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Genetic evidence for two distinct clades in a French Polynesian population of the coral reef three-spot damselfish *Dascyllus trimaculatus*

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Abstract Previous studies have shown that the three-spot damselfish species complex [*Dascyllus albisella* Gill, *D. auripinnis* Randall and Randall, *D. strasburgi* Klausewitz, *D. trimaculatus* (Ruppell)] is an assemblage of five geographically distinct clades. The one exception was a single *D. trimaculatus* from French Polynesia, which grouped with "Pacific Rim" individuals. In the present study, an additional 252 individuals from French Polynesia collected between June 1996 and January 2002 were analyzed using PCR amplifications, restriction fragment length polymorphisms, and DNA sequencing of the mitochondrial control region. The French Polynesian *D. trimaculatus* belong to two distinct clades. One clade comprising 96% of the individuals includes haplotypes found only in French Polynesia. The other clade (4% of the individuals) is comprised of haplotypes that cluster with "Pacific Rim" individuals, a clade with widespread distribution from Japan to the Line Islands and from Wallis to Palau. Present data suggest that a small number of larvae from northwestern reefs (possibly Line Islands) may have occasionally reached and colonized French Polynesian reefs.

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Introduction

Most coral reef fishes exhibit a bipartite life with a pelagic larval phase and a benthic adult phase. While the benthic phase is usually relatively sedentary, the pelagic larval stage provides the species with an opportunity for long-distance dispersal. It has been assumed that pelagic larval duration correlates with dispersal potential. Several findings, however, challenge this idea. For example, many species that are endemic to remote islands, such as the Hawaiian archipelago, have very long larval durations (e.g. *Thalassoma ballieui*; Victor 1986), while a short dispersal potential that maintains endemism would have been predicted. Furthermore, genetic studies that specifically examined the relationship between larval durations and gene flow levels (a proxy for dispersal) found conflicting results (Waples 1987; Doherty et al. 1995; Shulman and Bermingham 1995; Riginos and Victor 2001). However, genetic studies on species that lack pelagic larvae, such as spiny damselfish (*Acanthochromis polyacanthus*) and black surfperch (*Embiotoca jacksoni*), did show that gene flow was, as expected, extremely reduced (Doherty et al. 1995; Bernardi 2000; Planes et al. 2001). Data for these species were particularly interesting for oceanic island populations (Coral Sea Islands and California Channel Islands, respectively), where chance events caused unusual dispersal and resulted in strong founder effects.

There are four nominal species in the three-spot damselfish species complex: *Dascyllus albisella* Gill, *D. auripinnis* Randall and Randall, *D. strasburgi* Klausewitz, and *D. trimaculatus* (Ruppell). The larvae of *D. trimaculatus* have relatively short pelagic larval durations (22–24 days) (Wellington and Victor 1989) and might be expected to have low gene flow levels among geographically distant populations. In previous studies (Bernardi et al. 2001, 2002), members of the species complex were sampled from the Indian Ocean, the Pacific Rim (Japan to Wallis Island), French Polynesia, and Hawaii. Using DNA sequences of the mitochondrial control region we identified five clades. Clade 1 representing

putative *D. trimaculatus* from the western Indian Ocean. Clade 2 representing *D. trimaculatus* and *D. auripinnis* from localities ranging from Japan to Indonesia to the Line Islands. The recently described species *D. auripinnis* occurs in the Phoenix and Line Islands to the northwest of French Polynesia (Randall and Randall 2001) and in the Cook Islands to the west (Randall and Randall 2001; H. Debelius, personal communication). It is readily distinguished from *D. trimaculatus*, which is solid black with the characteristic three white spots, by its bright yellow fins and underbody. In our previous studies, individuals from this species did not form a monophyletic clade, but were intermixed with other individuals from clade 2, the "Pacific Rim" clade of *D. trimaculatus* sensu stricto. Clade 3 representing *D. albisella* from Hawaii. Clade 4 representing *D. trimaculatus* from the Society Islands and Tuamotu in French Polynesia. Clade 5 representing *D. strasburgi* from the Marquesas. Only 1 of 62 individuals from French Polynesia grouped with the widely distributed clade 2, while all others belonged to clade 4, which appeared to be restricted to French Polynesia. This raised the possibility that low frequency dispersal events might be responsible for transporting small numbers of clade 2 individuals from more northwestern localities to the southeastern extreme of the distribution of *D. trimaculatus* (clade 4) in French Polynesia (Bernardi et al. 2001, 2002).

