

Species boundaries, populations and colour morphs in the coral reef three-spot damselfish (*Dascyllus trimaculatus*) species complex

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Coloration patterns of tropical reef fishes is commonly used for taxonomic purposes, yet few studies have focused on the relationship between species boundaries and coloration types. The three-spot damselfish (*Dascyllus trimaculatus*) species complex comprises four species that vary both in geographical ranges and colour patterns making them an ideal model to study these relationships. We analysed the mitochondrial control region of 122 individuals from all four species collected from 13 localities. Individuals from two species (*Dascyllus albisella* and *D. strasburgi*) grouped into monophyletic clades, while the two other species (*D. trimaculatus* and *D. auripinnis*) were found to be paraphyletic. Coloration patterns were therefore not found to be good predictors of genetic isolation. In contrast, geographical origin was always consistent with the observed genetic pattern.

Keywords: *Dascyllus trimaculatus*; mitochondrial DNA; population genetics; phylogeography; species boundaries

1. INTRODUCTION

The relative role of sexual selection and colour patterns on speciation processes have been well documented in terrestrial (e.g. Endler & Thery 1996) and freshwater systems (e.g. Sturmbauer & Meyer 1992; Seehausen *et al.* 1997, 1999; Grether *et al.* 2001). In contrast, while clear tropical waters generally associated with coral reefs host a large number of brightly coloured marine fishes (McFarland 1991), few studies have focused on the evolutionary implications of colour patterns in coral reef fishes (McMillan *et al.* 1999). Yet the significance of fish coloration is implicitly highlighted when used as the main basis for taxonomic descriptions of typical coral reef fishes, such as parrotfishes (Scaridae), wrasses (Labridae), butterflyfishes (Chaetodontidae) and damselfishes (Pomacentridae) (Lieske & Myers 1999).

While marine reef fishes tend to exhibit large dispersal capabilities, generally via a long pelagic larval stage, geographically localized colour morphs do exist and have been described in a large number of coral reef species. The presence of these morphs has been attributed to local larval retention or local adaptation. In the Caribbean, approximately 10 different colour morphs of the serranid hamlets (genus *Hypoplectrus*) have been described as different species, although little genetic evidence supported it (Thresher 1978; Fischer 1980; Graves & Rosenblatt 1980;

Domeier 1994; Ramon 2000). In contrast, northern and southern Great Barrier Reef colour morphs of the spiny damselfish (*Acanthochromis polyacanthus*), which display assortative mating in contact zones and large genetic divergences, were not described as different species or even sub-species (Doherty *et al.* 1995; Planes & Doherty 1997*a,b*). In sympatric cases, colour morphs were shown to develop in ecological allopatry by occupying different depth zones. This was observed for gopher/black-and-yellow rockfish (*Sebastes carnatus*, *S. chrysomelas*), cabrilla seabass (*Serranus scriba*) (Larson 1980; DeMartini & Donaldson 1996; Medioni *et al.* 2001) and arc-eye hawkfish (*Paracirrhites arcatus*). However, no fixed genetic differences between colour morphs were found in any of these cases (Alesandrini & Bernardi 1999; Medioni *et al.* 2001; G. Bernardi and E. E. DeMartini, unpublished data). Similarly, McMillan and co-workers found very loose species boundaries with large incidences of colour-morph and genetic intermediates for Indo-Pacific butterflyfishes (*Chaetodon* spp.) (McMillan *et al.* 1999).

Thus, no clear relationship between colour morphs and genetic isolation has emerged from the studies described above. The general picture of marine speciation is further complicated by the apparently vast, usually undetected, presence of cryptic species, which only differ genetically but differ neither morphologically nor in coloration pattern (Knowlton 2000).

