ALLOPATRIC DIVERGENCE AND SPECIATION IN CORAL REEF FISH: THE THREE-SPOT DASCYLLUS, DASCYLLUS TRIMACULATUS, SPECIES COMPLEX

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Long pelagic larval phases and the absence of physical barriers impede rapid speciation and contrast the high diversity observed in marine ecosystems such as coral reefs. In this study, we used the three-spot dascyllus (Dascyllus trimaculatus) species complex to evaluate speciation modes at the spatial scale of the Indo-Pacific. The complex includes four recognized species and four main color morphs that differ in distribution. Previous studies of the group using mitochondrial DNA revealed a noncongruence between color morphs and genetic groupings; with two of the color morphs grouped together and one color morph separated into three clades. Using extensive geographic sampling of 563 individuals and a combination of mitochondrial DNA sequences and 13 nuclear microsatellites, we defined population/species boundaries and inferred different speciation modes. The complex is composed of seven genetically distinct entities, some of which are distinct morphologically. Despite extensive dispersal abilities and an apparent lack of barriers, observed genetic partitions are consistent with allopatric speciation. However, ecological pressure, assortative mating, and sexual selection, were likely important during periods of geographical isolation. This study therefore suggests that primarily historical factors later followed by ecological factors caused divergence and speciation in this group of coral reef fish.

KEY WORDS: Allopatry, coral reef fish diversity, microsatellites, phylogeny, speciation.

Species level phylogenies provide indirect records of the evolutionary history of speciation. A link between phylogeny and distribution range can then be used to infer the most parsimonious geographical mode of speciation (Barraclough and Vogler 2000; Barraclough and Nee 2001) under two major assumptions: (1) the current distribution range of species reflects their mode of diversification and (2) the gene tree is congruent with the species tree as substantiated by multiple independent molecular markers.

In addition, environmental characteristics, life-history traits, and biological interactions gained from field observations and experiments can inform the processes that drive diversification.

In the context of understanding speciation mechanisms, as described above, marine systems challenge conventional allopatric speciation. The paucity of physical barriers combined with the potential for long-range dispersal of early life-history stages makes the study of speciation in marine organisms a unique
challenge. This is particularly true for coral reef organisms, where extreme diversity seems to coincide with highly connected environments. Additional factors also play important roles: (1) Dispersal of planktotrophic larvae increase potential for connectivity. Most coral reef fish have a bipartite life cycle (Leis 1991) with sedentary adults and pelagic larvae that spend from a few days to several months in the water column (Wellington and Victor 1989). Although biotic (Planes 1993; Riginos and Victor 2001; Ovenden et al. 2004) and abiotic (Frith et al. 1986; Shulman and Bermingham 1995; Lessios et al. 1999) processes drive exchanges between patchily distributed reefs, evidence of current and historical long distance dispersion and colonization have already been documented for coral reef fish (Lessios et al. 1998). A number of taxa in various families have widespread ranges of distribution coupled with genetic homogeneity (Randall 1998; McMillan et al. 1999; Planes and Fauvelot 2002; Horne et al. 2008). (2) Besides the permanent hard continental barriers that have led to separation between ocean basins (e.g., the rise of the Isthmus of Panama between the Atlantic and Pacific Oceans, Lessios 2008), only a few barriers to coral reef fish dispersal such as oceanic currents (Lessios et al. 1999; Barber et al. 2000), open-ocean distances (Vermeij 1987; Lessios and Robertson 2006), and freshwater outflows (Rocha 2004; Floeter et al. 2008; Beldade et al. 2009) have been identified as important factors in marine speciation (but see Connolly et al. 2003 for critical assessment). (3) Barriers that are currently recognized have changed over time. Environmental variations such as sea level fluctuations related to climatic cycles are also likely to have impacted the effectiveness of these barriers (Fauvelot et al. 2003; Rocha et al. 2005a; Bowen et al. 2006; Robertson et al. 2006; Renema et al. 2008).

Due to all the factors mentioned above, dispersal abilities probably play only a modest role in allopatric speciation in the marine realm (Mayr 1954; Hellberg 1998). As a result, the concept of ecological speciation, already recognized in the terrestrial environment, was suggested as a potential explanation for coral reef fish diversity (Streelman et al. 2002; Rocha et al. 2005b; Choat 2006).

Selective pressure on color polymorphisms, so striking in coral reef fish, may result from ecological factors such as communication, competition, camouflage, habitat differences, and mating behavior (DeMartini and Donaldson 1996; Crook 1997; Marshall et al. 2003; Munday et al. 2003; Moland and Jones 2004). Although the role of color variation in speciation processes has not yet been very well established, Planes and Doherty (1997) and Puebla et al. (2007) demonstrated the potential for this phenotypic trait to drive diversification via assortative mating (prezygotic isolation). By contrast, Rocha et al. (2005b) found that ecological speciation was mediated by differential temperature between adjacent habitats and Munday et al. (2004) demonstrated the key role of competition for limited resources in host shift for sympatric species of Gobiodon. Taken together, these studies suggest a strong influence of ecological pressure in speciation processes in coral reef fish.