The goal of the present study was to determine whether the previous finding of a single, clade 2 type *D. trimaculatus* in French Polynesia (Bernardi et al. 2001, 2002) would be borne out by more intensive sampling. We used a combination of restriction fragment length polymorphism (RFLP) and sequencing of the mitochondrial control region in adult fish collected in the Society and Tuamotu Islands, and determined the phylogenetic relationships among members of the whole "*D. trimaculatus*" species complex using the combined data from the present and previous studies (Bernardi et al. 2001, 2002).

Materials and methods

Sample collection

From June 1997 to January 2002, 252 adult or subadult *Dascyllus trimaculatus* (Ruppell) were collected by spear or by hand net while free- or SCUBA-diving in the Tuamotu archipelago (Rangiroa)

Table 1 *Dascyllus trimaculatus*. Sampling sites, dates, and numbers of specimens. Only clade 2 and clade 4 individuals are listed, and their phylogenetic distribution is scored in the corresponding column. Moorea, Tetiaroa, and Maiao samples, as well as 8 samples from Rangiroa and 18 samples from Christmas Island were from the present study; all other samples are from Bernardi et al. (2001, 2002)

Locality	Clade 2 "Pacific Rim"	Clade 4 "French Polynesia"	Collection period	Phylogenetic distribution
Akajima and Okinawa, Japan	9			
Manado, Indonesia	10			
Palau	4			
Kingman Atoll, Line Islands	2			
Christmas Island, Line Islands	20			
Baker island, Phoenix islands	2			
Wallis Island	8			
Total	55	0		
Society + Tuamotu Islands				
Total	9	243		
Rangiroa				
Rangiroa Motu	1	12	Jun 1997	Motu, inside
Tiputa Pass	1	2	Jun 1997	Pass, outside
Total	2	14		
Tetiaroa				
	0	6	Aug 1997	North shore ($n = 1$), south shore ($n = 5$)
	1	9	Dec 2000	North shore
Total	1	15		
Moorea				
Cook's Bay	0	7	Jun 1997	Pinnacle, inside
Between bays	0	6	Jun 1997	Outside slope
West Opunohu Bay	0	15	Apr 2000	Outside slope
White house wall	1	15	Apr 2000	Fringing reef, inside
Tiaora	0	5	Apr 2000	Inside lagoon
Temae	0	25	Apr 2000	Inside lagoon
Temae	1	14	Dec 2001	Inside lagoon
Motus	0	23	Jan 2002	Inside lagoon
Between bays	1	13	Jan 2002	Outside slope
White house wall	0	20	Jan 2002	Fringing reef, inside
Opunohu channel	1	40	Jan 2002	Inside lagoon
Opunohu crest	0	5	Jan 2002	Inside lagoon behind Crest
Total	4	188		
Maiao				
Total	2	26	Sep 1997	

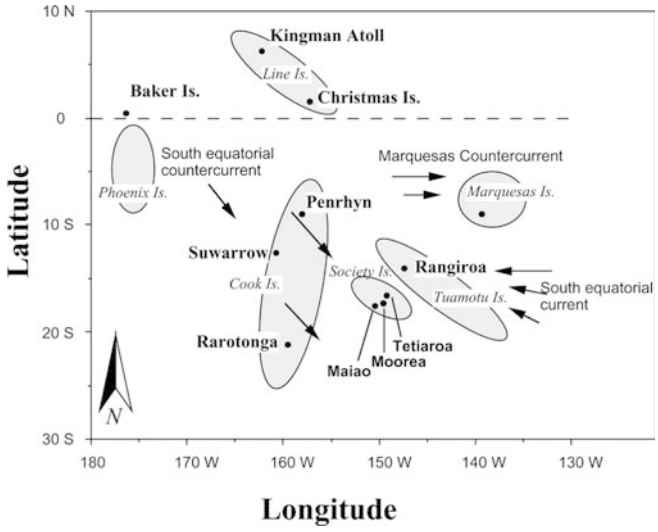

Fig. 1 *Dascyllus trimaculatus*. Sampling sites. Island groups are represented in *gray*. Oceanic currents discussed in the text are indicated with *arrows* (Rancher and Rougerie 1992)