Considering these conflicting data, our goal was to study the relationship between coloration pattern formation and speciation events in a widespread coral reef fish

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Figure 1. Sampling localities of *Dascyllus trimaculatus*-complex species. *trimaculatus*, circle; *albisella*, triangle; *auripinnis*, square; *strasburgi*, star.

species complex. Species definitions have been modified over the years and are still the subject of much debate, yet almost all include some level of reproductive isolation (Avisé 2000). This, in turn, results in concordant patterns of monophyletic genes (Avisé & Ball 1990; Baum & Shaw 1995; Palumbi *et al.* 2001). In the case of a match between colour morphs and speciation events, each colour morph should be associated with a reciprocally monophyletic assemblage, previously described as a species (see Shaw (1998), De Queiroz (1998) and Hey (2001) for species concept definitions). It is important to remember that this working definition is restrictive, especially for those incipient lineages where sorting has not had time to occur.

In order to approach our question, we studied a group of coral reef damselfishes in the genus *Dascyllus*. The genus *Dascyllus* comprises approximately 10 species (Randall & Allen 1977; Bernardi & Crane 1999). Among those 10 species, four large *Dascyllus* species are closely related: *D. albisella*, *D. strasburgi*, *D. trimaculatus* and the recently described *D. auripinnis* (Randall & Randall 2001). Because these species are very similar, we decided here to group them in what we call the *Dascyllus trimaculatus* complex. These species share life-history traits (Godwin 1995). During reproduction, eggs are laid on the bottom and fertilized externally. Larvae hatch after about three days (Garnaud 1957; Fricke & Holzberg 1974; Thresher 1984). After approximately 22–26 days in the water column (Wellington & Victor 1989), larvae settle primarily on anemones (*D. trimaculatus* and *D. auripinnis*) or branching corals (*D. albisella* and possibly *D. strasburgi*) (Fautin & Allen 1992; Schmitt & Holbrook 1996; Holbrook & Schmitt 1997, 1999; Schmitt & Holbrook 1999*a,b,c*) where they remain until the sub-adult stage. Adults then shelter in nearby reef crevices.

Three species have very restricted and non-overlapping geographical ranges. *Dascyllus albisella* is only found in the Hawaiian archipelago (and Johnston Atoll), *D. strasburgi* is endemic to the Marquesas Islands and *D. auripinnis* is found in Fiji, the Line Islands, the Phoenix Islands and the northern Cook Islands. The northern Cook Islands is the only region where two nominal species, *D. auripinnis* and *D. trimaculatus*, are found sympatrically (Randall & Randall 2001). In contrast to the restricted ranges described above, *D. trimaculatus* ranges broadly in the

Indo-West Pacific from the Red Sea to Japan and from East Africa to French Polynesia (figure 1). The four species show minor (but always overlapping) differences in morphometric and meristic characters (Randall & Allen 1977; Randall & Randall 2001). Their colour patterns, however, are distinct. *Dascyllus trimaculatus* is black with three white spots, one on the forehead and one on each side of the body, which tend to fade with age. The other species show colour deviations from this general pattern. *Dascyllus albisella* tend to have a whiter body with a large white patch on the side, *D. auripinnis* have yellow fins and belly, and *D. strasburgi* are overall brown with barely visible spots.

Molecular studies have proven useful on groups, such as marine cryptic species, which are difficult to study using classical approaches (Avisé 1994; Knowlton 2000). In this study, we used the mitochondrial control region (also called D-loop) as a molecular marker to evaluate the relationship between species, colour-morphs and the biogeography of the *D. trimaculatus* species complex.