In this study, we evaluated the geographic mechanisms of speciation in a group of coral reef fish, the three-spot dascyllus (Dascyllus trimaculatus) species complex where geographic distance, allopatry, coloration patterns, and ecological specialization are all encountered in differing degrees of intensity. To that end, we combined phylogenetic data (mitochondrial control region sequences and nuclear microsatellite genetic partitioning investigation), and range distributions to infer species boundaries and modes of speciation, as well as life-history traits and environmental characteristics to evaluate the role of ecological pressure on speciation.

Our results underscore the high resolution of nuclear microsatellites for investigations of macroevolutionary phenomena of speciation and reveal that in this complex, despite strong dispersal abilities, geographic isolation appears to be the key mechanism underlying initial speciation.

Materials and Methods

THE D. TRIMACULATUS COMPLEX

The complex comprises four species, D. trimaculatus (Rüppell 1828–30), D. albisella (Gill 1862), D. strasburgi (Klausewitz 1960), and D. auripinnis (Randall and Randall 2001). Dascyllus trimaculatus, a black fish with three small white spots, has the widest range of distribution, from the East African Coast to the Central Pacific, whereas the three remaining species have much more restricted distributions. Dascyllus albisella, a black fish with white flanks is endemic to Hawaii. D. strasburgi is a grayish fish that is restricted to the Marquesas Islands and the recently described D. auripinnis has yellow fins, a yellow lower body and is only found in the Line and Phoenix Islands (Fig. 1). All species have a nonoverlapping distribution with the exception of D. auripinnis that shows a parapatric distribution to D. trimaculatus with a small sympatric area in the Northern Cook Islands (Randall and Randall 2001; H. Debelius, pers. comm.). Some small local variation in morphology have been observed. For example, in Fiji, fish that are described as D. trimaculatus have some yellow on their body, potentially due to the presence of turbid waters (Allen 1991; Randall and Randall 2001). In Oman, D. trimaculatus tends to be brownish (G. Bernardi, pers. obs.). In Johnston Atoll, a few individuals, recorded as D. albisella, have yellow fin tips (E. DeMartini, pers. comm.). Molecular studies revealed that the four species of the D. trimaculatus complex, which display only small morphometric and meristic differences, have only recently diverged from the D. reticulatus complex (3.9 million years ago—Pleistocene) (Bernardi and Crane 1999; McCafferty et al. 2002). The four species present a mating system that involves conspecific
recognition using sound (Lobel and Mann 1995) followed by external fertilization of benthic eggs. After three or four days (Fricke and Holzberg 1974; Thresher 1984), larvae hatch and disperse in the water column for 22–26 days (Wellington and Victor 1989). Settlement substrata vary according to species. *Dascyllus trimaculatus*, *D. strasburgi*, and *D. auripinnis* recruit mainly on anemones where they remain protected for a few months whereas *D. albisella* use branching corals (Fautin and Allen 1992; Holbrook and Schmitt 1997; Ramon et al. 2008). On very rare occasions, however, *D. trimaculatus* have been observed to settle on corals, both in the Indian Ocean (Oman) and the Pacific (Moorea) (pers. obs.).

Recent divergence, unbalanced distribution range, extensive dispersal capabilities, small morphological differences, strong habitat association, and specific biological interaction, that characterize the members of the *D. trimaculatus* complex, make its members excellent candidates for studying speciation processes in coral reef fish. Several phylogenetic studies have already focused on the *D. trimaculatus* complex describing relationships among the species (Bernardi and Crane 1999; Bernardi et al. 2001, 2002, 2003; McCafferty et al. 2002). A study based on mitochondrial control region sequences found, as expected, that individuals of *D. albisella* and *D. strasburgi* were reciprocally monophyletic (Bernardi et al. 2002). In contrast, despite their morphological similarity throughout the Indo-Pacific, *D. trimaculatus* individuals clustered into three mitochondrial clades that were separated geographically. The Indian Ocean clade was considered as the ancestral population within the complex, the two other clades corresponded to the Tuamotu/Society Islands of French Polynesia and the “West-Central Pacific.” Mitochondrial control regions also clustered *D. auripinnis* individuals together with *D. trimaculatus* West-Central Pacific individuals, raising doubts regarding the validity of *D. auripinnis* as a species. In addition, Bernardi et al. (2003) found that 4% of *D. trimaculatus* individuals from Polynesia (termed OC3 haplotypes) also clustered with West-Central Pacific individuals (Fig. 2). Thus, although much information has already been gathered on the three-spot dascyllus
species complex, many questions regarding speciation processes and species relationships remained unanswered.

**SAMPLE COLLECTION AND DNA EXTRACTIONS**

To study the early speciation processes in the recently diverged *D. trimaculatus* species complex, 563 specimens were collected over the complete distribution range of the species (Table 1, Fig. 1). Individuals were collected using hand spears while free or scuba diving. Immediately after collection, fin clips were placed in 95% ethanol and stored at ambient temperature in the field, and then at 4°C in the laboratory. Total genomic DNA was prepared from 20 mg of fin tissue by proteinase K digestion in lysis buffer (10 mM Tris, 400 mM NaCl, 2 mM EDTA, 1% SDS) overnight at 55°C. This was followed by purification using phenol/chloroform extractions and alcohol precipitation (Sambrook et al. 1989).

