

# Historical biogeography and molecular systematics of the Indo-Pacific genus *Dascyllus* (Teleostei: Pomacentridae)

S. McCafferty,\* E. Bermingham,\*¶ B. Quenouille,\*† S. Planes,† G. Hoelzer‡ and K. Asoh§

\*Smithsonian Tropical Research Institute, Apdo 2072, Balboa, Republic of Panama, †Ecole Pratique des Hautes Etudes, ESA CNRS 8046, Université de Perpignan, 66860 Perpignan Cédex, France, ‡Department of Biology, University of Nevada Reno, Reno, NV 89557, USA, §Department of Zoology, University of Hawaii at Manoa, Honolulu, HI 96822, USA

## Abstract

The phylogeographical and systematic relationships among species in the tropical marine fish genus *Dascyllus* were inferred using mitochondrial DNA (mtDNA) sequence data. Although our results were generally consistent with previously published phylogenies based on both morphological and mitochondrial data, our broad taxonomic and geographical sampling design revealed novel insights into the phylogenetic history of *Dascyllus* that had escaped previous notice. These results include: (a) the polyphyletic nature of *D. reticulatus* mtDNAs, representing two divergent and geographically separated lineages, one shared with *D. flavicaudus* and the second forming the sister lineage of *D. carneus*; (b) the paraphyly of *D. trimaculatus* relative to the closely related *D. abisella*; and (c) phylogeographical structure within the widespread taxa *D. aruanus* and *D. trimaculatus*. Application of a molecular clock permits us to posit a causative role for tectonic and oceanic changes regarding some *Dascyllus* speciation events. Finally, we mapped body size and the presence or absence of protogynous sex change on the mtDNA tree, and tested published hypotheses regarding determinants of the evolution of mating system and protogyny in the genus. Our data rejected a model based on body size but not one based on phylogenetic inertia. The ability to change sex arose once in the ancestor to the entire genus, and was lost once in the ancestor of the *D. trimaculatus* complex. For taxa that are as geographically widespread as many Indo-Pacific genera, this study highlights the importance of adequate geographical sampling when inferring patterns of species diversification and life history evolution.

**Keywords:** damselfish, Indo-West Pacific, life history evolution, mitochondrial DNA, molecular systematics, phylogeography

Received 12 October 2001; revision received 10 April 2002; accepted 10 April 2002

