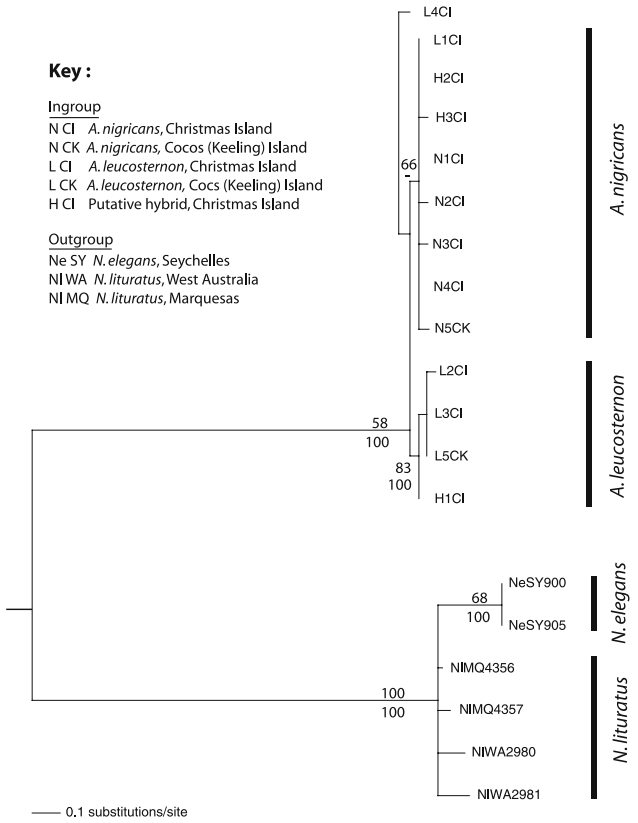

Fig. 2 The outgroup rooted COI phylogram generated by NJ analysis. *Branch lengths* show the number of substitutions between specimens. Majority rule support values (>50%) obtained from 1,000 bootstrap replicates are indicated *above* branches for NJ analysis and *below* branches are support values for the 50% majority rule MP consensus tree. Two clades are identified, an *Acanthurus nigricans* and an *Acanthurus leucosternon* clade. Individuals are identified by an alphanumeric code, the numeric part of the code uniquely identifies a specific fish while species and location are identified by the alphabetic part of the code as specified in the key to the figure

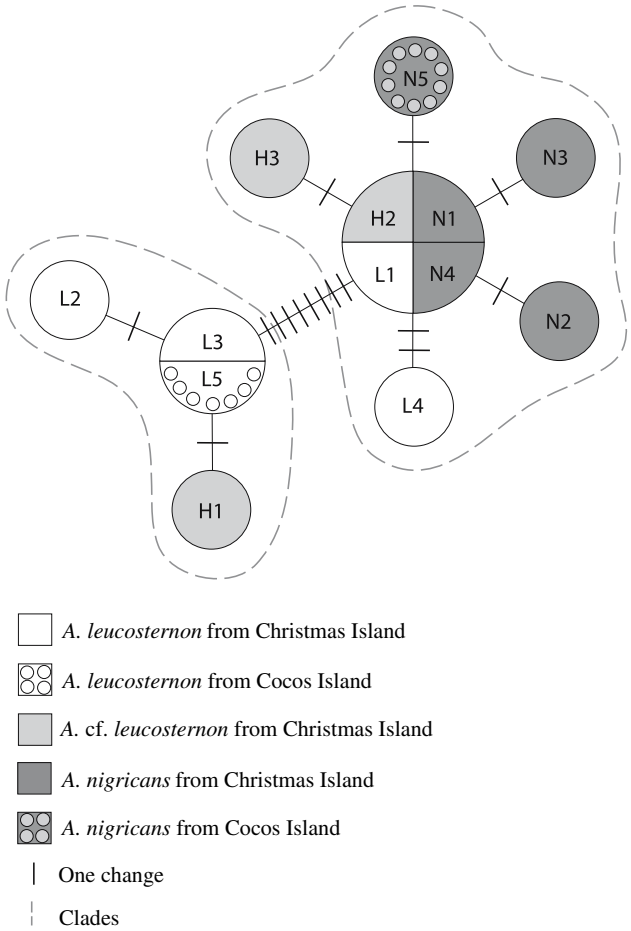

Fig. 3 Relationships between hybrids (*Acanthurus cf leucosternon*), *Acanthurus leucosternon* and *Acanthurus nigricans* haplotypes represented in an minimum spanning tree. Hybrid (*H*), *A. leucosternon* (*L*) and *A. nigricans* (*N*) specimens from Christmas and Cocos (Keeling) Islands are identified by different fills, as indicated in the figure key. The sizes of *circles* indicate the number of individuals sharing that particular haplotype. *Crossbars on the line* connecting haplotypes represent the number of substitutions separating them. The two clades are *encircled*