**DNA SEQUENCES AND MICROSATellite DATA**

Previously published sequences from the mitochondrial control region (Dloop) were included in this study (Bernardi and Crane 1999; Bernardi et al. 2001, 2002, 2003; Ramon et al. 2008), together with an additional 126 new samples (Table 1). These samples represent the entire distribution of the species complex except for the region comprised between western Australia and Cocos-Keeling. Using microsatellites markers allowed to compare nuclear genetic partitioning within the *D. trimaculatus* complex with the genetic partitioning observed using mitochondrial DNA sequences and reveal patterns and processes such as incomplete lineage sorting, hybridization, and introgression. Therefore, the selection of samples used for microsatellite data analysis was based on described morphological species and on the mitochondrial clades. At least 48 individuals per clade were randomly selected for screening and all the *D. auripinnis* available (49 individuals) were also genotyped. Within the West-Central Pacific and Hawaiian archipelago, where particular processes of interest seem to take place based on previous mitochondrial DNA analysis, the selection of samples included a few individuals from different locations chosen to integrate all the genetic variability. Finally, all Polynesian samples that cluster with the West-Central Pacific mitochondrial clade as previously described (OC3 samples of Bernardi et al. 2003) were genotyped.

**MITOCHONDRIAL GENE AMPLIFICATION AND MICROsatellite SCORING**

Sequence data of 366 bp for the mitochondrial control region were amplified for 126 new samples using previously detailed conditions (Bernardi et al. 2001) and primers (Lee et al. 1995). Mitochondrial sequences were aligned using Bioedit (Hall 1999). For microsatellite analyses, we tested 52 loci isolated from a *D. trimaculatus* individual sampled in Moorea (French Polynesia). Of these, 13 successfully amplified all samples and were found to be highly polymorphic. Leray et al. (2009) provide a full description of amplification procedures and microsatellite characteristics. GENEMAPPER3.7 Applied Biosystems (Foster City, CA) was used for scoring microsatellite genotypes.

**PHYLOGENETIC RELATIONSHIP AND GENETIC STRUCTURE**

To evaluate phylogenetic relationships based on mitochondrial control regions, 10 independent maximum likelihood (ML)
replicates were computed using the program GARLI (Zwickl 2006) that uses a genetic algorithm approach to simultaneously find the topology, branch lengths and model parameters that maximize the likelihood of the tree. Out of the 10 best trees, the one with the highest likelihood overall was selected. Branch support on the resulting best tree was assessed via 100 bootstraps implemented by GARLI. In addition to using a ML approach, pairwise genetic distances were estimated using Arlequin 3.0 (Excoffier et al. 2005) to quantify the divergence between previously identified groups. To infer which model of nucleotide substitution best fit the data, Modeltest (Posada and Crandall 1998) was used. To account for differences between gene divergence and population divergence due to the presence of ancestral polymorphisms (Edwards and Beerli 2000), average pairwise distances between populations were the mean number of pairwise differences between two populations minus the average distance between individual within those populations. Significance was tested with 10,000 replicates and a sequential Bonferroni correction for the level of significance was used.

GENETIC DIVERSITY

Samples were divided into groups previously identified based on the ML phylogenetic tree. Haplotype diversity, nucleotide diversity, and mean number of pairwise differences were calculated with DNASp (Rozas et al. 2003).

ANALYSIS OF THE NUCLEAR MICROSATELITES

DATA

Based on predefined groups (mitochondrial clades and morphological species), the number of alleles, observed and expected heterozygosities were computed per locus using Fstat (Goudet 1995). Linkage disequilibrium between loci and deviations from Hardy–Weinberg equilibrium (HWE) were also tested for each locus and group. Significance was tested with 10,000 replicates. Levels of significance for multiple comparisons of loci across samples were adjusted using a standard Bonferroni correction (Rice 1989). Departures from HWE can be caused by inbreeding, Wahlund effect, or technical causes such as null alleles, misscoring due to stuttering, and allelic dropout. The proportion of null alleles that could influence the genetic signal was evaluated using the algorithm implemented in FREENA (Dempster et al. 1977; Chapuis and Estoup 2007).

GENOTYPE ASSIGNMENT

To explore and decompose the genetic variability into gene pools without providing prior information on the geographical origin of the samples, a Bayesian clustering approach implemented in Structure 2.2 was used (Pritchard et al. 2000). The program simultaneously defines clusters and assigns individual multilocus genotypes to the defined clusters. Allele frequencies were presumed uncorrelated and null alleles were coded as recessive to take into account the presence of null alleles in the dataset (Falush et al. 2007). The most likely number of clusters in the dataset was identified based on posterior probabilities and 10 runs were implemented in Structure 2.2 to test for robustness of the results following Pritchard et al. (2000).