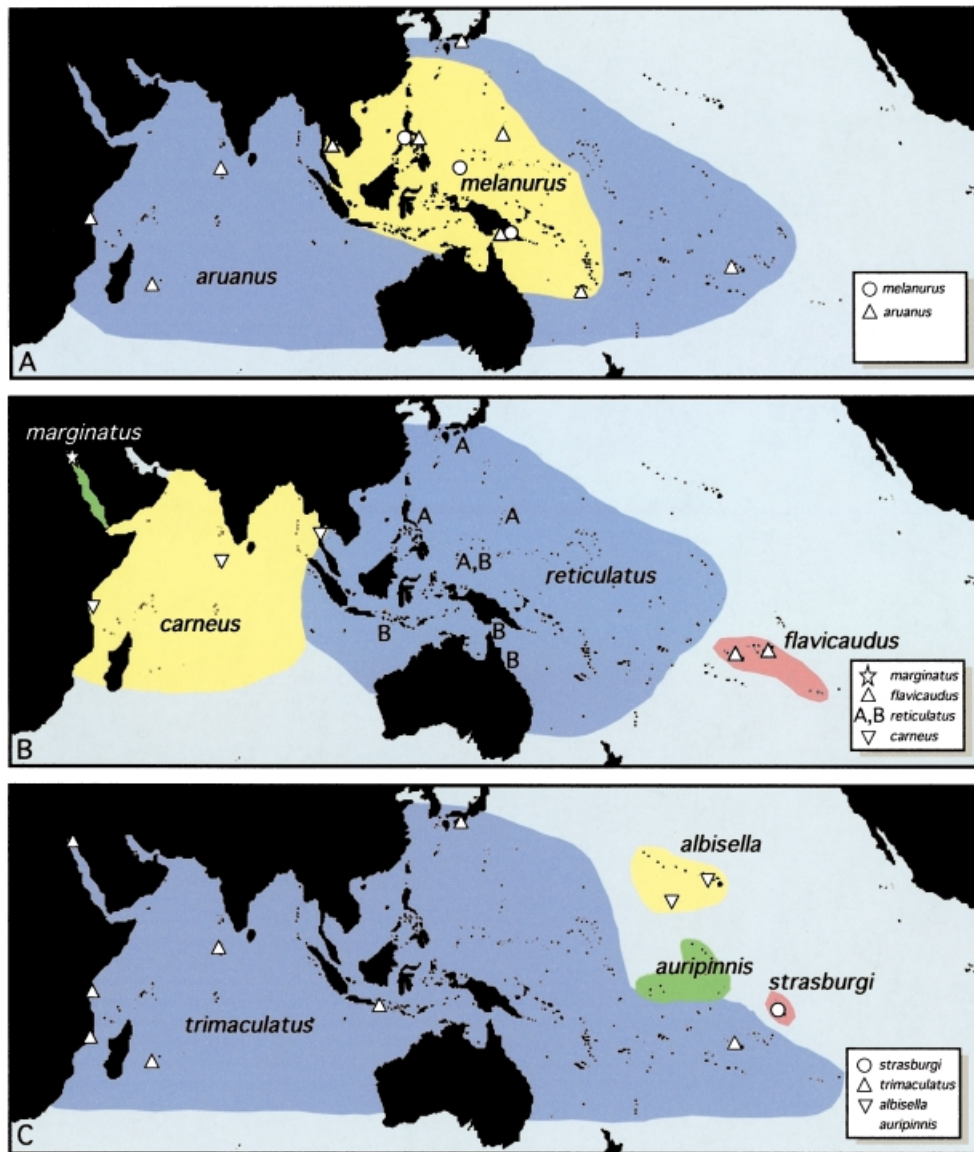
## Introduction

With an area spanning half the world's tropical marine belt, from East Africa to eastern Polynesia, the Indo-West Pacific (IWP) region is recognized as the most diverse marine biogeographical province (e.g. Rosen 1988; Paulay 1997). High diversity coupled with the exceptionally broad geographical range of many IWP species has created a biogeographical paradox: in the face of apparently high dispersal capabilities, how did species become sufficiently isolated to speciate (Palumbi 1992)? Although the mechan-

isms of speciation are often complex and difficult to tease apart, the resolution of this paradox will hinge ultimately on phylogenetic analyses from numerous taxa representing a broad taxonomic selection.

The most informative phylogenetic studies of IWP marine taxa have been those for which the relationships of closely related species could be determined clearly and unambiguously, where sister species tended toward allopatric distributions, and where the timing of speciation events could be estimated reliably (e.g. McMillan & Palumbi 1995; Bermingham *et al.* 1997a; Palumbi *et al.* 1997; Lessios *et al.* 1998; Bowen *et al.* 2001; Lessios *et al.* 2001). In addition, it has become clear that taxon sampling should be considered carefully, especially in situations where closely related taxa have broad geographical distributions (Lessios *et al.* 2001).

Correspondence: Eldredge Bermingham. ¶Present address: STRI, Unit 0948, APO, AA 34002–0948, USA. Fax: + 507 2128790; E-mail: eb@naos.si.edu



**Fig. 1** Distribution of the 10 species of *Dascyllus*. Following Godwin (1995), separate maps represent the named species complexes: (A) *aruanus*; (B) *reticulatus*; and (C) *trimaculatus*. Sample locations are mapped on Fig. 1 (see Table 1 for details). In the *reticulatus* species complex, the A and B symbols for *D. reticulatus* reflect the presence of two divergent mtDNA clades (see text for details). The range of *D. auripinnis* is shown for completeness, but no samples were collected.

As Funk (1999) has pointed out, failure to include closely related nominal taxa and adequate sampling across species' ranges can lead to deceptive phylogenies.

The genus *Dascyllus* (Pomacentridae) makes an excellent model for studying IWP biogeography. These primarily planktivorous damselfishes are found throughout the IWP with putative sister species typically retaining allopatric or parapatric distributions. The genus contains both widely distributed taxa (*D. aruanus*, *D. reticulatus* and *D. trimaculatus*) and more localized endemics (*D. albisella*, *D. auripinnis*, *D. marginatus* and *D. strasburgi*). Ten species are recognized currently (Randall & Allen 1977; Randall & Randall 2001)

and are thought to fall into three species complexes based on morphology, distribution and coloration (Godwin 1995): the *aruanus*, *reticulatus* and *trimaculatus* complexes (Fig. 1). Godwin's (1995) phylogenetic hypothesis placed the *aruanus* complex in a basal position, with the *trimaculatus* and *reticulatus* species groups as derived sister taxa.