2. MATERIAL AND METHODS

(a) *Sample collections*

Adult *Dascyllus* individuals were collected by divers using hand nets or spear. *Dascyllus trimaculatus* individuals were collected from Eilat (Israel), Wallis Island (French Territories) and Palau (Micronesia). *Dascyllus auripinnis* adults were collected at Kingman Atoll (Line Islands) and Baker Island (Phoenix Islands). *Dascyllus strasburgi* adults were collected in the Marquesas Islands (French Polynesia). Other individuals included in this study were: *D. trimaculatus* from Zanzibar (Tanzania), Moorea and Rangiroa (French Polynesia), Okinawa and Akajima (Japan), Manado (Indonesia), *D. auripinnis* from Kiritimati (Kiribati) and *D. albisella* from Hawaii. These latter individuals were from Bernardi *et al.* (2001) (figure 1). Sample numbers per locality are described in table 1. The closely related species *D. reticulatus* and *D. carneus* were used as outgroups, following Bernardi & Crane (1999). While all samples were used in the analysis, only 11 representatives from French Polynesia are shown here. A full resolution of all French Polynesia samples is given in Bernardi *et al.* (2001).

Table 1. Summary of sample numbers, number of haplotypes, number of variable sites and mean sequence divergence (and standard deviations) within species and clades of the *Dascyllus trimaculatus* complex.

group name	<i>n</i>	number of haplotypes	<i>n</i> of variable sites	mean pairwise sequence divergence
all samples	122	115	177	6.9% (3.8%)
<i>D. albisella</i> /clade 3	10	9	19	2.4% (1.5%)
<i>D. auripinnis</i>	6	6	14	1.8% (0.8%)
<i>D. strasburgi</i> /clade 5	5	5	29	3.6% (1.9%)
<i>D. trimaculatus</i>	101	97	154	6.5% (4.1%)
clade 1	8	8	48	4.9% (1.8%)
clade 2	38	37	52	2.1% (1.0%)
clade 4	61	56	62	2.5% (1.3%)

(b) Polymerase chain reaction and sequence analysis

DNA preparations followed Bernardi *et al.* (2001). Amplification of the mitochondrial control region (also called D-loop) was accomplished with universal primers CR-A and CR-E described in Lee *et al.* (1995). After sequencing 10 individuals, a conserved region was used to design a specific internal primer, DTPL (TTT GTT ACA GCA AAT TAT TTAT) and used in conjunction with CR-E. Sequencing and polymerase chain reaction (PCR) protocols with their respective computer analyses followed Bernardi *et al.* (2001). Population analyses of the sequences and the repeats observed on the 5' end of the D-loop was performed using the ARLEQUIN software package (Schneider *et al.* 2000).

3. RESULTS

(a) Sequences

The amplified mitochondrial control region was 410 base pairs (bp) long. The 5' end portion of the control region comprised a 23 bp repeat (TTA TAG CAA TGA TAG TTT TTA AT) that was present in up to nine copies. Repeated regions on the 5' end of the control region are not uncommon in fish species (reviewed in Lee *et al.* 1995; Faber & Stepien 1999). We did not find any heteroplasmic individuals (i.e. individuals containing several types of molecules differing in repeat copy numbers). This repeated region was almost always identical in sequence and variable in number of repeats between individuals, and therefore it was not included in the phylogenetic study but was analysed separately.

Out of the amplified 410 bp, some regions were excluded from the phylogenetic analysis. We removed 22 bp and 20 bp from the 3' and 5' ends of the sequence, corresponding to the primer sequences. We removed the multiples of 23 bp corresponding with the identical repeats described above. We also removed 10 bp that were found between the 5' end PCR primer region and the beginning of the repeats. We assumed that the substitution rate of this region could be affected by its neighbouring repeated region. The resulting sequence analysed was 358 bp long, which, once aligned with the outgroup (thus including gaps for best alignment) was 378 bp long.

We compared sequences from 122 individuals. Sample numbers per species and clade, numbers of haplotypes and variable sites, as well as average pairwise sequence divergences, are given in table 1. Haplotype diversity was found to be very high. Out of 122 individuals, 115 had

unique haplotypes. Alignments resulted in a 378 bp sequence with 177 variable sites and 122 phylogenetically informative ones. As expected, transitions were more frequent than transversions (ratio of transitions: transversions = 2.8). Phylogenetic relationships were estimated using equal weights between transitions and transversions, and also with transversions weighted three times as much as transitions. Major tree branchings using these two weighting schemes were identical. Phylogenetic relationships among individuals based on mitochondrial control regions are shown in figure 2. Consensus maximum parsimony bootstrapped trees were identical to the neighbour-joining ones (figure 2).