COALESCENCE ESTIMATES AND MIGRATION RATES

Historical demography parameters were examined for groups based on genetic partitioning and morphology to evaluate possible events of population fluctuation based on a coalescent approach. Estimates of \( \Theta = 2N\mu \) (where \( \mu \) is the mutation rate) were made for each genetic group as well as the whole species complex. The parameter \( \Theta \) was estimated under two conditions: an unconstrained exponential growth parameter and an assumption of constant \( N (g = 0) \). We used FLUCTUATE (Kuhner et al. 1998) to estimate the ML of the parameters \( \Theta \) and \( g \) (the exponential growth parameter in units \( \mu \cdot \text{year}^{-1} \)). Seeds for all analyses were generated randomly. Analyses were repeated 10 times per region to ensure stability of parameters estimates. Final analyses of each dataset employed 10 short Monte Carlo chains of 200 steps each and five long chains of length 20,000, with a sample increment of 20. Exchanges and range expansions (immigration) between groups were investigated with MIGRATE 2.0 (Beerli and Felsenstein 2001; Beerli 2003) to identify reproductively isolated entities, quantify gene flow, and evaluate the direction of gene flow between genetic partitions and morphological species. Multiple runs were computed with progressively increasing constraints and length of the analysis until reaching consistent results. The final parameters used 10 short Monte Carlo chains with 5000 recorded genealogies and six long chains with 50,000 recorded genealogies. To explore more genealogies, a method that allows swapping between chains running (in parallel) at different temperatures was implemented, the “colder” chain exploring less genealogy space than the “hotter” one. Again 10 replicates were realized to ensure the reproducibility and stability of the estimation.

The time of coalescence was estimated by assuming that coalescence was reached when the population size was reduced to 1% of its present day value, following Wares and Cunningham (2001). To estimate coalescence time, we used the mutation rate (\( \mu = \text{substitutions per site per generation} \)) that was determined for the mitochondrial control region for other damselfish species, the Panama Trans-Isthmian geminates *Chronis atrilobata* and *C. multilineata* (Dominues et al. 2005).

Results

MITOCHONDRIAL DNA ANALYSIS

Phylogenetic results

Samples partitioned into five well-supported major clades (Fig. 2). Although the dataset used here was more extensive, results were
consistent with previously published data (Bernardi et al. 2002, 2003). The Indo-Pacific separated into five distinct geographical areas: the Indian Ocean (clade 1), the West-Central Pacific (clade 2) which stretches from Japan to Fiji and Wallis to Vietnam, Society and Tuamotu islands, southern French Polynesia (clade 3), the Marquesas Islands, northern French Polynesia (clade 4), and the Hawaiian archipelago (clade 5) including Johnston atoll.

*Dascyllus strasburgi* and *D. albisella* each segregated into monophyletic clades, corresponding to Marquesas (clade 4) and Hawaii (clade 5) samples, respectively. The West-Central Pacific group (clade 2) included *D. trimaculatus* from West-Central Pacific locales, a small number of *D. trimaculatus* from Society and Tuamotu islands (OC3 samples), and all *D. auripinnis* individuals. The genetic partitioning across the rest of the Indo-Pacific did not match with taxonomically described species. Indeed, the wide-ranging *D. trimaculatus* separated into three deeply divergent groups (clade 1, 2, and 3, Fig. 2). Moreover, within the West-Central Pacific, where the two species *D. auripinnis* and *D. trimaculatus* occur, all sampled individuals grouped in a single well-supported clade (clade 2). Within clade 2, two subclades (weakly supported and not shown) were found, suggesting potential population structure within this clade. *D. auripinnis* individuals were found in both subclades.

**Genetic diversity and historical demography in the *D. trimaculatus* complex**

Genetic diversity reflected the historical demography of the different genetic entities of the *D. trimaculatus* species complex. Genetic diversity was highest in the Indian Ocean (Table 2). For example, the mean number of nucleotide differences between Indian Ocean haplotypes was twice that in all the other clades (Table 2). Conversely, the genetic diversity of the West-Central Pacific was the lowest despite the broad geographic origin of the individuals: all West-Central Pacific samples (two described species *D. trimaculatus* and *D. auripinnis*) and 25 samples from Society and Tuamotu islands (OC3 samples).

Overall coalescence for the *D. trimaculatus* complex occurred approximately between 560,000 and 630,000 years ago (ya). Coalescence estimates are more recent for the Indian Ocean (190,000–210,000 ya) than for the Pacific Ocean (470,000–530,000 ya). In addition, coalescence estimates for each clade, which ranged from 150,000 to 400,000 ya, were lower for the West-Central Pacific clade than for Society + Tuamotu, Marquesas, and even Hawaii clades (Table 2).

**Table 2.** Genetic characteristics of *Dascyllus trimaculatus* clades based on the mitochondrial control region. Genetic diversity and coalescence data are presented, with numbers in parentheses being standard deviations. Estimated coalescence times are given in millions of years (My).

