Hybridization in coral reef fishes: Introgression and bi-directional gene exchange in *Thalassoma* (family Labridae)

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Abstract

Hybrids in coral reef fishes have traditionally been described based on external features using meristic characters and colouration to identify putative parental contributors. This study utilised molecular genetic techniques to verify hybrid status and identify putative parental species for seven hybrid specimens (Labridae: *Thalassoma*) collected from Holmes Reef in the Coral Sea. Phylogenetic analyses support hybrid origins of the specimens. Mitochondrial COI gene, nuclear S7 (intron 1) and nuclear copy of mitochondrial (NUMT) D-loop region corroborate the identity of *T. quinquevittatum* as the maternal and *T. jansenii* as the paternal contributor. Backcrossing to parental species by hybrids and bi-directional gene exchange between the Holmes Reef populations of *T. jansenii* and *T. quinquevittatum* was detected, suggesting that hybrids are fertile and able to reproduce successfully. F1 hybrids display a mixture of the colouration attributes of the two parental species, but subsequent backcrossed individuals were unrecognisable as hybrids and displayed colouration of either parental species. A large numerical imbalance exists between the putative parental species at Holmes Reef, with *T. quinquevittatum* outnumbering *T. jansenii* by approximately 25:1. In this case study, hybridization appears to be driven by ecological rather than evolutionary factors.

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1. Introduction

A hybrid is formed through interbreeding between individuals from two populations, or groups of populations, which are distinguishable based on one or more heritable characters (Arnold, 1997). It then follows that a hybrid zone is an area of spatial overlap of two or more populations, which cross to form viable offspring of mixed ancestry (Arnold, 1997; Harrison, 1993). Hybridization and hybrid zones are of interest to evolutionary biologists because of the opportunity to observe the interplay of selection and gene exchange and its implications for the evolutionary process (Futuyma, 1998).

While there have been several reviews and books on the topic of natural hybridization and its role in the evolutionary process (Arnold, 1997; Barton and Hewitt, 1985; Harrison, 1990, 1993; Hewitt, 1988), the focus of these studies has been on terrestrial biota. Hybridization is traditionally thought to be a rare occurrence in the marine environment (Mayr, 1999; Randall et al., 1977). A review on hybridization in the marine environment (Gardner, 1997) and several recent papers on hybridization in marine taxa (Bierne et al., 2003; Frisch and van Herwerden, in press; Nielsen et al., 2003; Planes et al., 2001; van Herwerden et al., 2002; van Herwerden and Doherty, 2005; van Oppen et al., 2000, 2001, 2002) have highlighted the potential for hybridization in marine systems. Nevertheless, it is important to make the
A study on hybridization is most useful when the attributes of its model organisms are characteristic of a broader range of taxa. Wrasses (family Labridae) are a diverse group of more than 600 species in 82 genera and display a range of body shapes, sizes, colours, and habitat preferences (Parenti and Randall, 2000; Wainwright et al., 2004). They are quintessential reef fishes and make up a considerable proportion of the typical reef fish assemblage, second only to the gobies (family Gobiidae) on the Great Barrier Reef (Bellwood and Wainwright, 2002; Randall et al., 1996). Labrids also display life history characteristics that are shared by most other coral reef fishes (Leis, 1994). Wrasses of the genus *Thalassoma* (family Labridae, subfamily Julidini) have a circumboreal distribution and are characteristic inhabitants of both coral reefs and rocky shores. All 27 species within the genus share similar morphological and meristic characters but display a wide variety of color patterns. Species in this genus are distinguished largely based on colour patterns (Randall et al., 1996), and recent work on the molecular phylogenetic relationships between species has confirmed the validity of currently recognised species (Bernardi et al., 2004; Costagliola et al., 2004).

Both inter-specific and inter-generic hybrids have been documented in the genus *Thalassoma*. Hybrids have been documented from crosses between *T. lunare × T. rupPELLI* in the Red Sea (Randall and Miroz, 2001), *T. hardwicke × T. quinquevittatum* in Micronesia (Myers, 1999), *T. duperrey × T. lutescens* in Hawaii (Randall, 1996), *T. duperrey × T. quinquevittatum* at Johnston Atoll (Lobel, 2003), and between *Gomphosus varius × T. lunare* in north-western Australia (Randall and Allen, 2004). *Gomphosus* is a derived lineage within *Thalassoma*, and may be placed within the genus (Bernardi et al., 2004).

Between 2000 and 2004, five *Thalassoma* hybrid specimens were discovered on Holmes Reef, an isolated atoll in the Coral Sea. From an evolutionary standpoint, hybridization success is theoretically more likely between closely

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**Fig. 1.** Hybridization in nine coral reef fish families expressed as a percentage of the total number of species in each family reported as hybrid contributors. Numbers in parentheses are the total number of species in each family. Data combined from Pyle and Randall (1994), Gardner (1997), Allen et al. (1998), Kuiter (2002), and Fishbase (2005).
related species than more distantly related species because of the time since cladogenesis. Although there are other species of *Thalassoma* at Holmes Reef that can be considered putative parental species, based on the molecular phylogeny of *Thalassoma* (Bernardi et al., 2004), it is evolutionarily logical for the hybrids to be crosses between *T. jansenii* × *T. hardwicke* because they are sister taxa and belong to a distinct clade (Fig. 2). However, Walsh and Randall (2004) described *T. quinquevittatum* and *T. jansenii* as the parental species of the hybrid specimens based on meristics and colour patterns. Since all three of these species are sympatric throughout most of their distribution range from the Indian Ocean to Pacific Ocean (Fig. 3), we cannot discount *T. hardwicke* as a putative parental contributor to the hybrid specimens. To date, hybrids from this genus have been identified solely based on meristics and

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**Fig. 2.** A partial molecular phylogeny of Indo-Pacific *Thalassoma* (after Bernardi et al., 2004) showing the putative parental species in bold font. Note the basal position of *T. lunare* which represents a sister clade to the ingroup taxa used in this study.