and in the Society Islands (Tetiaroa, Moorea, Maiao), which lie between 15° and 18°S and 147° and 152°W. In order to avoid cohort and Hedgecock effects (Hedgecock 1994), all fish had a total length (TL) of >25 mm and most were longer than 75 mm. As many different habitats as possible were sampled (Table 1). None of the fish had the yellow fins and underbody of *D. auripinnis*. An additional 16 adult and 2 juvenile *D. auripinnis* were also collected by spear while scuba diving in December 2002 at Christmas Island, Kiribati (Fig. 1).

DNA extraction, PCR amplifications, RFLPs, and sequencing

All molecular protocols used in this study were identical to the protocols used by Bernardi et al. (2001, 2002). Using data generated from our previous studies, we identified a part of the mitochondrial control region that distinguishes the "Pacific Rim" clade from the "Society + Tuamotu" clade (clades 2 and 4 of Bernardi et al. 2002). This variable region is differentially cut by the restriction enzyme *Hin*f 1, which we used for an RFLP assay. Samples were PCR (polymerase chain reaction) amplified for the control region, and then digested with *Hin*f 1 for 2 h following the manufacturer's protocols (New England Biolabs). Positive (OC3) and negative controls were always used. All positive samples were then sequenced with an automated sequencer (Applied Biosystems, ABI 3100). Random negative samples were also digested overnight and/or sequenced, but we never found false negatives or false positives.

Statistical analysis

Phylogenetic reconstructions followed Bernardi et al. (2002). Briefly, the neighbor-joining method was used with Kimura 2-parameter distances. A total of 1,000 bootstrap replicates was used to assess the robustness of nodes. Gene flow levels were calculated using DNAsp 3.51 (Rozas and Rozas 1999) following Hudson et al. (1992).

Results

Population structure

In the Society and Tuamotu Islands that were sampled, *Dascyllus trimaculatus* samples of clade 2 type were found in low frequencies. Overall, we found that 3.6% of the individuals sampled were of the clade 2 type (9 individuals out of 252) (Table 1; Fig. 2). Absolute numbers of individuals per sampling site and island are given in Table 1. The overall frequency of clade 2 individuals in French Polynesia was not significantly different between different islands, between Society and Tuamotu Islands, between atolls and high islands, or between lagoons and the outer reef (Chi-squared tests). The rarity of "clade 2" haplotypes in French Polynesia reflects very low gene flow between the "Pacific Rim" clade and the "Society + Tuamotu Islands" clade, with an average number of 0.16 migrants per generation ($N_m = 0.16$; $F_{st} = 0.753$).

Phylogenetic relationships

Previous investigations showed that one individual, OC3, from Moorea, French Polynesia grouped with clade 2 individuals (Bernardi et al. 2001, 2002). All nine individuals from French Polynesia that clustered with "Pacific Rim" individuals grouped in a single monophyletic subclade, which also included the original OC3 sample, one individual from Manado, Indonesia (MAN9), one individual from Wallis Island (WAL3), and 15 *D. auripinnis* individuals from Baker Island (1), Kingman Atoll (1), and Christmas Island (13) (Fig. 2). This subclade was defined by two unique synapomorphic nucleotides (positions 169 and 251). Four of the Christmas Island (CHI2, XMA4, XMA11, XMA17) and four French Polynesian individuals (OC3, RM10, TPT1, MAI4) shared an identical haplotype (Fig. 2).