<table>
<thead>
<tr>
<th>Genetic diversity:</th>
<th>Clade 1 Indian Ocean</th>
<th>Clade 2 Pacific Rim</th>
<th>Clade 3 Tuam\Soc</th>
<th>Clade 4 Marquesas</th>
<th>Clade 5 Hawaii</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of sequences</td>
<td>40</td>
<td>142</td>
<td>267</td>
<td>37</td>
<td>105</td>
</tr>
<tr>
<td>Number of segregating sites</td>
<td>86</td>
<td>69</td>
<td>99</td>
<td>57</td>
<td>44</td>
</tr>
<tr>
<td>Haplotype diversity</td>
<td>0.997 (0.006)</td>
<td>0.972 (0.007)</td>
<td>0.986 (0.002)</td>
<td>0.974 (0.018)</td>
<td>0.933 (0.016)</td>
</tr>
<tr>
<td>Nucleotide diversity</td>
<td>0.031 (0.002)</td>
<td>0.013 (0.001)</td>
<td>0.020 (0.001)</td>
<td>0.019 (0.003)</td>
<td>0.020 (0.001)</td>
</tr>
<tr>
<td>Mean number of nucleotide differences</td>
<td>10.86 (4.9)</td>
<td>4.41 (2.17)</td>
<td>5.46 (2.60)</td>
<td>6.67 (3.13)</td>
<td>4.43 (2.18)</td>
</tr>
<tr>
<td>Coalescence</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Theta (variable)</td>
<td>0.281 (0.012)</td>
<td>0.230 (0.017)</td>
<td>0.827 (0.035)</td>
<td>0.085 (0.002)</td>
<td>0.203 (0.008)</td>
</tr>
<tr>
<td>Theta (constant)</td>
<td>2.716 (0.468)</td>
<td>1.264 (0.355)</td>
<td>2.518 (0.452)</td>
<td>0.173 (0.035)</td>
<td>0.731 (0.140)</td>
</tr>
<tr>
<td>g (growth)</td>
<td>267.17 (26.41)</td>
<td>329.08 (33.31)</td>
<td>154.07 (10.36)</td>
<td>144.38 (29.59)</td>
<td>244.65 (41.64)</td>
</tr>
<tr>
<td>Coalescence time (My)</td>
<td>0.19–0.21 (0.019–0.17)</td>
<td>0.32–0.36 (0.002–0.016)</td>
<td>0.34–0.39 (0.029–0.016)</td>
<td>0.20–0.23 (0.001–0.01)</td>
<td></td>
</tr>
</tbody>
</table>

**NUCLEAR DNA ANALYSIS**

**Genetic partitioning based on nuclear microsatellites**

Specific characteristics of the microsatellite data used here are provided in Table S1. A partition of our samples in seven clusters was found to best fit the observed microsatellite variability using the clustering method (Fig. 3, and Table S2). All individuals from the Indian Ocean, Hawaii, and the Marquesas Islands showed very high levels of reassignment to their own cluster (average: 0.995). Similarly, individuals from Society and Tuamotu islands were also assigned to their own cluster (reassignment = 0.996), including the OC3 samples that in the mitochondrial dataset clustered with
the West-Central Pacific haplotypes (Fig. 3). Within the Hawaii cluster, samples were divided in two different subclusters. One subcluster comprised all 20 sampled individuals from Johnston Atoll (JOHN) and five samples from the northwestern Hawaiian island of French Frigate Shoals (FFS), and one subcluster comprised all other remaining Hawaiian samples (this cluster included samples both from the main island group and from the Northwestern Hawaiian island of Kure, Table 1). West-Central Pacific individuals presented a mosaic picture. Unlike the mitochondrial results, genotype assignment divided D. auripinnis and D. trimaculatus in two clusters (Fig. 3). All D. auripinnis were assigned to a single cluster, except one individual (collected in Palmyra) that clustered with D. trimaculatus.

We specifically chose a suite of D. trimaculatus individuals from the West-Central Pacific that would span a region with varying distances to the contact zone between D. trimaculatus and D. auripinnis. Of these 48 samples, 19 clustered with D. auripinnis, and the presence of D. auripinnis genotypes occurred in a decreasing proportion with increasing distance from the Line Islands. Indeed, five of eight fish in Wallis and nine Fijian individuals of 15 displayed a D. auripinnis genotype. Although no coloration pattern was available for the Wallis samples, samples from Fiji displayed yellow anal and pelvic fins with streaks of orange in the caudal fin and the spinous part of the dorsal fin (Allen 1991, pers. obs.). This color variant, intermediate between the bright yellow D. auripinnis and the pure black D. trimaculatus, is consistent with a mixed D. auripinnis–D. trimaculatus genotype.

**Discussion**

**SPECIES BOUNDARIES**

Mitochondrial molecular markers have extensively been used to infer phylogenetic relationships and species boundaries. In the case of the D. trimaculatus complex, nuclear markers were mostly consistent with previously published results solely based on mitochondrial markers. However, the use of highly variable microsatellites allowed for additional information that could not be obtained with mitochondrial markers alone.