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**Fig. 3.** The overlapping and broadly sympatric species distribution ranges of the putative parental species: *Thalassoma quinquevittatum, T. jansenii* and *T. hardwicke*. A star denotes the approximate location where hybrids were collected at Holmes Reef in the Coral Sea.
intermediacy in colour patterns relative to putative parental contributors. No study has yet attempted to verify the genetic status of these hybrids.

The specific aims of this study therefore are: (1) to evaluate the status of the Holmes Reef hybrid specimens; (2) to identify the putative parental contributors and their evolutionary relationships; and (3) to examine the ecological factors that may have influenced hybridization.

2. Methods

2.1. Species selection and tissues collected

A total of 38 individuals of putative parental species Thalassoma hardwicke (11 individuals), T. janseni (8 individuals), T. quinquevittatum (14 individuals), and five hybrid specimens were used in this study (Table 1). All hybrid specimens of Thalassoma were collected from Holmes Reef (16°24′S, 147°54′E), an isolated oceanic atoll in the Coral Sea. Three other species (T. lutescens, T. duperrey, and T. genivittatum) were chosen to represent within-clade divergences (Fig. 2); T. lunare was selected as the outgroup species because it is a representative of the sister clade to the other species in this study based on the existing molecular phylogeny of the genus (Bernardi et al., 2004). All tissue samples were collected from freshly euthanised or recently collected specimens. Fishes were held in an ice-water slurry until they were dissected. Tissues (fin clip and muscle tissue) were collected and stored in a 70% ethanol solution and stored at −12°C until DNA extraction.

2.2. DNA extraction, PCR amplification, sequence preparation, and alignment

Tissues were washed in TE buffer and digested in a Proteinase K extraction buffer. Genomic DNA was extracted using a standard salt-chloroform extraction and ethanol precipitation procedure (Sambrook et al., 1989). Five partial genomic regions were amplified using polymerase chain reaction (PCR) as follows:

(i) Cytochrome oxidase c subunit I. A total of 689 bp of the cytochrome oxidase I (COI) region of the mitochondrial genome was amplified using the universal primers LCO 1490 and HCO 2198 (Folmer et al., 1994). A cycling profile of 30 s at 94°C, 30 s at 50°C and 90 s at 72°C for 35 cycles was used.

(ii) S7 nuclear gene. A total of 739 bp of the ribosomal S7 first intron region was amplified using the primers S7RPEX1F and S7RPEX2R (Chow and Hazama, 1998). The same cycling profile was used as for COI.

(iii) Nuclear copy of the mitochondrial genome control region. The nuclear copy of the mitochondrial genome (NUMT) was detected in all putative parental species and hybrids. NUMT fragments have been incorporated into the nuclear genome and are thus bi-paternally inherited. They are usually non-functional (but see Mabuchi et al., 2004, for an example of functional tRNA pseudogene). Based on a preliminary study, NUMT bands that were common to the hybrid and putative parental species were identified. These were amplified using universal fish primers for D-loop, LI5995F and HI6498 (Lee et al., 1995) under a touchdown cycling profile of 30 s at 94°C, 30 s at 57°C and 90 s at 72°C for 5 cycles followed by another 30 cycles as before, but with an annealing temperature of 55°C.

(iv) Aldoase B. A 270 bp partial sequence of the first intron of Aldoase B (Aldo B1) was amplified using primers AldoB1 F and AldoB1 R (Hassan et al., 2002) on a touchdown cycling profile of 30 s at 94°C, 30 s at 57°C and 90 s at 72°C for 5 cycles followed by another 30 cycles as before, but with an annealing temperature of 58°C.

(v) Gonadotropin releasing hormone gene (GnRH3-2). A 274 bp partial sequence of the GnRH gene was amplified using primers GnRH3-2 F and GnRH3-2 R (Hassan et al., 2002) on a cycling profile of 30 s at 94°C, 30 s at 60°C and 90 s at 72°C for 5 cycles followed by another 30 cycles as before, but with an annealing temperature of 58°C.

All PCR (20 μl) contained 2.5 mM Tris–Cl (pH 8.7), 5 mM KCl, 5 mM (NH4)2SO4, 2.5–6.5 mM MgCl2, 200 μM each dNTP, 10 μM each primer, 1.0 U of Taq polymerase (Qiagen), and 15 ng template DNA. PCR products were separated in a 1.5% agarose gel, excised, and purified using

Table 1 Material examined with collection locations, number of samples (n) from each location and GenBank accession numbers for the ribosomal S7 gene. GenBank accession numbers are DQ443838–DQ443879 for the COI gene and DQ443760–DQ443793 for the D-loop NUMTs respectively.