*Dascyllus* also offers an excellent system for studying the evolution of mating system and protogyny (a form of sequential hermaphroditism where individuals function first as female and later in life change sex, functioning exclusively as male). The members of the *aruanus* and *reticulatus* complexes are small-bodied with a maximum

standard length (SL) of 50–65 mm (except *D. flavicaudus* with a maximum SL of 90 mm). The species in these two complexes are associated strongly with arborescent corals throughout their lives and form typically single-male polygynous groups composed of small numbers of individuals (Sale 1970; Fricke & Holzberg 1974; Fricke 1977, 1980; Randall & Allen 1977; Allen 1991; Godwin 1995). It has been reported or suggested that all species in the *aruanus* and *reticulatus* complexes are protogynous (Coates 1982; Shpigel & Fishelson 1986; Schwarz & Smith 1990; Godwin 1995; Cole 2002; Asoh and Yoshikawa in review). The members of the *trimaculatus* complex are large-bodied fish with a maximum SL of 90–110 mm (Allen 1991). Juveniles in the *trimaculatus* complex are associated closely with live coral colonies (and with sea anemones in the case of *D. trimaculatus*), whereas adults form large feeding groups over the reefs (Randall & Allen 1977; Allen 1991). *D. albisella* and *D. trimaculatus* are reported to be gonochoristic, while the sexual pattern of *D. strasburgi* is unknown (Godwin 1995; Asoh *et al.* 2001; Asoh & Kasuya 2002). All *Dascyllus* species are demersal spawners. Males prepare nests by removing debris from an area of substratum. Females travel to a male-guarded nest to lay eggs, which are tended subsequently by the male until hatching (Thresher 1984).

Godwin (1995) proposed alternative models for *Dascyllus* regarding the determinants of mating system and the presence or absence of protogyny: the body-size model (originally termed the 'monopolization hypothesis') and the phylogenetic inertia model. The body-size model states that an evolutionary increase in *Dascyllus* body size has permitted increased female mobility, leading in turn to a decrease in the degree of female monopolization by large males and loss of the single-male polygynous mating system, resulting eventually in the loss of protogyny. The phylogenetic inertia model applied to *Dascyllus* posits that descendant species will retain the mating system and sexual pattern of their ancestors, regardless of body size. Using a morphology-based phylogeny for the genus *Dascyllus*, Godwin rejected the body-size model in favour of the phylogenetic inertia model based on histological evidence for protogyny in *D. flavicaudus*, the only species in the genus for which the predictions from the two models differ. Subsequently, Bernardi & Crane (1999) presented an alternative hypothesis for the relationship among *Dascyllus* species based on mtDNA sequences (cytochrome B and 16 s rRNA) and concluded that their mtDNA-based phylogeny supported the body-size model over the phylogenetic inertia model.

Here we present the results of a more detailed phylogeographical study of the molecular systematics of nine of the 10 currently described species of *Dascyllus* sampled from locales representative of the species' ranges. We address the following questions: what are the phylogenetic relationships among the various species of *Dascyllus*? Is there evidence for significant phylogeographical structure within

widespread species? Does a geographically enriched taxon sampling design shed additional light on IWP biogeography and the evolution of mating system and protogyny in the genus *Dascyllus*?

## Materials and methods

### *Taxon sampling, voucher specimens and tissue preservation*

Samples of *Dascyllus* were collected throughout the IWP region. Sampling locations are summarized in Fig. 1 for each of the species complexes. Table 1 presents the relevant locale information for the samples used in this study. Tissue samples were collected either as fin clips preserved in EtOH or DMSO/NaCl buffer (Seutin *et al.* 1991), gill tissue stored in DMSO/NaCl or whole frozen samples. Voucher specimens have been deposited in the STRI Museum (Birmingham *et al.* 1997b). The current classification of damselfishes (Allen 1991) places *Dascyllus* in the subfamily Chrominae within the Family Pomacentridae. We therefore used two additional taxa within the same subfamily, *Chromis atrilobata* and *C. multilineata*, as outgroups.