(b) Phylogenetic analysis

Individuals partitioned into five major clades (numbered 1 to 5 in figure 2). Each of these clades was well supported by both reconstruction methods (figure 2). Genetic distances between clades averaged 9.87% sequence divergence (Kimura 2 distances, table 2). Clade 1 included the Indian Ocean–Red Sea populations. Pacific Ocean individuals separated into the four following clades: Japan, Indonesia, Wallis, Palau, Phoenix and Line islands (clade 2), Hawaii (clade 3), Society Islands–Tuamotus (clade 4), and Marquesas (clade 5). A polytomic grouping that included clades 3, 4 and 5 was also well supported (figure 2). Clade 2 was further divided into two sub-clades. One sub-clade comprised all Indonesian individuals. The other sub-clade included all individuals from Palau and most individuals from Wallis (8 out of 9). Individuals from Japan and the Line Islands were shared among those two sub-clades. *Dascyllus auripinnis* individuals were also found in both clades.

(c) Testing alternative topologies

In order to test alternative topologies, we forced samples to partition according to the four recognized species (*D. trimaculatus*, *D. auripinnis*, *D. strasburgi*, *D. albisella*). The alternative topology that forced these four groups to be monophyletic augmented the tree length by 33 steps and was found to be significantly worse than the tree presented in figure 2 (Kishino–Hasegawa test, $p < 0.0001$).

As mentioned above, clade 2 comprises both *D. trimaculatus* and *D. auripinnis* individuals. When *D. trimaculatus* and *D. auripinnis* individuals from clade 2 were constrained into two monophyletic clades, an additional seven steps were added to the tree length, and