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*a Note. The southwest Pacific variant of T. janseni has recently been described as a separate species, T. nigrofasciatus by Randall (2003). There was no support for this division in the molecular data collected herein; the species division is therefore not recognised and the original designation of T. janseni is retained.*
Qiagen PCR Purification Kit (as per manufacturer’s protocol) and sequenced directly in both directions using BigDye TM Terminator (version 3.1) by Macrogen Sequences were assembled and edited using Sequencher v4.5 software (Gene Code Corporation). Where forward and reverse sequences could not be consolidated, they were excluded from the analyses for that dataset. Forward and reverse sequences of each primer were aligned manually in SeAl v2.0a11 (Rambaut, 2002) and adjusted by eye through the insertion or deletion of gaps in the sequences as required.

2.3. Data analysis

Sequence data from each of the five datasets were analysed separately because mitochondrial and nuclear datasets highlight unique aspects of the genetic relationships between species in a hybridization study. It is crucial to identify the maternal genetic contribution to the hybrid offspring based on the mtDNA and to identify both parental contributions from each nuclear marker independently, because each nuclear marker may have a different signal, depending on the level of hybridization and introgression.

Phylogenetic relationships were assessed by analysing gene fragments using maximum likelihood (ML) and maximum parsimony (MP) methods in PAUP* v4.0b10 (Swofford, 1998) for each separate dataset (COI, S7, D-loop NUMT, Aldo B1, and GnRH3-2). Bayesian inference (BI) methods were also applied to the COI and D-loop NUMT datasets, using Mr. Bayes v3.0b4 (Huelsenbeck and Ronquist, 2001). An optimal model of sequence evolution was determined using the Akaike information criterion with a probability (BI) support for nodes (Fig. 4A). There was strong support for the structure both within the putative parental clades as well as the remaining species (Fig 4A). All Thalassoma hardwicke individuals form a distinct and highly supported clade (HW clade), with T. jansenii (JN) as its sister clade and T. quinquevittatum (Q) as a basal clade to both T. jansenii and T. hardwicke. Seven T. quinquevittatum specimens fell into the JN clade along with six of the eight T. jansenii individuals in this study. There was strong support for the structure both within the putative parental clades as well as the remaining species (Fig 4A).

3. Results

3.1. Cytochrome oxidase I

The COI gene fragment provided good resolution of the relationships between species. There were 161 variable characters, of which 116 characters were parsimony informative (16.8%). Majority rule consensus trees were based on 22 best MP, 150 ML, and 9854 post-‘burn-in’ BI trees. All the above majority rule trees showed similar topologies and strong bootstrap (MP and ML) and strong posterior probability (BI) support for nodes (Fig. 4A).

All analyses, trees were rooted only with Thalassoma lunare, T. lutescens, T. duperrey, and T. genivittatum were not included in the outgroup rooting so as not to enforce a false topology on the tree. Excluding these species from the outgroup gave a clearer picture of the relationships between the species in the clade and between putative parental species and the hybrid. No outgroup or rooting was specified for the D-loop NUMT dataset in both ML and BI analyses because there were no NUMT bands amplified in any outgroup species at the given cycling profile.

ML analyses were performed with the subtree-pruning–regrafting (SPR) algorithm for 100 bootstrap replicates and Shimodaira–Hasegawa likelihood based topology tests were used to confirm that the topologies of retained trees were not significantly worse than the single best tree. A 50% majority rule consensus tree was computed from the best trees or from best trees with identical log likelihood (−ln L) scores, if too many best trees were retained. BI analyses utilised a Markov chain Monte Carlo search with four chains for 1,000,000 generations, with trees sampled every 100 generations. Initial trees that preceded stabilization were discarded as ‘burn-in.’ MP analyses utilised a full heuristic search with tree–bisection–reconnection (TBR) branch swapping algorithm and random addition of taxa. For AldoB1 and GnRH3-2 datasets, variable sites that were not informative about the relationships between species were manually excluded from the analysis. A 50% majority rule consensus tree was constructed for retained trees.

Finally, relationships between COI mitochondrial haplotypes were inferred using a minimum spanning tree (MST) in Arlequin v2000 (Schneider et al., 2000) using the same substitution model as identified by Modeltest v3.06.

Fig. 4. (A) Best maximum likelihood (ML) tree of the mitochondrial cytochrome Oxidase I (COI) region, with branch lengths showing the number of substitutions between specimens. Topmost values on branches indicate majority rule support values (>50%) for maximum parsimony (MP) followed by maximum likelihood (ML) analyses. Values below branches indicate majority rule support values from 100 pseudoreplications for the Bayesian inference (BI) analysis. The tree is rooted with T. lunare. Clades were identified and corroborated based on an existing molecular phylogeny of the genus (Bernardi et al., 2004). Numbers in brackets next to specimens indicate the number of individuals sharing the same genotype. Note the presence of T. quinquevittatum specimens in the T. jansenii clade and vice-versa. (B) Relationships between hybrids, T. jansenii and T. quinquevittatum haplotypes represented in a minimum spanning tree. Hybrid specimens are indicated by a striped fill and T. jansenii and T. quinquevittatum specimens are indicated by a black fill and white fill respectively. The sizes of the circles indicate the number of individuals sharing that particular haplotype. Crossbars on the line separating haplotypes represent the number of nucleotide substitutions separating them. Haplotype (h) and nucleotide (r) diversities are shown for each clade. Note the large number of T. quinquevittatum individuals with a T. jansenii mitochondrial haplotype.
T. quinquevittatum individuals in this clade all originate from outside of Holmes Reef, while the five hybrids and two T. jansenii individuals originate from Holmes Reef (Fig. 4A).