which are *Acanthurus nigricans* (N2, N3 and N5), one of which is a hybrid (H3) and one *Acanthurus leucosternon* (L4). The three *Acanthurus nigricans* specimens may be purebred, since, like N1 and N4, they have *Acanthurus nigricans* colouration. L4 is a descendant of either *Acanthurus leucosternon* (e.g. L1) or a hybrid (e.g. H2), as it has *Acanthurus leucosternon* colouration, despite having *Acanthurus nigricans* mtDNA from its mother. Both putative hybrids, H2 and H3, have intermediate colouration, but have *Acanthurus nigricans* mtDNA.

The *Acanthurus leucosternon* clade was strongly supported and contained the remaining three *Acanthurus leucosternon* individuals and one of the three putative hybrids. No *Acanthurus nigricans* occurred in the *Acanthurus leucosternon* clade. There were only two substitutions, one transition and one transversion. There was one shared haplotype (shared by two *Acanthurus leucosternon* individuals) and a total of three unique haplotypes in this clade.

The *N. lituratus* and *N. elegans* outgroups were also partitioned by species, as the *N. elegans* (NeSY) were strongly supported as a distinct clade from all of the *N. lituratus*, irrespective of location (NlMQ and NlWA).

The haplotype diversity, h, within clades was relatively high ($h = 0.83$ for both *Acanthurus nigricans* and *Acanthurus leucosternon* clades), with low nucleotide diversities ($\pi = 0.19 \pm 0.15\%$ and $\pi = 0.14 \pm 0.14\%$) for each clade respectively (Fig. 3). Both clades contained individuals from both locations (Christmas and Cocos Islands).

S7 intron 1

S7 intron 1 sequences were highly variable, with a relatively conserved block of 352 bp at the 5′ end and 293 bp at the 3′ end. Seventy-four base pairs were eliminated from the sequence centre, as they were too variable and polymorphic to align among individuals with confidence. Two hundred and twenty eight sites were variable, of which 122 were parsimony informative. Nucleotide frequencies were: $A = 23.56\%$, $C = 19.30\%$, $G = 25.05\%$ and $T = 32.09\%$.

The optimal substitution model was GTR + G, $G = 0.4982$ and produced an NJ phylogram of broadly similar topology to that of the MP tree. Therefore, an outgroup rooted NJ phylogram is presented with support from 1,000 bootstrap replicates and MP majority rule consensus support values indicated (Fig. 4). There were 22 gaps, which were treated as a complete deletion. Despite the reasonable number of parsimony informative sites, there is little supported structure in this nuclear data (Fig. 4). Two major lineages were resolved, one of which contains only one individual: The only representative of the allopatric *Acanthurus leucosternon* population (L3298SY) from the Seychelles, in the west Indian Ocean, for which data was obtained. This is proposed to be a west Indian Ocean *Acanthurus leucosternon*