Nuclear and mitochondrial molecular markers were consistent in genetically identifying the two species D. strasburgi and
**D. albisella** endemic to the Marquesas and Hawaii, respectively. Within **D. albisella**, mitochondrial sequences put Johnston Atoll individuals in a single cluster within the remaining Hawaiian samples (Ramon et al. 2008), whereas nuclear microsatellites, with more segregating power, grouped Johnston Atoll individuals, with five samples collected at FFS (Northwestern Hawaiian islands) and separated them from the remaining Hawaiian individuals. Based on the mitochondrial control region, strong phylogenetic partitions that separate the Indo-Pacific biogeographical regions seem well defined genetically, complex processes seem to take place within the West-Central Pacific. *Dascyllus auripinnis* had been considered a phenotypic variant of *D. trimaculatus*, and only recently was described as a new species based on striking different color patterns but “modest” morphometric differences measured on a small number of samples (Randall and Randall 2001). These include a larger size, one fewer gill raker, and a shorter average length of the paired fins. Although Bernardi et al. (2002) could not determine whether *D. auripinnis* was a valid species, an emerging species, or an isolated population of *D. trimaculatus*, based on mitochondrial DNA sequences, the combination of both nuclear and mitochondrial DNA in the present dataset provided new insights in the geographic dynamic of introgression in the West-Central Pacific, with a clinal zone between yellow and black phenotypes. These results are consistent with “directional” mating that, very likely, is driving introgression from *D. auripinnis* into *D. trimaculatus* through repeated backcrossing. In the contact zone, where both species live in sympatry, at the northern Cook Islands of Penhryn (Tongareva) and Suwarrow Islands (Randall and Randall 2001), the yellow form is much more abundant than the black form (H. Debelius, pers. comm.). Therefore, if hybridization occurs at this location, rare chance events of introgression of the yellow on the black fish are more likely than in the opposite direction. Directional hybridization in species with different abundances has also been observed in other coral reef species (Yaakub et al. 2006). Although assortative mating based on coloration pattern is likely to be present, as seen in other damselfish (Planes et al. 2001; van Herwerden and Doherty 2006), a direct examination of mating behavior in this area (sneak mating was observed in Yaakub et al. 2006), coupled with paternity analysis, would allow a test of this hypothesis. An alternative to this ecological explanation, that would require further testing, is that a selective sweep may be responsible for the clinal distribution of this haplotype (e.g., Galtier et al. 2000).

**GEOGRAPHY OF SPECIATION**

The use of multiple markers to infer the phylogeny of a very recently diverged group such as the *D. trimaculatus* species complex allows inferences about geographical modes of speciation using comparison of present day distribution range between sister species (Barrachlough and Vogler 2000; McCafferty et al. 2002). Recently allopatrically diverged taxa will likely have a nonoverlapping distribution. Both *D. albisella* and *D. strasburgi* coloration patterns coincide with genetic partitions even though a cryptic divergence appears to be occurring within *D. albisella*.

In contrast, no simple relationship between color morphs and genetic partitioning was found in *D. trimaculatus* (Bernardi et al. 2002). Although intraspecific morphological, behavioral, or ecological variation have not been specifically investigated (Fishelson 1966; Fricke 1973), both mitochondrial and nuclear markers divided the widespread *D. trimaculatus* into three deeply divergent genetic partitions that separate the Indo-Pacific biogeographical region in three distinct zones: Indian Ocean, West-Central Pacific, and southern French Polynesia.

Although Indian Ocean and southern French Polynesian regions seem well defined genetically, complex processes seem to take place within the West-Central Pacific. *Dascyllus auripinnis* was described as a new species based on striking different color patterns but “modest” morphometric differences measured on a small number of samples (Randall and Randall 2001). These include a larger size, one fewer gill raker, and a shorter average length of the paired fins. Although Bernardi et al. (2002) could not determine whether *D. auripinnis* was a valid species, an emerging species, or an isolated population of *D. trimaculatus*, based on mitochondrial DNA sequences, the combination of both nuclear and mitochondrial DNA in the present dataset provided new insights in the geographic dynamic of introgression in the West-Central Pacific, with a clinal zone between yellow and black phenotypes. These results are consistent with “directional” mating that, very likely, is driving introgression from *D. auripinnis* into *D. trimaculatus* through repeated backcrossing. In the contact zone, where both species live in sympatry, at the northern Cook Islands of Penhryn (Tongareva) and Suwarrow Islands (Randall and Randall 2001), the yellow form is much more abundant than the black form (H. Debelius, pers. comm.). Therefore, if hybridization occurs at this location, rare chance events of introgression of the yellow on the black fish are more likely than in the opposite direction. Directional hybridization in species with different abundances has also been observed in other coral reef species (Yaakub et al. 2006). Although assortative mating based on coloration pattern is likely to be present, as seen in other damselfish (Planes et al. 2001; van Herwerden and Doherty 2006), a direct examination of mating behavior in this area (sneak mating was observed in Yaakub et al. 2006), coupled with paternity analysis, would allow a test of this hypothesis. An alternative to this ecological explanation, that would require further testing, is that a selective sweep may be responsible for the clinal distribution of this haplotype (e.g., Galtier et al. 2000).