A haplotype minimum spanning tree (MST) of T. quinquevittatum, T. jansenii, and hybrid individuals grouped them into two distinct clusters, separated by 53 nucleotide substitutions (Fig. 4B). These clusters correspond exactly to the substitutions between haplotypes in both clades, T. quinquevittatum and T. jansenii clades identified in our phylogenetic analyses (Fig. 4A). Haplotype diversity, h, within clades was relatively high (h = 0.859 for Q clade; h = 0.795 for J clade), with low nucleotide diversities of 1–2 nucleotide substitutions between haplotypes in both clades, π = 0.36% (±0.24) and 0.25% (±0.17) for Q and JN clades respectively (Fig. 4B). In the T. quinquevittatum haplotype group, eight different haplotypes were identified, with 5 of 13 individuals sharing an identical most common haplotype. The shared haplotype was present in a hybrid, and two of each of the parental species (Fig. 4B). A similar trend is observed in the T. jansenii haplotype group, with seven different haplotypes identified and 6 of the 13 individuals in that group sharing the single most common haplotype (Fig. 4B). In this instance, six of the seven T. quinquevittatum individuals shared a single haplotype of T. jansenii derivation. All these individuals originated from Holmes Reef, with the exception of one individual in the T. jansenii clade originating from Cocos Keeling Island.

3.2. S7 ribosomal intron 1

In a 739 bp sequenced fragment, 101 characters (13.7%) were parsimony informative. Of these, 23 sites in the hybrid specimens were polymorphic and showed clear evidence of an allele inherited from each parental species, T. quinquevittatum and T. jansenii, which had fixed differences between them. Majority rule consensus trees were based on 2,122,108 MP, 168 ML trees, and 9000 post-‘burn-in’ BI trees. All specimens of T. quinquevittatum, T. jansenii, and T. hardwicke fell into strongly supported and distinct species-specific clades at the S7 nuclear ribosomal intron (Fig. 5).

While the maximum parsimony (MP) analysis provides strong support for the node uniting T. quinquevittatum, T. jansenii, and the hybrids, which form a lineage that is a sister clade to the remaining species in this study, these nodes were not strongly supported in the majority rule consensus trees from maximum likelihood (ML) and Bayesian (BI) analyses (Fig. 5). The underlying phylogenetic signal in the data favours a T. jansenii–T. hardwicke sister relationship, which is responsible for the lack of resolution between, and support for, the clades in the ML and BI analyses. On the other hand, the signal from the hybridization from the MP analysis clearly favours a T. jansenii and T. quinquevittatum sister relationship, within which the hybrids are intermediate to the two.

3.3. Nuclear copy of mitochondrial D-loop

Two NUMT fragments of different sizes were amplified, both of which were present in the hybrids, whilst only one of the fragment sizes was present in each of the three potential putative parental species. The two NUMT fragments (300 and 400 bp, respectively) were analysed as a combined dataset of 404 characters. Of those, 104 were variable and 101 (25%) were parsimony informative. Majority rule consensus trees were based on 453 MP, 646 ML, and 9916 post-‘burn-in’ BI trees. MP and ML majority rule trees showed similar topologies and strong bootstrap support for all nodes (Fig. 6). There was strong posterior probability (BI) support (>95%) for nodes of the Q 400 clade and the node separating HW 300 from Q 400 and JN 300 (Fig. 6).

The 300 bp NUMT fragments from the hybrids and T. jansenii individuals formed a separate clade from the T. hardwicke 300 bp fragment. The 400 bp NUMT fragments from the hybrids and T. quinquevittatum individuals formed a clade separate to either of the T. hardwicke 300 bp or the hybrid and T. jansenii 300 bp clades (Fig. 6). Good sequence data could not be obtained for one hybrid and four T. quinquevittatum 400 bp NUMT fragments and was thus left out of the analysis.

3.4. SNPs: GnRH3-2 and Aldo B1

In a 274 base pair sequenced Gonadotropin Releasing Hormone gene fragment (GnRH3-2), 21 characters were variable with eight characters (2.9%) parsimony informative. Majority rule consensus trees were based on 2862 MP and 14,941 best of 62,780 possible ML (−ln L = 503.11988718) trees. The consensus MP and ML trees had similar topologies and strong bootstrap support for all branches (Fig. 7A). A 270 bp sequenced fragment of the Aldoase B1 (AldoB1) region was amplified. Of the 270 characters, 24 were variable and 19 (7%) were parsimony informative. Majority rule consensus trees were constructed from 2198 MP and best 402 of 17,145 retained ML (−ln L = 506.98744) trees. The consensus MP and ML trees had similar topologies and nodes within the clade were poorly supported (Fig. 7A). A 270 bp sequenced fragment of the Aldoase B1 (AldoB1) region was amplified. Of the 270 characters, 24 were variable and 19 (7%) were parsimony informative. Majority rule consensus trees were constructed from 2198 MP and best 402 of 17,145 retained ML (−ln L = 506.98744) trees. Both MP and ML consensus trees had similar topologies with similar bootstrap support for branches (Fig. 7B). In both cases, relationships between lineages were poorly resolved and the tree topologies were incongruent.