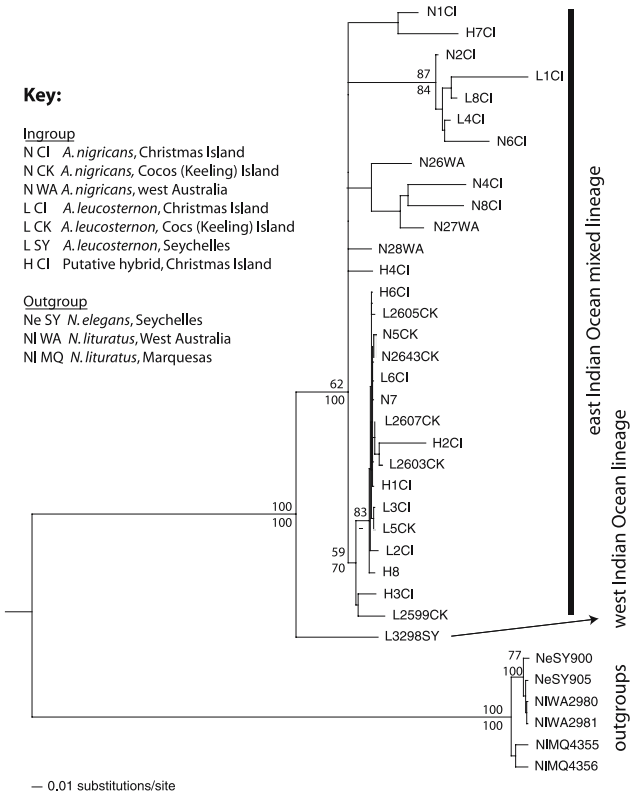

Fig. 4 An outgroup rooted S7 intron 1 phylogram generated by NJ analysis. *Branch lengths* show the number of substitutions between specimens. NJ bootstrap support values (>50%) of 1,000 bootstrap replicates are indicated *above* branches for NJ analysis and *below* branches are support values for the 50% majority rule MP consensus tree. Individuals are identified by an alphanumeric code, the numeric part of the code uniquely identifies a specific fish while species and location are identified by the alphabetic part of the code as specified in the key to the figure

lineage. The second clade contains all remaining samples of both species from the east Indian Ocean, including the west Australian *Acanthurus nigricans* (N26WA, N27WA and N28WA). Two subclades were identified within this lineage, neither of which was species-specific either. Putative hybrids were dispersed throughout this mixed species lineage. The MP majority rule consensus tree of 370 equally likely trees was 21 steps in length, with CI, RI and rescaled consistency indices of 0.904762, 0.857143 and 0.77551 respectively. The same two lineages were identified as before, but only one of the subclades was resolved by the MP analysis (Fig. 4).

Like the ingroup, the *N. lituratus* and *N. elegans* outgroup sequences also failed to form species-specific lineages, unlike the case for the mtDNA. The *N. elegans* (NeSY) and *N. lituratus* from west Australia (NlWA) formed a strongly supported clade, which excluded the conspecific *N. lituratus* samples from the Marquesas in the Pacific Ocean (NlMQ).

Discussion

Relationship between colour pattern and genetic divergence

This study tested the hypothesis that *Acanthurus* cf. *leucosternon* is a hybrid of *Acanthurus leucosternon* and *Acanthurus nigricans* as suggested by meristics, colour pattern and also by the fact that *Acanthurus* cf. *leucosternon* is restricted to the area where its putative parents are sympatric and the relative abundances of all three at these sites (Table 1). The results of this study support hybridization, since mtDNA of the parental species (and sister species of the outgroup) are genetically differentiated, although there is evidence of some mtDNA introgression from the most abundant, *Acanthurus nigricans* into the much rarer *Acanthurus leucosternon*. Furthermore, mtDNA from both parental species is present in the putative hybrids, *Acanthurus cf leucosternon*. However, due to incomplete lineage sorting of the nuclear marker in both the study species (and the sister species of the outgroups), conclusions from this work require confirmation from additional nuclear markers which do not suffer from incomplete lineage sorting.

In another study, the presence of hybridization between meristically indistinguishable fish species with differences in colouration was tested using genetics (Yaakub et al. 2006) and confirmed that the wrasses *Thalassoma quinquevittatum* and *T. jansenii* hybridize to produce the intermediate colour morphs. These wrasses are sympatric through much of their distribution ranges, but hybrids have only been found at an isolated coral reef in the Coral Sea, where the species abundances are very different (Yaakub et al. 2006). Different relative abundances of hybridizing species are considered one of the hallmarks of hybridization (Arnold 1997). In contrast, seven colour morphs of the genus *Hypoplectrus* (hamlet fish) that co-occur on the same Caribbean reefs do not appear to be genetically distinguishable (e.g. Ramon et al. 2003), despite strong colour pattern-based assortative mating. However, closer examination by McCartney et al. (2003) identified a complex mosaic of genetic differentiation among Hamlet colour morphospecies, providing evidence for reproductive isolation at some, but not other study sites. In the Hamlets, which are a very young group of morphospecies, haplotype and microsatellite genotype frequency differences among the morphospecies were highly significant at specific locations, despite a lack of concordance between haplo-, geno- and morphotypes (McCartney et al. 2003). Another example is presented in the study of *Pseudochromis fuscus*, which has more than six colour morphs at various locations. Genetic analyses variably identified differences between morphs (Messmer et al. 2005) suggesting that colour alone is not a reliable indicator of distinct species. All these examples of coral reef fish colour variants/species indicate that descriptions based on meristics and colour patterns alone, may not be sufficient to resolve the question of whether species are distinct, whether they hybridize or are simply colour variants. Moreover, such studies do not provide information on the genetic contribution of each putative parent, which is an essential factor to understand what ecological and behavioural conditions permit hybridization amongst coral reef fishes.