**ALLOPATRIC DIVERGENCE AND SPECIATION IN CORAL REEF FISH**

The use of multiple markers to infer the phylogeny of a very recently diverged group such as the *D. trimaculatus* species complex allows inferences about geographical modes of speciation using comparison of present day distribution range between sister species (Barrachlough and Vogler 2000; McCafferty et al. 2002). Recently allopatrically diverged taxa will likely have a nonoverlapping distribution. Both *D. albisella* and *D. strasburgi* group in monophyletic clades and cluster based on mitochondrial and nuclear DNA. Bernardi et al. (2002) suggested that these species emerged as the result of rare chance long-distance dispersal of propagules, which once they settled in remote locations diverged in allopatry as the result of low level of gene flow. The congruent biogeographical and genetic patterns shared by several taxa and independent genetic markers support the allopatric speciation scenario in the Marquesas and Hawaiian archipelagos (Planes and Fauvelot 2002). Although the Hawaii separation is mainly explained by its geographical isolation, the separation between the Marquesas and Society + Tuamotu islands is likely related to the barrier of larval exchange created by the South Equatorial (Marquesas) Countercurrent flowing from east to west in opposite direction to the South Equatorial Current (Vermeij 1987; Planes 1993). Importantly, although such an oceanographic barrier may potentially prevent any larval crossing, an interchange of a few propagules may still be possible. In this case, however, the relative success of the recruits is probably the cause of the observed partition.

At a smaller scale, emerging divergence was detected within the *D. albisella* distribution range based on nuclear and mitochondrial DNA. Because all samples from the most remote location, Johnston Atoll, group together, the phylogenetic structure is very likely the outcome of reduced gene flow between islands within the Hawaiian archipelago. Larval transport simulations described in Ramon et al. (2008) also support this hypothesis. Indeed, several dispersion corridors were identified between Johnston Atoll and the Hawaiian archipelago but only for organisms with a pelagic larval duration (PLD) longer than 40 days (Kobayashi 2006). However, although PLD is short in *D. albisella* (approximately 25 days), the presence of five samples from the Hawaiian archipelago (FFS) in the Johnston Atoll microsatellite cluster suggests long distance, but rare, larval exchange.
Allopatric speciation, as observed within D. trimaculatus, match numerous previous genetic comparisons between Western Pacific and Indian Ocean populations of marine invertebrates and fish (e.g., Lacson and Clark 1995; McMillan and Palumbi 1995; Chenoweth et al. 1998; Duda and Palumbi 1999; Lessios et al. 1999; Barber et al. 2000; Hobbs et al. 2009). The generally accepted explanation for this observed pattern in many taxa is that sea level fluctuations culminating during the last Pleistocene glaciation, exposed the Sunda shelf, between Malaysia and northern Australia, thus limiting water exchange between the two oceanic provinces. In the case of D. trimaculatus, the Pacific and Indian Oceans cryptic species are genetically partitioned for both mitochondrial DNA and nuclear markers, indicating a current absence of gene flow, despite their adjacent distribution. The contact zone, represented in our samples by the Cocos-Keeling population, does not seem to indicate the presence of gene flow in the region of secondary contact either. However, defining the precise boundary between the Indian Ocean and West-Central Pacific clades will require additional sampling in the Western Australia–Cocos-Keeling area. Indeed, as for other genetic studies, it is likely that Western Australia samples may group with West-Central Pacific samples rather than Indian Ocean ones, and additional samples from Cocos-Keeling/Christmas Island may reveal some level of gene exchange in the region (Hobbs et al. 2009).

The second genetic boundary within the D. trimaculatus species, between the Tuamotu\Society Islands and the West-Central Pacific results from a different process. Mitochondrial DNA indicates that few Polynesian (Society and Tuamotu Islands) individuals (OC3) group with the West-Central Pacific clade, whereas microsatellite analysis clustered those same individuals exclusively with the Polynesian group. Bernardi et al. (2002) suggested that the presence of two distinct clades in Polynesia was the result of larval transport from the West-Central Pacific to South Polynesia. The present study, with new information provided by highly polymorphic microsatellites, is consistent with such an ecological explanation combined with introgression. Alternatively, it may also be indicative of incomplete lineage sorting with a lack of resolution for the mitochondrial DNA. Using the mitochondrial control region, genetic structure between South Polynesia and West-Central Pacific has previously been observed for the widespread Lutjanus kasmira ($F_{ST} = 0.12−0.25$, S. Planes, unpubl. data) and D. auripinnis ($F_{ST} = 0.20−0.63$, S. Planes, unpubl. data), two species that have a comparable PLD to D. trimaculatus (Juncker et al. 2006). In comparison Acanthurus triostegus, a species with a PLD twice as long, 60–70 days (Randall 1961; Juncker et al. 2006), does not show significant genetic structure (S. Planes, unpubl. data). Therefore, these patterns suggest that highly restricted gene flow (which may be linked to the PLD or to differential survival of the propagules) between the two geographical areas is consistent among different species, resulting in a strong genetic break between South Polynesia and the West-Central Pacific.

Within the West-Central Pacific, D. auripinnis and D. trimaculatus have very different sizes of geographic range. The range of D. auripinnis is less than 5% of the range of D. trimaculatus. In itself, this may suggest peripatric speciation, as divergence of a peripheral population in allopatry (Losos and Glor 2003). The small overlapping range observed in the Northern Cook Islands would also be an indication of a range shift. The hypothesis of peripatric speciation assumes the temporary emergence of a barrier that isolated a peripheral population in the Line and Phoenix Islands. It is possible, however, that a parapatric ecological mode of speciation (Wu and Ting 2004), or rapid sexual selection, may have played a role in the divergence of this species.