4. Discussion

4.1. Genetic evidence for hybridization

Phylogenetic analyses support the hybrid origins of the five specimens collected from Holmes Reef. In all instances, the maternally inherited COI marker identified T. quinquevittatum as the maternal contributor to the hybridization. The COI marker also indicates that backcrossing by hybrids to both parental species has led to introgression of the mitochondrial genomes between T. quinquevittatum and T. jansenii. The nuclear S7 marker confirms that only T. quinquevittatum and T. jansenii are involved in the hybridizations. This is independently corroborated by the nuclear NUMTs. The results of all three analyses exclude T. hardwicke from any role in the hybridization. These find-
ings confirm the proposed parental contributors, which were identified based on meristics and colour patterns, proposed by Walsh and Randall (2004).

It is evident in this example that determining hybrid status based on meristic and colour pattern descriptions can be accurate and, in this case, is an important diagnostic tool. However, these descriptions invariably fall short of identifying which of the two parental species contribute to the maternal and paternal make-up of the hybrids. It is important to be able to identify both the paternal and maternal contributions because it provides insights into the processes and conditions that lead to hybridization.

The S7 nuclear marker gave the best resolution of relationships between the hybrids and its parent species with strong support for reciprocally monophyletic species clades. There was no evidence of introgression of this nuclear marker between T. jansenii and T. quinquevittatum (cf. Schelly et al., 2006). Although T. quinquevittatum was not found to be the sister species to T. jansenii in a previous phylogenetic analysis (Bernardi et al., 2004), the inclusion of hybrids in the analyses have resulted in these two species appearing to be sister taxa in both nuclear loci (S7 and NUMT). This is a further indication of the intermediate genetic standing of the hybrids compared to T. quinquevittatum and T. jansenii, which concurs with the other findings of this study. It also highlights the conflict between the underlying phylogenetic signal and the hybridization signal and emphasises the utility of maximum parsimony analyses as a method for detecting species-relationships in a hybridization context.
4.2. Backcrossing and introgression in Thalassoma

Some fish that exhibit *T. quinquevittatum* colouration were shown to have *T. jansenii* mitochondrial haplotypes and vice-versa. There are two possible interpretations for this: (1) viable and fertile offspring of F₁ hybrids have backcrossed to both *T. quinquevittatum* and *T. jansenii* parents; or (2) it is a case of incomplete lineage sorting. Mitochondrial gene inheritance is extranuclear and occurs only through the female parent, with few exceptions (Fairbanks and Andersen, 1999). As such, the offspring carries only the maternal mitochondrial haplotype. Hence, the pattern observed in this study is most likely the result of bi-directional mitochondrial gene exchange between *T. jansenii* and *T. quinquevittatum*, facilitated by hybrids that have backcrossed to both parental species (Fig. 8).

Two things are immediately apparent from this process. The first is that F₁ hybrids must have some degree of fertility and reproductive success to be able to cross back to their parent species. The second is that the hybrids produced from backcrosses are not outwardly recognisable as hybrids and must themselves be fertile for further backcrosses. These hybrid backcrosses display the patterning and colouration of one of the two parents, while possessing the mitochondrial haplotype of the other species. For discussion purposes, these backcross hybrids will henceforth be referred to as look-alike hybrids. Although F₁ hybrids may display a combination of parental traits, it is not unusual for a backcross hybrid to have similar colouration and morphological traits as one of its parent species (Mallet, 2005). This occurs because a backcross hybrid would have inherited about 3/4 of its nuclear genomic make-up from one of its parental species. Other factors that could explain this phenomenon include the effect of epistasis on hybrid gene combinations (Burke and Arnold, 2001; Naisbit et al., 2003), although this process is not well understood in fishes.

An alternative explanation for the pattern of mitochondrial lineages observed may be attributed to incomplete lineage sorting (Avise, 2004; Moran and Kornfeld, 1993), which occurs when two species-specific lineages are not fixed for all individuals of either species. This is unlikely to...
Fig. 7. Best maximum likelihood (ML) tree of (A) the nuclear intron GnRH3-2 and (B) the nuclear AldoB1 intron, with branch lengths showing the number of substitutions between specimens. Majority rule support values (>50%) are indicated above the branches for maximum parsimony (MP) analysis and below the branches for maximum likelihood (ML) analysis. Trees are rooted with *T. lunare*.
be the case for *Thalassoma* for two reasons. First, a robust molecular phylogeny has been developed for the genus (see Bernardi et al., 2004) and our results corroborate these phylogenetic relationships with strong node support for all putative parent clades, as well as the remaining ingroup clades, in all three methods of analysis. Second, *T. quinquevittatum* and *T. jansenii* specimens from outside of Holmes Reef fall into the expected species clades, displaying normal colour patterns and mitochondrial make up of their respective species. On the other hand, look-alike hybrids are largely confined to Holmes Reef. The look-alike hybrid individual collected from Cocos Keeling suggests that a similar pattern of hybridization between *T. jansenii* and *T. quinquevittatum* is occurring at Cocos Keeling; another remote and isolated atoll, similar to Holmes Reef.