Discussion

The phylogenetic groupings of three-spot damselfishes into five divergent and well-supported clades has been interpreted to represent five separate species (three of them being cryptic species), or as a cluster of five genetic populations of a single species (Bernardi et al. 2001, 2002). If we consider the five clades as separate species, the results described above would indicate the presence in French Polynesia of two sympatric cryptic species at very different frequencies. This possibility should be tested further by carefully examining large numbers of individuals. Indeed, it has been shown in other systems that previously unnoticed subtle morphological differences become apparent once genetic studies identify cryptic species (Knowlton 2000). The *Dascyllus trimaculatus* complex is morphologically very uniform. For example, besides coloration, *D. auripinnis* differs from *D. trimaculatus* by an apparently larger size and by only two non-diagnostic morphological characters: modally 1 fewer gill raker and a shorter average length of the paired fins (Randall and Randall 2001). Randall and Randall (2001) were able to detect such differences using

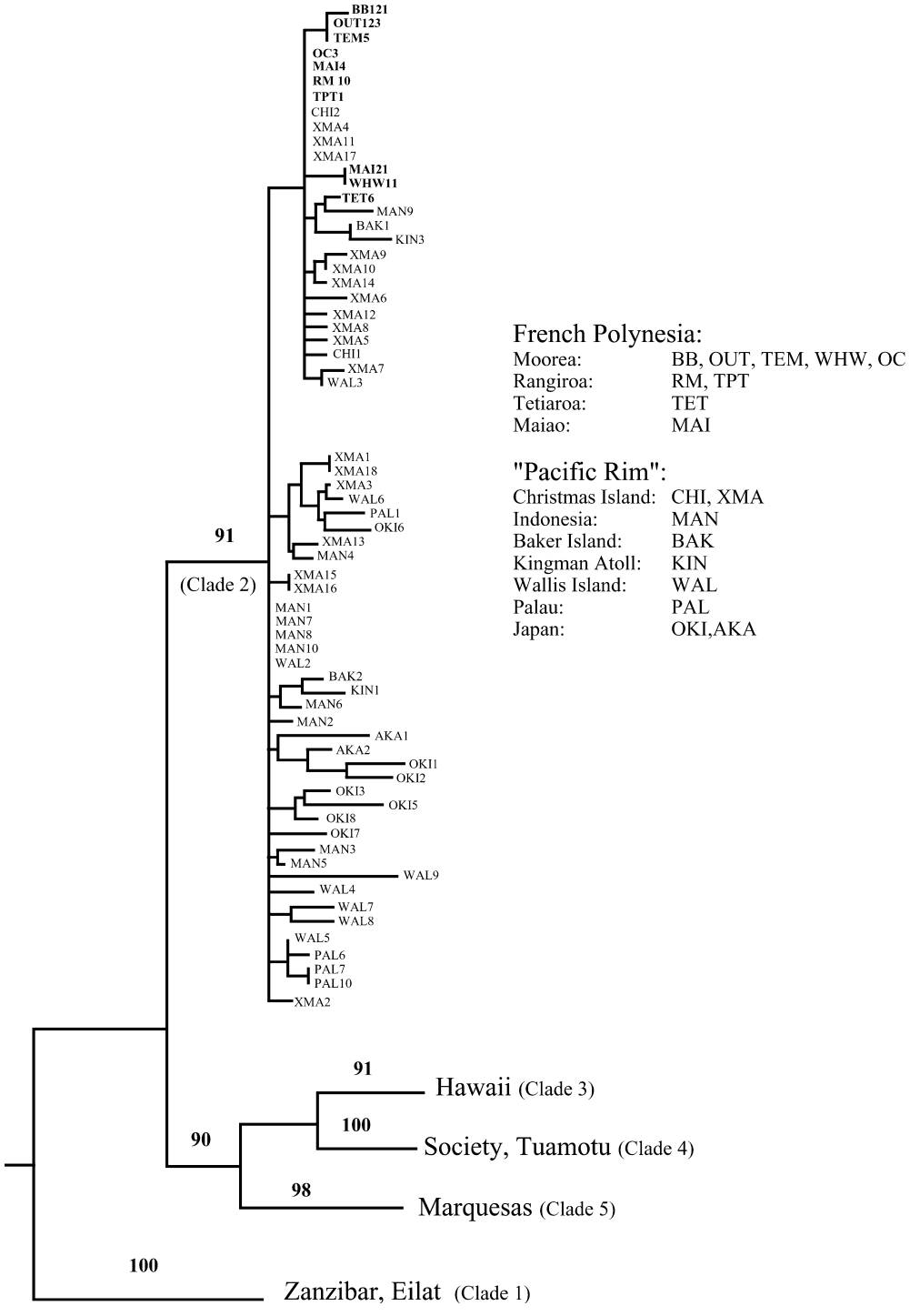

Fig. 2 *Dascyllus trimaculatus*. Phylogenetic relationships based on the mitochondrial control region. Phylogenetic reconstruction used the neighbor-joining method with numbers above major nodes indicating bootstrap support. All available individuals were used to establish this tree ($n = 178$), but only clade 2 (Pacific Rim) individuals are represented. *Right panel* indicates a list of abbreviations used in the tree. French Polynesia (except OC3) and XMA (Christmas Island) sequences are from the present study. All other sequences are from Bernardi et al. (2002)

18 individuals, which in our case would correspond to a screening of approximately 500 French Polynesian individuals.

The alternative interpretation is that the five clades correspond to geographically partitioned populations. For both interpretations, the phylogeographic approach described above sheds unique light on two aspects of our question: how often does long-distance dispersal occur, and what is the evolutionary origin of the clade 2 type French Polynesian population. It is likely that clade 2 individuals from French Polynesia are the result