Putative parental contributors

MtDNA

This study identified two mtDNA clades, one containing all *Acanthurus nigricans* samples and a few *Acanthurus leucosternon* individuals, the other containing only *Acanthurus leucosternon* and no *Acanthurus nigricans* individuals. Putative hybrids were present in both clades.

This data suggests that *Acanthurus nigricans* is the mother in hybrid matings with *Acanthurus leucosternon* more often than not, but that *Acanthurus leucosternon* females can also mate with *Acanthurus nigricans* males or hybrids. This may be due to *Acanthurus nigricans* being at least eightfold more abundant than *Acanthurus leucosternon* and twofold more abundant than putative hybrids at the study site (Table 1). In several other studies of reef fish hybridization, only one of the parents has acted exclusively as the mother during hybridization (e.g. coral trout, van Herwerden et al. 2006; wrasses, Yaakub et al. 2006; the damselfish *Acanthochromis polyacanthus* from the "southern hybrid zone", van Herwerden and Doherty 2006). However, like the Acanthurid species presently studied, some species of butterflyfish (McMillan et al. 1999) and some morphs of *Acanthochromis polyacanthus* from the "northern hybrid zone" (van Herwerden and Doherty 2006) can hybridize using either species or colour morph as the hybridizing mother.

Incomplete lineage sorting is an alternative interpretation for the lack of absolute partitioning between species at the mtDNA marker (e.g. see van Herwerden et al. 2006). However, this is much less likely, given: (1) the relative abundances in the contact zone, (2) meristics, (3) colouration, (4) biogeography (see below), (5) ecology of these species at the study sites (see below), (6) resolution obtained with this marker for the outgroup sister species and (7) that COI has been successfully and extensively used to differentiate congeneric species of tropical coral reef fish in phylogenetic studies (e.g. Bernardi et al. 2004; Read et al. 2006, Yaakub et al. 2007). Additional more comprehensive phylogeographic data based on larger sample sizes is required to discriminate between these alternative hypotheses: hybridization vs. incomplete lineage sorting. Partitioning of the lineages from allopatric populations of

these two species into the two clades identified in this study would support hybridization, whilst a similarly inter-mixed genetic signal from distant allopatric populations to that observed here in the zone of sympatry, may favour incomplete lineage sorting (see McMillan et al. 1999; van Herwerden et al. 2006).

Nuclear DNA

The nuclear data suggests either incomplete lineage sorting or introgressive hybridization, since there is no partitioning of this marker in the parental species sampled within the east Indian Ocean. The allopatric west Indian Ocean *Acanthurus leucosternon* does however appear to be genetically distinct, although this is only based on a single individual and requires additional data for confirmation. Support for incomplete lineage sorting (rather than hybridization) at this marker is provided by the genetic structure within the outgroup, since well-established sister-species (Klanten et al. 2004) are not differentiated by this marker, but share a well-supported lineage. This is in marked contrast to the clear genetic structure revealed for these outgroup species using the mtDNA marker.

Hybridization, introgression, ecology and evolution

Hybridization can be facilitated when parental species, which have similar ecological and morphological traits, come into contact, especially if there is a numerical imbalance and one of the species is rare relative to the other, as is the case for *Acanthurus nigricans* and *Acanthurus leucosternon* at Christmas and Cocos (Keeling) Islands. *Acanthurus nigricans* is generally found on hard substrates and seaward reefs from the lower surge zone, whilst *Acanthurus leucosternon* is generally found on reef flats and along upper seaward slopes (Kuiter and Debelius 2001). In the contact zone studied here, these species (and the putative hybrids) were all found in shallow water (1–4 m), feeding in the same area (J. P. Hobbs, personal observation). Furthermore, *Acanthurus leucosternon* was rare relative to the putative hybrids (F1 and post-F1) and *Acanthurus nigricans*, thereby providing an incentive for inter-breeding by *Acanthurus leucosternon*. Although, mating behaviour of these species has not been documented to date, it is noted that most of the putative hybrids are post-F1, which is eightfold more abundant than F1 hybrids, suggesting that de novo F1 hybrids are generated or survive to adulthood less frequently than do subsequent generations of hybrids.