**4.3. Hybridization in an ecological context**

Only initial abundance data was available for Holmes Reef. However, the pattern reflects that seen in the distribution and abundance of labrid fishes on three outer-shelf reefs (Hicks, Yonge, and Day Reefs) on the Great Barrier Reef (Bellwood and Wainwright, 2001), except that the abundance of *T. quinquevittatum* individuals was about one order magnitude greater than on the Great Barrier Reef (GBR) (F. Walsh, pers. obs.). On the GBR, it is apparent that there is some degree of habitat separation between *T. hardwicke* and the other two species (Fig. 9). This has been attributed to the differing swimming abilities of the three species, which in turn correlates with water movement and wave energy (Bellwood and Wainwright, 2001; Fulton et al., 2001). Holmes Reef is an oceanic atoll and like the outer GBR reefs, is exposed to oceanic swells. On the GBR, *T. jansenii* and *T. quinquevittatum* are predominantly reef flat species, whereas *T. hardwicke* is a predominantly reef
crest species (Bellwood and Wainwright, 2001). Habitat overlap between T. jansenii and T. quinquevittatum provides ample opportunity for interactions during daily activities such as foraging and feeding. While the distributions are similar, there is disparity in the abundance of the three species on Holmes Reef. T. quinquevittatum is more abundant at this location, outnumbering both T. jansenii and T. hardwicke by approximately 25 to 1 in both cases (F. Walsh, pers obs.).

Ecological factors, such as abundance disparities (Arnold, 1997) and environmental selection pressures (Grant and Grant, 1994, 1996; Grant et al., 2004), have been cited as important factors in both initiating hybridization and affecting its evolutionary outcomes (Schluter, 2001; Seehausen, 2004). While it is important to know how exogenous selection impacts on hybridization (Burke and Arnold, 2001), the role of ecology in bringing about hybridization in the first place is often overlooked. In coral reef fish taxa with widespread sympatric distributions, reported hybridizations that are restricted to one or a few areas is increasing (Gardner, 1997; Liao et al., 2004; Lobel, 2003; Myers, 1999; Walsh and Randall, 2004). This contrasts with the terrestrial paradigm of a hybrid zone that coincides with an area of contact or overlap in species distributions (although marine equivalents of this do occur; see McMillan and Palumbi, 1995; Nielsen et al., 2003; Planes and Doherty, 1997; van Herwerden et al., 2002).

At Holmes Reef, habitat overlap combined with a numerical disparity in abundance may be facilitating hybridization between the parental species, T. jansenii and T. quinquevittatum. At low population numbers or densities, species are less likely to encounter conspecifics, thus reducing their probability of finding a mate. Natural hybridizations as a result of numerical imbalance between parent species has long been recognised and is well documented in cases of hybridization in fishes (Arnold, 1997; Hubbs, 1955; Rao and Lakshmi, 1999). Under such circumstances, the rarer species is less likely to encounter another individual of its own species with which to mate and may be left without any choice but to mate with another species. Disparity in abundance such as the one at Holmes Reef means that a T. jansenii individual is more likely to encounter and interact with a T. quinquevittatum than one of its own species. Studies have shown that fishes are able to retain and process information and are able to learn from the behaviour of other (usually conspecific) individuals (Brown and Laland, 2003; Laland et al., 2003). For example, in another species of Thalassoma (T. bifasciatum), new females learn mating site locations from more experienced females (Warner, 1988). Since new recruits of T. jansenii are more likely to interact with and observe T. quinquevittatum individuals, it is possible that they may take cues for behaviour, such as migrating to a spawning site, from them rather than from a member of its own species. Also, studies on other species of Thalassoma have shown that these fishes maintain high fidelity to a specific spawning location, to which they migrate over long distances to mass spawn on a daily basis (Ross, 1984; Warner, 1995). Again, it is possible that a species in the minority (T. jansenii) may learn behaviour, such as where to spawn, from them rather than from a member of its own species. Although evolutionary (and genetic) closeness is an important factor in determining a successful outcome from

4.4. Why hybridize?

Although evolutionary (and genetic) closeness is an important factor in determining a successful outcome from
hybridization, it does not appear to be the underlying basis that drives hybridization between T. jansenii and T. quinquevittatum. Although these two species are closely related, T. hardwicke is the sister species to T. jansenii (Bernardi et al., 2004). Furthermore, T. jansenii and T. quinquevittatum have not been reported previously to hybridize anywhere else in their largely sympatric ranges. This points to the need for an alternative explanation for the underlying reasons for hybridization. As previously discussed, given the overlapping habitat preferences of the two species and the difference in abundance of T. quinquevittatum over T. jansenii, it is plausible that ecology overrides evolutionary relationships in this case of hybridization.