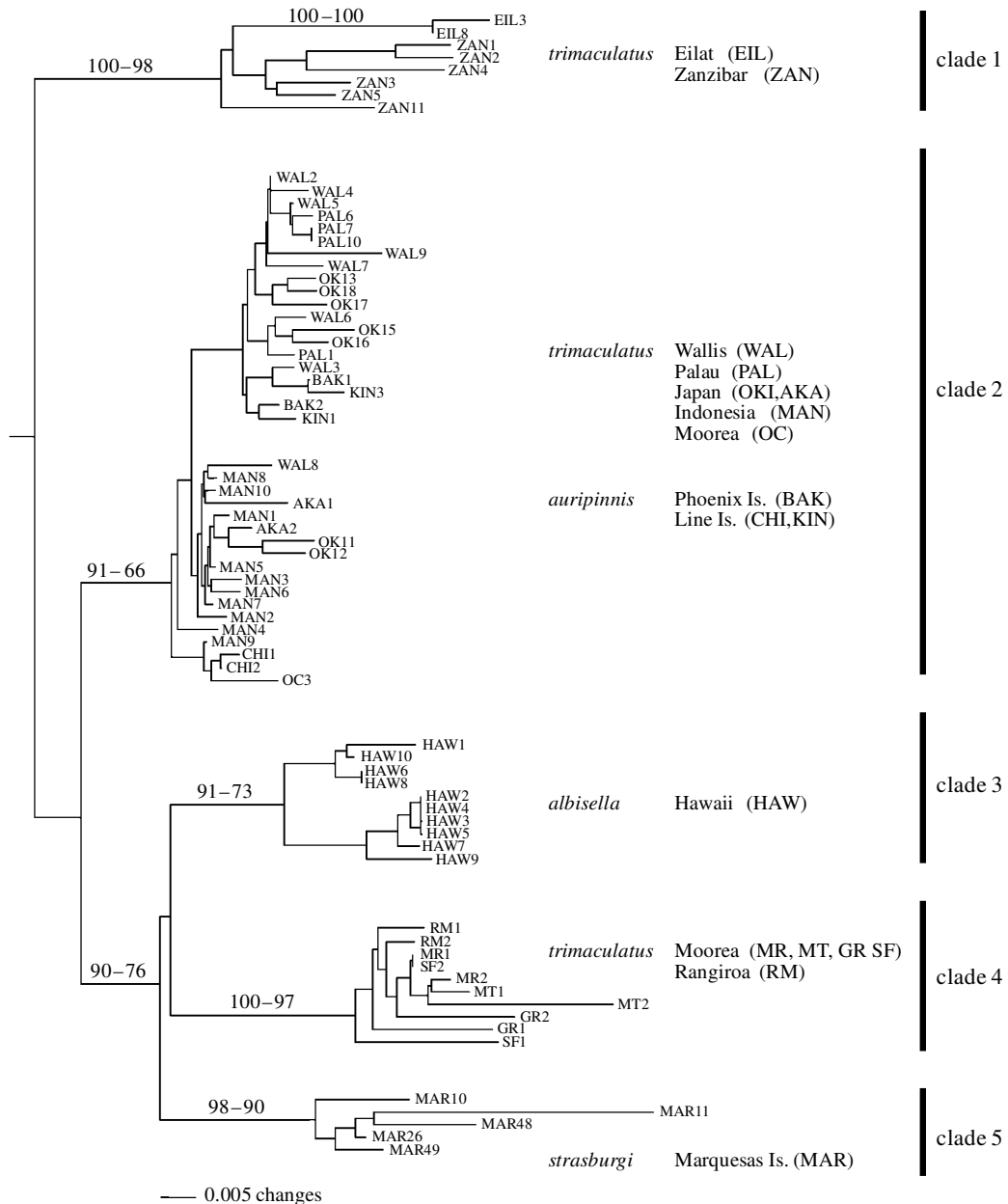


Figure 2. Phylogenetic relationships of *Dascyllus trimaculatus*-complex individuals using the neighbour-joining method. Kimura 2 parameter method was used to calculate distance. Abbreviations used in the tree are listed on the right. Sampling localities in Moorea (MR, MT, GR, SF, OC) are described in Bernardi *et al.* (2001). Numbers above the major nodes indicate bootstrap support using the neighbour-joining and the maximum parsimony methods (heuristic search), respectively. In all cases, 500 bootstrap replicates were used.

this augmentation was found to be marginally significant (Kishino-Hasegawa test, $p = 0.08$).

(d) Population structure

An analysis of molecular variance (AMOVA) on the five clades revealed that gene flow between clades was very low with an average $F_{st} = 0.67$ (s.d. = 0.04), $N_m = 0.25$ (s.d. = 0.05) (table 2). All F_{st} pairwise comparisons were found to be statistically significant at the 0.001 level. Furthermore, 66.4% of the overall variance of the data could be explained by the variance among the clades ($\Phi_{ct} = 0.6639$).

The distribution of the 23 bp-repeats was also analysed by grouping distributions in the five clades from the phylogenetic analysis (figure 3). An AMOVA showed that F_{st}

values between clades were also high, average $F_{st} = 0.209$ (s.d. = 0.178) and statistically significant ($p < 0.001$) in all pairwise comparisons but for one population pair (clade 2 and clade 3, Pacific and Hawaii).