It is possible that hybridization will re-orient evolution (at this location at least), because eventually the already relatively rare *Acanthurus leucosternon* may disappear from this location (as per Rhymer and Simberloff 1996; Grant et al. 2005). Furthermore, hybrid offspring may disperse from the hybrid zone leading to more widespread introgression as recorded for hybridizing butterflyfishes (McMillan et al. 1999). An alternative, but less likely consequence of hybridization may be the merging of both parental species due to hybridization ("reverse speciation") as has recently been documented for a pair of lake inhabiting sticklebacks following the introduction of an exotic crayfish (Taylor et al. 2006). However, additional data is required from additional nuclear markers that do not suffer from incomplete lineage sorting, to further evaluate this.

Little is known of the fitness of reef fish hybrids compared to their parents, but the hybrids are often confined to the hybrid zone, suggesting that they are less fit than both parent species outside of the hybrid zone (e.g. wrasses in Yaakub et al. 2006; damselfish in van Herwerden and Doherty 2006; Acanthurids in this study), but see McMillan et al. (1999) for a case in contrast, where butterflyfish mtDNA introgression extends across nearly 12,000 km. Some authors affirm that hybrids are as fit or fitter than their parents in the hybrid zone at least, which is often intermediate to the respective parental habitats (e.g. Arnold and Hodges 1995). This suggests that hybrids can contribute to adaptation in spite of the genetic barriers (Barton 2001). The present study suggests that hybrids in the hybrid zone are at least as fit as the fourfold rarer parent, *Acanthurus leucosternon*. Other authors suggest that the fitness of hybrids relative to parents can be reduced (e.g. Barton and Hewitt 1985). Hybrid abundance is difficult to evaluate in the natural environment, because unlike F1 hybrids, post-F1 hybrids may have colouration that is difficult to distinguish from the parental colouration. This study, like the Yaakub et al. (2006) and van Herwerden et al. (2006) studies, has shown that reef fish hybrids can be much more abundant than is apparent from colouration alone. This may eventually lead to the loss of the rarer parental species from the hybrid zone. Only through genetic analyses can the presence of such introgressive hybridization be determined.

Finally, the observed introgressive hybridization suggests that there is no assortative mating behaviour, at least not in the rare *Acanthurus leucosternon* or the F1 hybrids, nor prezygotic isolation or habitat partitioning between species in the hybrid zone, since these species co-occur and probably spawn concurrently. Hybridization in these species appears to be due to secondary contact at a biogeographic border and the absence of prezygotic isolating mechanisms (behavioural and gamete compatibility). A behavioural study is necessary to determine if hybridization between the parental species is due to active behavioural responses of mating individuals of both species, sneak mating by the rarer *Acanthurus leucosternon* males or accidental fertilization by compatible gametes. Data from other hybridizing coral reef fish species suggest that sneak mating

by males of the numerically rare species is likely to be more common (Frisch and van Herwerden 2006; Yaakub et al. 2006). Regardless, this study identifies a definite risk that *Acanthurus leucosternon* may go locally extinct as a consequence of its relative rarity and possibly extensive introgressive hybridization at the Cocos (Keeling) and Christmas Islands (as per Rhymer and Simberloff 1996). This may ensure that these species are maintained in allopatry, however, if hybrid larvae successfully disperse from the hybrid zone, then introgression may be more widespread and eventually result in the merging of two species into one, as has been documented for some sticklebacks (Taylor et al. 2006). Given the relative isolation of these islands, it is unlikely that hybrids will populate and "pollute" distant purebred stocks of the parental species, e.g. *Acanthurus leucosternon* at the Seychelles, rather, they are likely to remain confined to the hybrid zone and adjacent reefs, e.g. Indonesia and Reefs off the northwest Australian coast. Further genetic analyses, involving widespread phylogeographic sampling is necessary to determine the geographic extent of introgression in these species.