The reproductive behaviour of Thalassoma species is very complex and can vary widely between species. Two main mating behaviours have been reported in the genus. The first mating behaviour is mass spawning, where individuals gather at spawning sites to mate (Ross, 1984; Warner, 1988, 1995). These aggregations usually involve smaller initial phase (IP) males that group-spawn with multiple females, where individuals rise rapidly in the water column and release gametes simultaneously at the apex of the rise (Ross, 1984; Warner, 1995). The second mating behaviour involves pair spawning, where a female enters a site maintained by a single terminal phase (TP) male to mate (Ross, 1984). Even within species, mating behaviour can vary between group spawning and pair spawning depending on the density of individuals and the sex and phase of the individuals (Sara et al., 2005). While there is nothing in the literature on the reproductive behaviour of T. quinquevittatum, T. jansenii or T. hardwicke, we consider both the mass spawning and pair forming strategies for discussion purposes. It is very likely that all three species exhibit both reproductive modes, although on the GBR, territoriality and putative haremic structures are most often seen in T. jansenii and T. hardwicke (D.R. Bellwood, pers obs.), suggesting that pair spawning is possible in these three species, at least in conspecific matings. There are three possible mechanisms by which hybridization may have transpired based on the abundance disparity between T. quinquevittatum and T. jansenii. These are outlined below:

(i) Sneak mating. Given the low probability of encountering a female of its own species, a T. jansenii male may choose to exploit the presence of mass spawning aggregations of T. quinquevittatum individuals by being opportunistic and engage in sneak spawning to maximize its reproductive output. This phenomenon of sneak spawning is not uncommon in fishes (Frisch and van Herwerden, in press; Jamieson and Colgan, 1992; Koseki and Maekawa, 2002) and has been implicated in hybridizations in some commercial salmonid species (Garcia-Vazquez et al., 2002). It is probably more applicable to smaller IP males (Scagianti et al., 1999) than to larger TP males, as TP males tend to have specific spawning sites where they exert territoriality (Warner and Schultz, 1992). Sneak mating is consistent with the genetic data presented herein (mtDNA and NUMT), because T. jansenii males are the rarer of the two hybridising species, whilst the most abundant T. quinquevittatum are the females in all five hybrids investigated. Furthermore, each hybrid is likely to be the product of an independent mating episode with different T. quinquevittatum females, as none of the hybrids share haplotypes with each other.

(ii) No choice. A no choice situation involves the rarer species being compelled to mate with a heterospecific. Although brought about by similar circumstances to the scenario described earlier, this situation is more likely to apply to gravid T. jansenii females looking for a conspecific but unable to find one. However, it can also apply to T. jansenii TP males aggressively courting a gravid T. quinquevittatum female. Given that the maternal parent in all five of the hybrid specimens was T. quinquevittatum, the latter situation would be required.

(iii) Accidental fertilization. Most species of Thalassoma migrate to specific spawning locations on a daily basis (Warner, 1995). These spawning sites are usually located up current on the reef crest or upper slope so released gametes are transported off reef, to minimise egg predation. At Holmes Reef, which is an oceanic atoll, the reef crest drops off suddenly into deep waters of more than 600 m (Leis, 1994). This limits the available area for spawning aggregations and may increase the potential for accidental fertilization, resulting in hybridization. In the case of T. jansenii and T. quinquevittatum, the chances of T. jansenii gametes coming in contact with T. quinquevittatum gametes is further intensified by the numerical disparity between the two species.

Of the three alternatives, the fact that all five of our hybrid specimens have T. quinquevittatum mitochondrial haplotypes, sneak mating by IP males of T. jansenii appear to be the most likely situation under which hybridization between the two species occurred. However, the situation at Holmes Reef requires a further step involving the backcrossing of an F2 hybrid to a T. quinquevittatum male (Fig. 8) as the majority of T. quinquevittatum individuals at Holmes Reef were found to be hybrids with T. jansenii mitochondria. This probably occurs in a mixed mass-spawning aggregation where F2 hybrid females with T. jansenii mitochondria mate with T. quinquevittatum IP males, resulting in offspring that show T. quinquevittatum colouration but with T. jansenii mitochondria (Fig. 8). An alternative possibility is that the initial hybridization producing the F1 hybrids occurred between a T. jansenii female and a T. quinquevittatum IP male. However, there is no evidence to support this scenario, as first generation hybrids with T. jansenii mitochondria have not been found at Holmes Reef to date. Both situations require two separate hybridization events but either one or both must have occurred because of the abundance of T. quinquevittatum individuals with T. jansenii mitochondria at Holmes Reef.

4.5. Look-alike hybrids: the beginning or the end?

The presence of look-alike hybrids is problematic. Their overwhelming presence in the sample of individuals taken
from Holmes Reef (8 of 11 specimens were look-alike hybrids) suggests that the majority of *T. quinquevittatum* and *T. jansenii* individuals at Holmes Reef may be hybrids. However, beyond indicating that the F1 hybrids have a reasonable degree of reproductive success, we know nothing more about them. Based on the large size and gaudy colouration of the five F1 hybrid specimens collected (Walsh and Randall, 2004), it is highly likely that these specimens are terminal phase (TP) males as opposed to initial phase (IP) individuals. So while there is evidence of the F1 hybrids backcrossing to parental species, whether or not the F2 look-alike hybrids are able to breed is unclear. Depending on the fertility of look-alike hybrids and their mate choices, there are three possible scenarios.

