



Molecular phylogeny of the humbug damselfishes inferred from mtDNA sequences

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The damselfish genus *Dascyllus* comprises nine species of both large- and small-bodied fishes distributed over the entire Indo-West Pacific. Most members of the genus have polygynous mating systems with protogynous sex change, while others are promiscuous with no sex change. Hypotheses linking presumed phylogenetic relationships with body size, sex change and mating structure have been proposed previously. However, lack of a strong phylogenetic hypothesis has prevented the careful testing of such hypotheses. In this study, the phylogenetic relationships between *Dascyllus* species based on mitochondrial DNA sequences (cytochrome b and 16SrRNA) have been established. The data also shed light on the relationship between mating structure and body size, as well as on the complex biogeographical patterns of the genus.

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Key words: mtDNA; *Dascyllus*; molecular phylogeny; mating structure.

INTRODUCTION

The damselfish genus *Dascyllus* (Pomacentridae) comprises nine species restricted to the coral reefs in the Indo-West Pacific. The distribution of each species is variable: *D. aruanus* (L.) and *D. trimaculatus* (Rüppell) are distributed widely, while *D. strasburgi* Klausevitz is found only in the Marquesas Islands. Thus, the evolutionary history of the genus may reveal interesting biogeographical patterns. Several species have been the focus of a number of ecological, behavioural, physiological and intraspecific genetic studies (Sale, 1971, 1972, 1976; Holzberg, 1973; Schpigel & Fishelson, 1983; Jones, 1988; Planes *et al.*, 1993; Schmitt & Holbrook, 1996). In reviewing the mating system of the genus, Godwin (1995) proposed a tentative phylogeny based on meristic characters described by Randall & Allen (1977) (Fig. 1). This phylogeny separates *Dascyllus* into three complexes: the aruanus complex with *D. aruanus* and *D. melanurus* (Bleeker), the trimaculatus complex with *D. trimaculatus*, *D. albisella* Gill, and *D. strasburgi*, and the reticulatus complex with *D. reticulatus* (Richardson), *D. flavicaudus* Randall & Allen, *D. carneus* Fischer, and *D. marginatus* Rüppell. While this phylogeny did not address the species relationships within each complex, it allowed Godwin (1995) to propose a hypothesis about the evolution of *Dascyllus* mating structure (Fig. 1).

Mating systems in *Dascyllus* are mostly polygynous with protogynous sex change, and/or functional gonochorism (Godwin, 1995). While small-bodied

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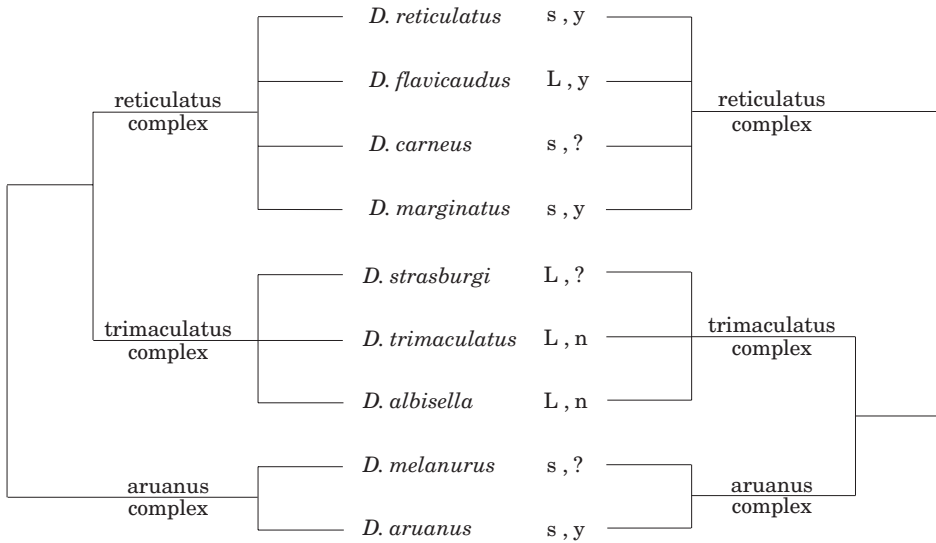