4. DISCUSSION

(a) Pacific-Indian Ocean biogeography

The results presented here add to our previous biogeographical inferences (Bernardi *et al.* 2001). The *D. trimaculatus* complex is separated into two major groups, the Indian Ocean (and Red Sea) and the Pacific. Within the Pacific, four biogeographical regions of vastly different sizes stand out. The basal group is formed by a very large area that comprises individuals from Japan to

Table 2. Gene flow levels and DNA sequence divergence between clades of the *Dascyllus trimaculatus* complex (as shown in figure 2). Values of F_{st} are given above the diagonal. Below the diagonal, DNA sequence divergences are given in percentages (Kimura 2 method) with standard deviations between parentheses.

	clade 1	clade 2	clade 3	clade 4	clade 5
clade 1	—	0.62	0.68	0.75	0.61
clade 2	9.81 (1.27)	—	0.65	0.71	0.64
clade 3	12.42 (1.66)	8.12 (1.19)	—	0.67	0.64
clade 4	12.41 (1.80)	9.03 (1.95)	8.17 (2.02)	—	0.71
clade 5	12.82 (1.55)	9.16 (1.62)	7.58 (1.26)	9.16 (2.21)	—

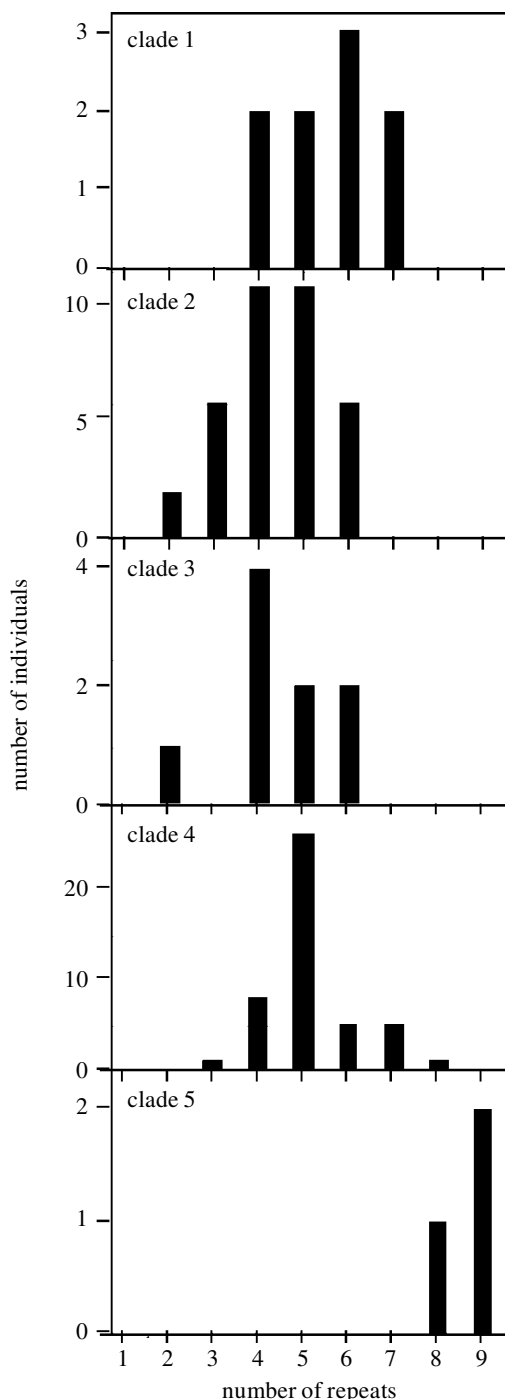


Figure 3. Histograms represent the number of individuals per size class of 5'-end 23 bp repeats.

Wallis. This group is divided into two subgroups. More samples and sites will be necessary to identify the substructure of this group. The three remaining groups form a trichotomy with equidistant clades (Hawaii, Society-Tuamotu Islands and Marquesas Islands). While the Marquesas are much closer to the Tuamotus than to Hawaii, this was not reflected by the data. Furthermore, individuals from Kiritimati and Kingman Atoll, which are islands that lie between the Marquesas and Hawaii, belong to the basal Pacific clade (clade 2). One possible explanation for these patterns is that clade 3, 4 and 5 are the result of rare chance events of propagules being transported to remote locations. Once in these locations, local retention results in low levels of gene flow and strong founding effects.