(i) **Look-alike hybrids are fertile and backcross with either parental species.** If the look-alike hybrids are fertile and backcross to either pure *T. jansenii* or *T. quinquevittatum* individuals, then there will be a flow of genes of one of the parental species gene pool to the other species gene pool, otherwise known as introgression (Arnold, 1997). This is likely given that they are unrecognisable as hybrids. Persistence of one species mitochondrial lineage in the genome of the other depends on whether the look-alike hybrids breed as males or females. Breeding as males will mean the termination of the mitochondrial haplotype, whereas breeding as females will pass the mitochondrial genome on to the next generation. If the offspring from these backcrosses continue to be fertile and breed with parental species, then eventually the genetic introgression from the other species will be diluted and the species will continue to remain separate at nuclear genome loci, but the mitochondria may persist in the other species if introgression is by female hybrids. This introduces the mitochondria of one species, displacing the mitochondria of the “purebred” parent. It has been shown that species are able to maintain their integrity in the face of limited amounts of gene flow from another species (Arnold, 1997). This is likely if hybridization is a rare event. However, if hybridization is frequent and parental species continue to be assailed by gene flow from the other parent species via the hybrids, it is possible that they may merge and, with time, may become indistinguishable (Rhymer and Simberloff, 1996).

(ii) **Look-alike hybrids are fertile and breed true to other look-alikes.** Look-alike hybrids that are fertile and only breed true to other look-alike hybrids is a step towards becoming reproductively isolated from both parental species (Coyne and Orr, 2004). This is an important requisite for speciation by hybridization, whereby the new true breeding lineage is no longer a part of the evolutionary trajectory of its parental species (Arnold, 1997; Coyne and Orr, 2004). In reef fishes, this depends largely on the ability to identify conspecífics (e.g. based on colour and mating behaviour) to mate with (Randall et al., 1996). Since these look-alike hybrids are indistinguishable from either of the parent species (as far as we can see), a primary mechanism for isolation is missing, making this scenario unlikely.

(iii) **Look-alike hybrids are infertile.** Several studies have shown that while some F1 hybrids are fertile and able to breed, infertility tends to manifest itself in the F2 generation resulting from crosses between hybrid parents (Burke and Arnold, 2001; Price and Bouvier, 2002). If look-alike hybrids are infertile, they will not pass on their genes and thus be an evolutionary dead end (Mayr, 1999). If these infertile look-alike hybrids were the down-the-line result of an exceptional one-off hybridization event, their genetic contribution is stopped and eventually their genes will die out from the population. However, the repercussion of this gene sink for the two parental species is greater if hybridization and production of fertile F1s and the subsequent infertile backcrosses was a persistent event. It could for example, lead to the eventual loss of the rarer *T. jansenii* population at Holmes Reef because no offspring are being generated to carry on the gene lineage.

The look-alike hybrids represent an intriguing aspect of this study but interpretations on the likely outcomes have to be made with caution, especially in light of how little we know about them. However, the fact that so many of the individuals collected from Holmes Reef were look-alike hybrids, and that it was a reciprocal exchange, suggests that backcrossing may have happened repeatedly. There is also evidence to suggest that while the initial hybridization producing F1 hybrids happened between a *T. quinquevittatum* female and a *T. jansenii* male, in the back-cross hybrids, it is more likely that the maternal contribution is coming from a *T. jansenii* female crossing with a hybrid TP male. This is evident from the fact that all *T. quinquevittatum* specimens collected from Holmes Reef turned out to be look-alike hybrids. To fully understand the consequences of hybridization, we need a thorough knowledge of the genetics of hybridization, such as how the break-up of favourable gene combinations affects hybrid fertility and viability and how epistasis affects the manifestation of certain traits in the hybrid phenotype.

### 4.6. Re-thinking hybridization in the marine environment

In a recent study of hybridization in coral reef fishes, van Herwerden and Doherty (2005) postulate that the formation of two hybrid zones, one in the Northern GBR and the other in the Southern GBR, between different morphs of *Acantochromis polycanthus* is the result of a recent divergence and secondary contact following Pleistocene sea-level change. The example of the Southern GBR hybrid zone particularly, where there is little introgression between morphs, represents a clear example of a recent vicariance event (Coyne and Orr, 2004; Mayr, 1963). Studies on other coral reef fish taxa such as the butterflyfishes (*Chaetodon* sp.) (McMillan and Palumbi, 1995; McMillan et al., 1999) have also hypothesised recent origins of coral reef fish lineages as a result of Pleistocene sea-level changes.

These studies are currently in the minority. *Thalassoma*, like many other lineages, is a relatively old genus with the origins of most component species pre-dating
the Pleistocene (Bernardi et al., 2004). Recent studies have highlighted the ancient origins of reef fishes, with most taxa having pre-Pleistocene origins (Barber and Bellwood, 2005; Klanten et al., 2004; McCafferty et al., 2002; Read et al., 2006). If it is true that hybridization is common in young sister taxa (Mallet, 2005), then this could explain the pattern of hybridization observed in most coral reef fish taxa, where “younger” species (e.g., Chaetodon spp.) are observed to hybridize more readily and more frequently than “older” species. While the ages of reef fish taxa may explain why some species hybridize more frequently than others, it does not explain the occurrence of hybrids in sympatric taxa at some locations but not at others. This is the case in the present study where hybridization has only been reported at Holmes Reef, and possibly Cocos Keeling, within the widespread and overlapping distributions of the parental species. Since sympatry is a common characteristic of many coral reef fish taxa (Connolly et al., 2003; Hughes et al., 2002), applying models and theories on hybridization and hybrid zones developed for terrestrial systems to marine examples of hybridization should be done with care. While examples of marine hybrid zones comparable to terrestrial hybrid zones undoubtedly do exist, the emerging pattern of hybridization within the overlapping distributions of sympatric taxa forces us to re-think and re-examine the factors underlying hybridizations in the marine environment.

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References