FIG. 1. Hypothesized relationships between *Dascyllus* species based on meristic characters, redrawn from Godwin (1995). The left tree is based on modal numbers of pectoral rays and soft dorsal rays. The right tree is based on gill raker counts on the first arch. Large-bodied fish are labelled L, small-bodied species are labelled s. Species that exhibit sex change (y) or which do not exhibit sex change (n) are noted. Species that were not studied are labelled (?). Data are from Godwin (1995).

species were shown to be polygynous and exhibit sex change, larger-bodied species (*D. trimaculatus*, *D. albisella* and *D. strasburgi*) which do not exhibit resource defence polygyny also do not exhibit sex changes (Godwin, 1995). Godwin's phylogeny places *D. trimaculatus*, *D. albisella* and *D. strasburgi* in a single clade, thus making it difficult to tease apart the effect of phylogeny as opposed to the effect of larger body size in the loss of sex change in this group of fishes.

However, Godwin suggested that the phylogeny itself may not be very robust since a different selection of meristic characters could change the tree topology. Indeed, the use of modal number of pectoral and soft dorsal rays suggests a closer relationship between the reticulatus complex and the trimaculatus complex, while using gill raker counts puts the trimaculatus complex closer to the aruanus complex (Fig. 1). These two possible tree topologies would require a different number of evolutionary events to explain the presence or absence of sex change in the different clades (Godwin, 1995). In this respect, the study of *D. flavicaudus*, a large-bodied species found in a small-bodied species clade, which also exhibits sex change, becomes crucial to understanding the influence of phylogeny on mating structure in the genus.

The geographical distribution of *Dascyllus* is also peculiar. Species ranges within the reticulatus and trimaculatus complexes (*sensu* Godwin, 1995) do not overlap. The trimaculatus complex includes three species found in the Hawaiian archipelago (*D. albisella*), the Marquesas Islands (*D. strasburgi*), and most of the rest of the Indo-West Pacific (*D. trimaculatus*). The reticulatus complex includes four species found in the Red Sea (*D. marginatus*), the Indian Ocean (*D. carneus*), the West Pacific (*D. reticulatus*), and the South-west Pacific (*D. flavicaudus*).

Within complexes, species are ecologically very similar, and have been suggested to replace each other in different regions.

Ecological and biogeographical studies in this group have developed largely in the absence of a strong phylogenetic framework. In this study, molecular markers (cytochrome b and 16SrRNA sequences have been used) to determine the phylogenetic relationships between *Dascyllus* species. Our goal was to establish evolutionary relationships between *Dascyllus* species and lay the basis for future comparative studies.

MATERIALS AND METHODS

SAMPLES AND DNA EXTRACTION

Specimens were caught by spear while free or scuba diving except for *D. marginatus*, *D. reticulatus* and *D. melamurus* which were purchased at a local pet store. *Dascyllus aruanus*, *D. flavicaudus* and *D. trimaculatus* were collected in Rangiroa Atoll, French Polynesia. *Dascyllus carneus* was collected in Zanzibar, Tanzania. *Dascyllus albisella* was collected in Kona, Hawaii, by E. DeMartini. One outgroup, *Chromis chromis* (L.) was selected for its close relationship with *Dascyllus* (both genera are grouped in the same sub-family, Chrominae) (Nelson, 1994). *Chromis chromis* was collected in Orbetello, Italy, by P. Stipa. Species were identified using Randall & Allen (1977). Two individuals of each species were sequenced. No differences were found between individuals of the same species.

Liver tissue was extracted immediately from collected specimens and preserved in 95% ethanol at ambient temperature in the field then stored at 4° C in the laboratory. Tissues were digested overnight at 55° C in 500 ml of extraction buffer (NaCl 400 mM, Tris 10 mM, EDTA 2 mM, SDS 1%). We purified the DNA by standard chloroform extraction and isopropanol precipitation (Sambrook *et al.*, 1989).

POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION

Amplification of the mitochondrial 16S ribosomal RNA gene (16SrRNA) and the protein encoding mitochondrial gene cytochrome b (cytb) was accomplished with the following primers: 16SAR, 16SBR for the 16SrRNA, and Cyt1 and CB3H for the cytochrome b (Meyer, 1994). Each 100- μ l reaction contained 10–100 ng of DNA, 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 2.5 units of Taq DNA polymerase (Perkin-Elmer, Norwalk, CT, U.S.A.), 150 mM of each dNTP, and 0.3 mM of each primer, and was amplified with a cycling profile of 45 s at 94° C, 45 s at 48° C, 1 min at 72° C, for 35 cycles. After purification following the manufacturer's protocol (ABI, Perkin-Elmer), sequencing was performed in both directions with the primers used in the PCR amplification on an ABI 373 automated sequencer (Applied Biosystems, Foster City, CA, U.S.A.).

SEQUENCE ANALYSIS

We used the computer program Clustal V implemented by Sequence Navigator (Applied Biosystems) to align the sequences. Phylogenetic relationships were assessed using the neighbour-joining (NJ) method (uncorrected distances) as well as the maximum parsimony (MP) method implemented by the software package PAUP (Phylogenetic Analyses Using Parsimony, version 4.0, Swofford, 1998). Robustness of trees was evaluated using 2000 bootstrap replicates (Felsenstein, 1985; Hedges, 1992). Alternate tree topologies were statistically compared using the topology-dependent tail permutation tests (T-PTP, Faith, 1991) with 1000 replicates.

RESULTS

Eight *Dascyllus* species were sampled and sequenced. The only species not sampled, *D. strasburgi* is restricted to the Marquesas Islands, French Polynesia.

TABLE I. Sequence divergence and transition/transversion ratios in the genus *Dascyllus*

	<i>alb.</i>	<i>aru.</i>	<i>car.</i>	<i>flav.</i>	<i>marg.</i>	<i>mela.</i>	<i>reti.</i>	<i>trim.</i>
<i>D. albisella</i>	—	3.6	3.5	4.3	5.9	4.1	4.0	4.3
<i>D. aruanus</i>	10.3	—	3.8	3.5	4.8	5.9	4.1	3.5
<i>D. carneus</i>	4.1	9.3	—	4.4	6.1	4.7	3.7	3.2
<i>D. flavicaudus</i>	8.0	10.0	7.1	—	5.3	4.0	5.0	4.9
<i>D. marginatus</i>	7.0	9.3	6.2	6.1	—	5.1	8.0	5.1
<i>D. melanurus</i>	10.5	6.5	9.6	9.9	8.7	—	4.4	3.7
<i>D. reticulatus</i>	3.0	9.8	2.8	7.1	6.1	9.4	—	3.8
<i>D. trimaculatus</i>	2.5	10.6	4.6	7.1	7.3	10.5	3.9	—

Sequence divergences, measured as uncorrected distances, are given in per cent below the diagonal. Transition/transversion ratios are shown above the diagonal.

The validity of this species has been questioned (Randall & Allen, 1977) as it resembles and could be a sub-species of *D. trimaculatus*.

A total of 1171 nucleotides were sequenced for all species studied (GenBank accession numbers AF119392–AF119409). Out of these 1171 nucleotides, 686 bp were a portion of the protein encoding mitochondrial cytochrome b gene, and 485 were a portion of the mitochondrial 16S ribosomal gene. Out of the 1171 characters, 30 (from the 16S rRNA gene) correspond to insertions or deletions (indels) in the outgroup as compared to *Dascyllus*. Since these positions are well aligned in *Dascyllus*, they were kept for the subsequent phylogenetic analysis and were given an unknown status for the outgroup. Out of the aligned characters, 286 were variable, and 156 were phylogenetically informative. To reconstruct a molecular phylogeny, the phenetic NJ method and the cladistic MP method were used. As expected, transitions were more frequent than transversions (average ratio=4.2) and sequence divergences ranged between 2.5 and 10.6% (Table I). Plots of sequence divergence against transition/transversion ratios showed that substitutions were not saturated (Table I), thus we decided not to weight the characters (plots not shown).