of a single invasion event followed by subsequent diversification, as they cluster in a monophyletic clade. Their evolutionary (and geographic) origin is likely to be in the Line Islands region. Fourteen individuals sequenced from the Line Islands cluster with the French Polynesian samples. Furthermore, identical haplotypes were found for four French Polynesian and four Christmas Island samples. This is particularly remarkable when taking into consideration the overall heterogeneity of *D.*

trimaculatus haplotypes. In a previous survey of 122 individuals, we found 115 unique haplotypes and never found more than two individuals sharing a single haplotype (Bernardi et al. 2002). The presence of individuals from Wallis Island, Baker Island, and Manado (Indonesia) in this same group may correspond to the presence of low levels of gene flow among these localities. As large numbers of individuals were necessary to understand the situation in French Polynesia, we would also need large sample sizes from these localities to fully describe the situation there.

Local currents may provide a passive transport mechanism for *D. trimaculatus* clade 2 type larvae that occasionally reach French Polynesia. South equatorial currents were shown to act as an effective barrier to gene flow between the Society + Tuamotu Islands and the Marquesas, and south equatorial countercurrents were seen to bring propagules to Society + Tuamotu Islands from the north-west, in the convict surgeonfish *Acanthurus triostegus* (Planes 1993). It is likely that *D. trimaculatus* larvae are similarly subjected to those currents (Fig. 1). Indeed, we find restricted gene flow between Society + Tuamotu and Marquesas Islands in *D. trimaculatus* as well (Fig. 2; Bernardi et al. 2002). The south equatorial countercurrent flows at approximately 0.5 knots (Rancher and Rougerie 1992), which would allow passive *D. trimaculatus* propagules to cover, in 22–24 days (the species' pelagic larval duration), approximately half of the distance between the Line Islands and French Polynesia. While larvae have been shown to exhibit strong swimming and orientation behaviors (Leis and Carson-Ewart 1997; Armsworth 2001; Armsworth et al. 2001), it is likely that stepping stone populations are necessary to account for the genetic connection between these regions.