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References

Arnold ML (1997) Natural hybridization and evolution. Oxford University Press, New York

Arnold ML, Hodges SA (1995) Are natural hybrids fit or unfit relative to their parents? Trends Ecol Evol 10:67–71

Barton NH (2001) The role of hybridization in evolution. Mol Ecol 10:551–568

Barton NH, Hewitt GM (1985) Analysis of hybrid zones. Annu Rev Ecol Syst 16:113–148

Bernardi G, Holbrook SJ, Schmitt RJ (2001) Gene flow at three spatial scales in a coral reef fish, the three-spot dascyllus, *Dascyllus trimaculatus*. Mar Biol 138:457–465

Bernardi G, Holbrook SJ, Schmitt RJ, Crane NL, DeMartini E (2002) Species boundaries, populations and colour morphs in the coral reef three-spot damselfish (*Dascyllus trimaculatus*) species complex. Proc R Soc B Biol Sci 269:599–605

Bernardi G, Bucciarelli G, Costagliola D, Robertson DR, Heiser JB (2004) Evolution of coral reef fish *Thalassoma* spp. (Labridae). 1. Molecular phylogeny and biogeography. Mar Biol 144:369–375

Brown LD (1995) Genetic evidence for hybridization between *Haliotis rubra* and *H. laevigata*. Mar Biol 123:89–93

Chow S, Hazama K (1998) Universal PCR primers for S7 ribosomal protein gene introns in fish. Mol Ecol 7:1255–1256

Excoffier L, Laval G, Schneider S (2005) Arlequin ver. 3.0: an integrated software package for population genetics data analysis. Evol Bioinformatics Online 1:47–50

Folmer O, Black M, Hoen W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3:294–299

Frisch A, van Herwerden L (2006) Field and experimental studies of hybridization between coral trouts, *Plectropomus leopardus* and *Plectropomus maculatus* (Serranidae), on the Great Barrier Reef, Australia. J Fish Biol 68:1013–1025

Garcia-Machado E, Chevalier Monteagudo P, Solignac M (2004) Lack of mtDNA differentiation among hamlets (*Hypoplectrus*, Serranidae). Mar Biol 144:147–152

Gardner JPA (1996) The *Mytilus edulis* species complex in southwest England: effects of hybridization and introgression upon interlocus associations and morphometric variation. Mar Biol 125:385–399

Grant PR, Grant BR, Petren K (2005) Hybridization in the recent past. Am Nat 166:56–67

Hall TA (1999) Bioedit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Harrison RG (1993) Hybrid zones and the evolutionary process. Oxford University Press, Oxford

Hutchinson WF, Carvalho GR, Rogers SI (2001) Marked genetic structuring in localised spawning populations of cod (*Gadus morhua*) within the North Sea and adjoining waters as revealed by microsatellites. Mar Ecol Prog Ser 223:251–260

Jansson H, Holmgren I, Wedin K, Andersson T (1991) High frequency of natural hybrids between Atlantic salmon, *Salmo salar* L., and brown trout, *S. truta* L., in a Swedish river. J Fish Biol 39:343–348

Klanten S, van Herwerden L, Choat JH, Blair D (2004) Patterns of lineage diversification in the genus *Naso* (Acanthuridae). Mol Phylogenet Evol 32:221–235

Knowlton N (2000) Molecular genetic analyses of species boundaries in the sea. Hydrobiologia 420:73–90

Kuiter RH, Debelius H (2001) Surgeonfishes, rabbitfishes and their relatives. A comprehensive guide to Acanthuroidei (marine fish families). TMC Publishing, Chorleywood

Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analyses and sequence alignment. Briefings in Bioinformatics 5:150–163

Lacson JM (1994) Fixed allele frequency differences among Palauan and Okinawan populations of the damselfishes *Chrysiptera cyanea* and *Pomacentrus coelestis*. Mar Biol 118:359–365

Lacson JM Clark S (1995) Genetic divergence of Maldivian and Micronesian demes of the damselfishes *Stegastes nigricans*, *Crysiptera biocellata*, *C. glauca* and *C. leucopoma* (Pomacentridae). Mar Biol 121:585–590

Lacson JM, Nelson SG (1993) Genetic distances among fishes of the genus *Siganus* (Siganidae) from the western Pacific Ocean. Mar Biol 116:187–192