When the complete unweighted dataset was used, a single most parsimonious tree of 364 steps, with a consistency index of 0.744 was obtained. This tree was identical to the one obtained using the NJ method. The robustness of these trees was evaluated with bootstrapping (Fig. 2) and by using alternative datasets which comprised the following: only cytochrome b; only first, second, third, and first+second codon positions of the cytochrome b; and only 16SrRNA data. For all these datasets, transversions were weighted equally, twice, or three times that of transitions. In all situations, phylogenetic trees were topologically identical to the one presented in Fig. 2.

The two species, *D. melanurus* and *D. aruanus* grouped together in a monophyletic and ancestral clade (100% bootstrap support). The sister clade of this group comprised two clades. One clade includes *D. flavicaudus* from French Polynesia and *D. marginatus* from the Red Sea, while the other clade includes the closely related domino damselfishes *D. trimaculatus* and *D. albisella* (which group together in 100% of the bootstrap replicates), as well as *D. carneus* from the Indian Ocean and *D. reticulatus* from the Western Pacific. This arrangement

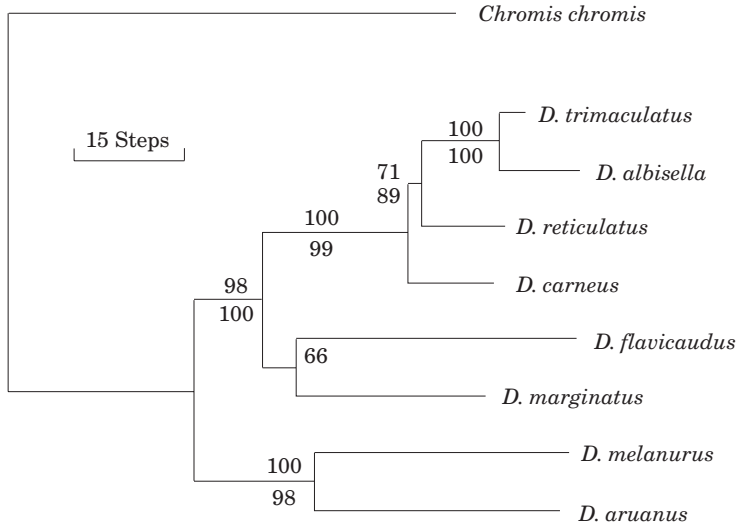


FIG. 2. Phylogenetic relationships based on portions of the cytochrome b and 16SrRNA sequences. Both neighbour-joining and maximum parsimony gave the same tree topology. Numbers by the nodes indicate the percentage of trees (when above 50%) out of 2000 bootstrap replicates for the NJ (above the node) and the MP (below the node) methods. Branches are proportional to the number of steps, scale bar indicates 15 steps. The outgroup, *Chromis chromis* is a member of the sub-family Chrominae, and is a presumed close relative of the in-group (same sub-family).

is different than that which was proposed earlier, based on morphology, ecology and behaviour (Godwin, 1995). Godwin proposed to group the two domino damselfishes in one clade, and the remaining four species in their sister clade. In order to test for this alternate topology, we forced it on our data to see if we could reject it statistically, or if it were compatible with our data. The most parsimonious tree that could accommodate the alternate topology had a length of 380 steps, which is 16 steps longer than the most parsimonious tree. Furthermore these 16 steps were found to be statistically significant when using a T-PTP ($P=0.02$) (Faith, 1991) indicating that the alternate topology could be rejected.