Three-spot damselfish from the Phoenix and Line Islands region belong to the recently described *D. auripinnis* (Randall and Randall 2001). This species differs from *D. trimaculatus* in that the fins and underside of the body are bright yellow. In a previous study, we found that individuals from this species do not group in a monophyletic clade, but are rather inter-mixed with other *D. trimaculatus* from the "Pacific-Rim" clade (clade 2 of Bernardi et al. 2002). This result may possibly be due to technical problems (not enough resolution), to incomplete lineage sorting (recent speciation event), or to the fact that *D. auripinnis* is not a bona fide species. Regardless of the ultimate cause, all *D. auripinnis* clustered in the "Pacific Rim" clade. With their bright yellow markings, *D. auripinnis* are phenotypically very easy to distinguish from the solid black (except for three white spots) *D. trimaculatus*. Black (*D. trimaculatus*) and yellow (*D. auripinnis*) forms are found sympatrically in the northern Cook Islands at Penhryn (Tongareva) and Suwarrow Islands (H. Debelius, personal communication; Randall and Randall 2001; Fig. 1). While assortative matings that would isolate yellow and black forms are possible, it is likely that hybridization in these islands occurs at least occasionally, as was seen in a color morph contact zone in another damselfish, *Acanthochromis polyacanthus* (Planes and Doherty 1997). In Penrhyn and Suwarrow, yellow forms are much more abundant than black forms (H. Debelius, personal communication), so if hybridization does occur, it is more likely for yellow (clade 2) haplotypes to introgress black fish than the reverse. These islands lie approximately half way between the Phoenix Islands and French Polynesia (Fig. 1). It is therefore possible that this "hybrid zone", or others, serves as a stepping stone for migrating introgressed individuals that are phenotypically black, but with "Pacific Rim" haplotypes. Further studies in the northern Cook Islands would be necessary to settle this question.

Long-distance dispersal and founding effects

Coral reef fishes with relatively short pelagic larval durations are sometimes restricted to small geographic locations (Taylor and Hellberg 2003). In the case of *D. trimaculatus*, the species is found over a vast geographic range, from the Red Sea to southern Africa and from Japan to French Polynesia. Yet these geographic populations are genetically quite distinct, as they were found to group in distinct clades (Bernardi et al. 2002; Fig. 2). Are edge populations colonized by rare episodes of long-distance dispersal? The presence of a rare genetic marker in French Polynesia that is shared with northern individuals seems to indicate that this is the case. Data show that all clade 2 haplotypes that are found in French Polynesia are descendants of a single lineage, possibly the consequence of a single dispersal event.

The presence of only two widely divergent lineages in French Polynesia suggests that long-distance dispersal is uncommon. In fact, dispersal may have happened more than twice, but we find evidence of successful colonization for only two such events. The fact that the two groups include 96% and 4% of the French Polynesian individuals may either reflect an old and a recent dispersal event, or that the two genetic groups have different degrees of fitness. The fact that the most common haplotypes from French Polynesia are found nowhere else does suggest that it corresponds to an old lineage. Conversely, the less common haplotypes are shared with the Pacific Rim, indicating a relatively recent colonization event. This sharing of haplotypes is either due to on-going gene flow, or, more likely, due to not enough time for lineage sorting. When considering a molecular clock for the mitochondrial control region for fishes (5.8–14.5% per million years; Bernardi et al. 2001), we found that the two lineages from French Polynesia originally diverged approximately 600,000 to 1.5 million years ago (sequence divergence is 9%). This is, however, an estimate of divergence time between evolutionary lineages and not between dispersal events. A demographic study combined with coalescence simulations may provide a more accurate estimate for the temporal origin of these colonizing events.

D. trimaculatus individuals in French Polynesia belong to two distinct clades. One clade includes the vast majority of individuals (96.4%), with haplotypes that are exclusively found in French Polynesia. The other clade (3.6% of individuals) is represented by haplotypes that cluster with ''Pacific Rim'' individuals that probably originated in the Line Islands, and reached French Polynesia via stepping stone populations, possibly located in the northern Cook Islands. Samples analyzed in this study were adult individuals, yet the original sample that led to this investigation (OC3) was a new recruit (13 mm TL). It will be very interesting to study the relative recruitment success of the two French Polynesian genetic types. The low frequency of the ''Pacific Rim'' haplotypes provides a natural molecular marker for this type of investigation.

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