Lessios HA, Kessing BD, Robertson DR, Paulay G (1999) Phylogeography of the pantropical sea urchin *Eucidaris* in relation to land barriers and ocean currents. Evolution 53:806–817

Lieske E, Meyers R (1999) Coral reef fishes. Princeton University Press, Princeton

Mallet J (2005) Hybridization as an invasion of the genome. Trends Ecol Evol 20:229–237

McCartney MA, Acevedo J, Heredia C, Rico C, Quenoville B, Bermingham E, McMillan O (2003) Genetic mosaic in a marine species flock. Mol Ecol 12:2963–2973

McMillan WO, Weight LA, Palumbi SR (1999) Color pattern evolution, assortative mating, and genetic differentiation in brightly colored butterflyfishes (Chaetodontidae). Evolution 53:247–260

Messmer V, van Herwerden L, Munday P, Jones GP (2005) Phylogeography of colour polymorphism in the coral reef fish *Pseudochromis*

fuscus, from Papua New Guinea and the Great Barrier Reef. Coral Reefs 24:392–402

Nei M (1987) Molecular evolutionary genetics. Columbia University Press, New York

Planes S, Doherty P (1997) Genetic and color interactions at a contact zone of *Acanthochromis polyacanthus*—a marine fish lacking pelagic larvae. Evolution 51:1232–1243

Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. Bioinformatics 14:817–818

Ramon ML, Lobel S, Sorenson MD (2003) Lack of mitochondrial genetic structure in hamlets (*Hypoplectrus spp.*); recent speciation or ongoing hybridization. Mol Ecol 12:2975–2980

Read C, Bellwood D, van Herwerden L (2006) Ancient origins of Indo-Pacific coral reef fish biodiversity: a case study of the leopard wrasses (Labridae: *Macropharyngodon*). Mol Phylogenet Evol 38:808–819

Rhymer JM, Simberloff D (1996) Extinction by hybridization and introgression. Annu Rev Ecol Syst 27:83–109

Ruzzante DE, Taggart CT, Lang S, Cook D (2000) Mixed-stock analysis of Atlantic cod near the Gulf of St Lawrence based on microsatellite DNA. Ecol Appl 10:1090–1109

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Swofford DL (1999) PAUP*: phylogenetic analysis using parsimony. Sinauer Associates, Champaign

Taylor EB, Boughman JW, Groenenboom M, Sniatynski M, Schluter D, Gow JL (2006) Speciation in reverse: morphological and genetic evidence of the collapse of a three-spined stickleback (*Gasterosteus aculeatus*) species pair. Mol Ecol 15:343–356

Taylor MS, Hellberg ME (2003) Genetic evidence for local retention of pelagic larvae in a Carribbean reef fish. Science 299:107–109

van Herwerden L, Doherty P (2006) Contrasting genetic structures across two hybrid zones of a tropical reef fish, I (Bleeker 1855). J Evol Biol 19:239–252

van Herwerden L, Choat JH, Dudgeon CL, Carlos G, Newman SJ, Frisch A, van Oppen M (2006) Contrasting patterns of genetic structure in two species of the coral trout *Plectropomus* (Serranidae) from east and west Australia: introgressive hybridization or ancestral polymorphisms. Mol Phylogenet Evol 41:420–435

van Oppen MJH, Gates RD (2006) Conservation genetics and the resilience of reef-building corals. Mol Ecol 15:3863–3883

Wilkins NP, Courtney HP, Gosling E, Linnane A, Jordan C, Curatolo A (1994) Morphometric and meristic characters in salmon, *Salmo salar* L., trout, *Salmo truta* L., and their hybrids. Aquaculture and Fisheries Management 25:505–518

Williams` ST, Benzie JAH (1998) Evidence of a biogeographic break between populations of a high dispersal starfish: congruent regions within the Indo-West Pacific defined by color morphs, mtDNA, and allozyme data. Evolution 52:87–99

Yaakub SM, Bellwood DR, van Herwerden L, Walsh FM (2006) Hybridization in coral reef fishes: introgression and bi-directional gene exchange in *Thalassoma* (family Labridae). Mol Phylogenet Evol 40:84–100

Yaakub SM, Bellwood DR, van Herwerden L (2007) A rare hybridization event in two common Caribbean wrasses (genus *Halichoeres*; family Labridae). Coral Reefs. doi: 10.1007/s00338-007-0240-z