DISCUSSION

PHYLOGENETIC RELATIONSHIPS

Phylogenetic relationships based on morphological characters and molecules are mostly concordant. Species that are morphologically and meristically almost identical, *D. aruanus* and *D. melanurus* on one hand, and *D. albisella* and *D. trimaculatus* on the other hand, are sister clades in both trees (Figs 1 and 2). The aruanus complex is ancestral in both the molecular tree (Fig. 2) and in the meristics tree based on fin counts (Fig. 1, left). As noted by Godwin (1995), it is likely that gill raker counts are not a very useful character since they are directly influenced by body size. The main difference between the tree based on meristics and the tree based on molecular data lies in the placement of species within the trimaculatus and reticulatus complexes. In the meristics tree, relationships within a complex were not resolved. Furthermore, the reticulatus complex is

considered monophyletic and is distinct from the trimaculatus complex. In contrast, when considering molecular data, these species are separated in a clade that includes *D. flavicaudus* and *D. marginatus*, and another clade that includes *D. carneus*, *D. reticulatus*, *D. albisella*, and *D. trimaculatus*, these latter two species being in a derived sister clade. As mentioned earlier, the difference between these two topologies was statistically significant.

The phylogenetic relationships described above give us a framework to test ecological and biogeographical hypotheses. Since little work has been done on this genus, future research will be necessary to understand fully the evolutionary significance of changing mating strategies in this group. However, our results may shed light on some hypotheses already proposed, as discussed below.

MATING STRUCTURE

Several hypotheses have been proposed to explain the pattern of sex change and mating strategy in *Dascyllus* (Fricke, 1980; reviewed in Godwin, 1995). The generally accepted idea is that *Dascyllus* males monopolize small coral heads, thus limiting resources and forcing females into a harem (in the case of small female sizes). This size advantage hypothesis (Ghiselin, 1969; Warner, 1975) predicts a protogynous sex change. In the case of domino damselfishes *D. albisella* and *D. trimaculatus* which are both large bodied, female mobility increases and species do not undergo sex change. Godwin (1995) proposed an alternate hypothesis, where phylogenetic inertia would prevail, and space use and sexual pattern is simply a reflection of phylogenetic history of the species complex.

Dascyllus albisella and *D. trimaculatus* (large bodied and gonochoristic) are most closely related to *D. reticulatus* (small bodied and undergoing sex change) (Fig. 2). Furthermore, *D. flavicaudus* which is large bodied, is most closely related to the small-bodied *D. marginatus*. In order to test for a possible phylogenetic inertia factor, we forced our phylogenetic reconstruction in placing in sister clades the large-bodied species (*D. albisella*, *D. trimaculatus*, *D. flavicaudus*). The constrained phylogeny could be rejected by T-PTP testing at the $P < 0.01$ level. Taken together, these two results indicate that mating structure and body size in *Dascyllus* may not be the result of phylogenetic inertia, but are more likely to follow the size advantage hypothesis.

BIOGEOGRAPHIC AND EVOLUTIONARY PATTERNS

Molecular data showed that *D. melanurus* and *D. aruanus* are sister clades. This result is not particularly surprising since habitat ranges of these species overlap. *Dascyllus aruanus* is widespread throughout the Indo-West Pacific, while *D. melanurus* has a range embedded in the aruanus range, within the Indonesian Archipelago and south to the Great Barrier Reef. These two species tend to live in shallow water inside lagoons. It has been argued that the habitat of these species has been exposed to drastic changes during the last glaciation, due to lower sea levels and loss of lagoon habitats. It is therefore likely that the modern distribution of these species differs from their original distribution (Planes *et al.*, 1993). It is also likely that historically these species have shared similar types of habitats and overlapping ranges. The wide habitat range of these

species is also consistent with the idea that this complex is ancestral to species with more restricted ranges.

The grouping of *D. trimaculatus* with *D. albisella*, and of *D. reticulatus* close to *D. carneus* is also not surprising as these species have adjacent ranges and probably have evolved in allopatry. The grouping of *D. flavicaudus* with *D. marginatus* is more puzzling since their habitat range is the South Pacific and the Red Sea, respectively. It is possible that an intermediate species may have been used as a stepping stone, and was replaced later by a more successful species.

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