Taken together, the geography of speciation in the D. trimaculatus species complex points toward allopatric and parapatric modes of speciation that led to the diversification of the complex with no evidence for sympatric speciation given the overall lack of overlap of the distribution range of the genetic partitions (Fig. 4). Our data cannot distinguish whether the divergence was due to natural selection, drift, or both. Besides simple geographic partitioning, it is possible that additional ecological pressure on speciation may also have played an important role in the diversification process (e.g., Hemmer-Hansen et al. 2007).

**ECOLOGICAL SPECIATION**

Rocha et al. (2005b) argued that the paradox of reef fish diversity and their high dispersal capabilities may be solved by ecological speciation. They demonstrated that differential selection would operate in adjacent habitats with contrasting environments and promote diversification. Habitat structure, climate, resource availability, predation, and competition are also thought to promote divergent or disruptive selection leading to speciation via character displacement (increasing morphological polymorphism), habitat, or behavioral changes with or without geographic isolation (Schluter 2000; Rundle and Nosil 2005). Munday et al. (2004) presented a case of speciation via host shift in highly specialized species of coral dwelling fish (genus Gobiodon), and proposed a key role for competition in the divergence. In general, D. trimaculatus remain protected from predators within anemones during their juvenile phase (12 months). Yet, D. albisella, a species endemic to the Hawaiian Archipelago, where suitable anemones are virtually absent, recruit on branching corals. Although species in the D. trimaculatus complex most likely arose in allopatry, early host shift may therefore have played an important role in their divergence. Occasionally, D. trimaculatus settles on branching corals or on Diadema urchins (G. Bernardi, pers. obs.), indicating that ecological divergence in the D. trimaculatus species complex is observed. An additional ecological signal may be uncovered.
in the *D. auripinnis*–*D. trimaculatus* hybrid zone. Indeed, the presence of sound production during mating, the variation in coloration patterns, and the imbalance between *D. auripinnis* versus *D. trimaculatus* haplotypes and genotypes suggest that female choice, assortative mating, and sexual selection may play an important, yet unexplored role in the genetic partitioning of individuals.

**CONCLUSIONS AND PERSPECTIVES**

The combined analysis of mitochondrial and nuclear markers presented here provides interesting insights on early processes of speciation in the *D. trimaculatus* species complex. Mitochondrial markers allowed us to broadly define five genetic clades that served as the basis of understanding of the complex relationship between established taxonomy, distribution, and coloration patterns of the species. Highly variable nuclear markers refined this view by settling alternative scenarios (the presence of unsorted OC3 haplotypes in south Polynesian samples), and uncovering subtle differences (Johnston Atoll individuals, *D. trimaculatus–D. auripinnis* hybrid zone). The study of speciation processes in marine organisms is complex, yet a combination of extensive geographic sampling and use of different molecular markers provides an opportunity to shed light on such intricate systems.

**ACKNOWLEDGMENTS**

We would like to thank R. Galzin and C. Fauvelot for comments and discussion, and Editor M. Hellberg and three anonymous reviewers for comments. We would like to very much thank G. Lecaillon for providing so many samples over the years from Mayotte, Philippines, and Vietnam. We would also like to thank P. Nelson, D. R. Robertson, J. H. Choat, and J. McIlwain for providing samples. NSF MCR LTER Award OCE 04-17412, and the Gordon and Betty Moore Foundation provided funding for this research.

**LITERATURE CITED**


Lobel, P. S., and D. A. Mann. 1995. Spawning sounds of the damselfish, Dascyllus aequipinnis and D. aruanus, a m o n g w e s t e r n I n d i a n O c e a n a n d w e s t e r n (Pisces: Pomacentridae), and relationship to male size. Bioacoustics 6:187–198.


Zwickl, D. J. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. Ph.D. dissertation, The Univ. of Texas at Austin, TX.
Supporting Information

The following supporting information is available for this article:

**Figure S1.** Phylogenetic relationship between 563 individuals sampled in the *D. trimaculatus* species complex based on mitochondrial control regions using the Maximum Likelihood method. A simplified tree is provided in the inset with size of pie charts being proportional to the number of individuals.

**Table S1.** Number of alleles (*N_a*), expected heterozygosity (He), and observed heterozygosity (Ho) across 13 loci and six groups.

**Table S2.** Proportion of membership of each predefined population in each of the seven clusters which best partition the microsatellite variability.

Supporting Information may be found in the online version of this article.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.
Table S1. Number of alleles ($N_a$), Expected heterozygosity (He) and Observed heterozygosity (Ho) across 13 loci and 6 groups. * significant deviation of Hardy Weinberg equilibrium after standard Bonferroni correction. The average gene diversity among loci $\theta$ (and its standard error) for each group as well as the total number of alleles per loci are also presented.

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$\theta$ 0.74 (0.38) 0.8 (0.41) 0.55 (0.31)
Table S2. Proportion of membership of each pre-defined population in each of the 7 clusters which best partition the microsatellite variability. \( n \): number of samples in each group pre-defined on a geographical baseline. Colours match with the genetic clusters.

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