(b) *Species boundaries*

As mentioned in the introduction, our working hypothesis was that matching species and coloration patterns should result in a concordant phylogeny. This is the case for *D. albisella* and *D. strasburgi*. Indeed, in both cases, all individuals collected in Hawaii and the Marquesas grouped in well-supported monophyletic groups. The genetic divergence within these groups also corresponded with the level of genetic divergence expected for a fish species (McCune & Lovejoy 1998). For the remaining two species, however, the situation was different. Data could statistically reject the placement of *D. trimaculatus* in a monophyletic assemblage ($p < 0.001$). *Dascyllus trimaculatus* individuals did not group in a single monophyletic clade, but in a paraphyletic assemblage of three monophyletic clades. These three clades consisted of one clade from the Indian Ocean and the Red Sea (where the type specimen of *D. trimaculatus* originated), one clade from a broad region in the Pacific and one clade from the Society-Tuamotus. These clades showed high bootstrap support and levels of genetic divergence more typical of between-fish species divergences (McCune & Lovejoy 1998). Thus these groups may be considered three different cryptic species. Alternatively the species denominations *D. albisella*, *D. strasburgi* and *D. auripinnis* may not correspond with *bona fide* species but to population variants of *D. trimaculatus*.

In the case of *D. auripinnis*, data also rejected its monophyletic status, but only marginally ($p = 0.08$). *Dascyllus auripinnis* data may be interpreted as individuals with colour variations within another species. If this is the case, this phenotype may have evolved in parallel at different locations. Alternatively, our data may be a case of introgression of mitochondrial DNA in closely related species, or of a very recent speciation event with incomplete lin-

age sorting. The main argument in favour of *D. auripinnis* being a genuine species is that it is found sympatrically with *D. trimaculatus* in the northern Cook islands (Randall & Randall 2001).

(c) Colour patterns and speciation

Are colour patterns good indicators of speciation events? The four colour morphs observed in the *D. trimaculatus*-complex individuals separate into five clades. Two of these clades match a single colour morph each—one clade includes two colour morphs and one colour morph is shared among three clades. Thus colour morphs do not strictly partition in natural groups. This may be due to the presence of previously undetected cryptic species. This is likely to be the case for *D. trimaculatus*, a species with a huge geographical range. If the tempo of marine speciation is linked to the pelagic larval duration, as was suggested before (Palumbi 1992, 1994), it is likely that the same mechanisms apply for all four species under consideration as they all have similar life histories. Since three species (*D. auripinnis*, *D. albisella*, *D. strasburgi*) have very small geographical ranges, it is likely that the currently recognized *D. trimaculatus* is a collection of entities with small geographical ranges rather than a single species with a huge geographical range. These entities display large genetic divergences but no morphological (including coloration) differences. On the other hand, *D. auripinnis* coloration patterns (yellow lower body and fins) may either be reflective of recent speciation with little genetic divergence (or lineage sorting), or due to ecological adaptation to turbid waters (Randall & Allen 1977).

Further work should focus both on molecular markers and sampling locations. It is likely that nuclear markers (introns and microsatellites) will provide insight on issues of introgression as well as lineage sorting. Expanding sampling localities and numbers, particularly at the contact zone between *D. auripinnis* and *D. trimaculatus* in the northern Cook Islands, will improve our understanding of speciation mechanisms in the *D. trimaculatus* complex.

We would like to thank all the people who provided us with samples: D. R. Robertson (Marquesas, Christmas Island, Akajima), A. Abelson (Eilat), L. Martin (Palau), S. Planes (Wallis), M. Erdman and E. Maloney (Indonesia) and S. Sato (Okinawa). We would also like to thank J. E. Randall, P. Raimondi, M. Carr and D. Siciliano for comments on our work. This research was funded by a Faculty Development Award (UCSC Natural Sciences) to G.B., an NSF OCE 9503305 grant to R.S. and S.H. and a Multicampus Research Incentive Fund, University of California, to G.B., S.H. and R.S.

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As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.