### *Laboratory procedures*

We followed routine methods for DNA extraction, PCR amplification and sequencing, as described elsewhere (Banford *et al.* 1999). An 842-bp region of the mitochondrial genome, which contained the complete ATP synthase subunits 6 and 8 (ATP6/8), was amplified from total genomic DNA using primers that are described at <http://nmg.si.edu/bermlab.htm>. The entire ATP6/8 region was sequenced using KlenTaqenase (Rematech) or the PRISM (ABI) cycle sequencing systems and analysed using an ABI 373 or 377 Automated Sequencer following the manufacturer's recommendations. Light and heavy strand sequence was determined for single individuals of each species in order to improve the accuracy of the reported sequences.

### *Phylogenetic and statistical analyses*

Sequences were aligned by eye and contigs made using SeqEdit (ABI). We reduced the aligned sequence data set by eliminating identical mitochondrial sequences using the program MacClade (Maddison & Maddison 1992). The resulting data set was analysed using minimum evolution/maximum likelihood (ME/ML) methods as implemented in the program PAUP\* (Swofford 2001). Differences in the nucleotide composition among OTUs were analysed using the *t*-test implemented in PAUP\*. Saturation effects at first, second and third codon positions and fourfold degenerate sites were assessed graphically.

**Table 1** Geographic locales, sample identities and GenBank accession numbers for the *Dascyllus* and outgroup *Chromis* specimens used in this study

Species-locale	Label on Figure 2	STRI ID	GenBank accession number
<i>albisella</i>			
Hawai'i	<i>albisella</i> , Hawai'i 1	stri-x-1993	AF489741
	<i>albisella</i> , Hawai'i 2	stri-x-1994	AF489742
	<i>albisella</i> , Hawai'i 3	stri-x-1995	AF489740
	<i>albisella</i> , Hawai'i 16	stri-x-1992	AF489743
	<i>albisella</i> , Hawai'i 18	stri-x-1975	AF489744
	Johnston Island	<i>albisella</i> , Johnston Atoll 1	stri-x-2000
<i>albisella</i> , Johnston Atoll 2		stri-x-2001	AF489746
<i>aruanus</i>			
Kenya	<i>aruanus</i> , Kenya 1	stri-x-2079	AF489749
Maldives	<i>aruanus</i> , Maldives 1	stri-x-2082	AF489750
	<i>aruanus</i> , Maldives 2	stri-x-2083	AF489751
Reunion Island	<i>aruanus</i> , Reunion 1	stri-x-2131	AY103259
	<i>aruanus</i> , Reunion 2	stri-x-2132	AF489747
Thailand	<i>aruanus</i> , Thailand 2	stri-x-2145	AF489752
	<i>aruanus</i> , Thailand 3	stri-x-2147	AF489753
	<i>aruanus</i> , Philippines 9	stri-x-2130	AF489762
Japan	<i>aruanus</i> , Japan 1	stri-x-2074	AF489758
	<i>aruanus</i> , Japan 2	stri-x-2075	AF489759
New Guinea	<i>aruanus</i> , New Guinea 1	stri-x-2116	AF489760
	<i>aruanus</i> , New Guinea 2	stri-x-2117	AF489761
Guam	<i>aruanus</i> , Guam 16	stri-x-2069	AF489756
New Caledonia	<i>aruanus</i> , Guam 19	stri-x-2072	AF489757
	<i>aruanus</i> , New Caledonia 5	stri-x-2101	AF489763
French Polynesia	<i>aruanus</i> , New Caledonia 7	stri-x-2103	AF489764
	<i>aruanus</i> , Moorea 5	stri-x-2055	AF489754
	<i>aruanus</i> , Moorea 6	stri-x-2056	AF489755
<i>carneus</i>			
Kenya	<i>carneus</i> , Kenya 1	stri-x-2158	AF489769
	<i>carneus</i> , Kenya 2	stri-x-2159	AF489770
Maldives	<i>carneus</i> , Maldives 1	stri-x-2161	AF489771
	<i>carneus</i> , Maldives 2	stri-x-2162	AF489772
Thailand	<i>carneus</i> , Thailand 10	stri-x-2167	AF489773
	<i>carneus</i> , Thailand 15	stri-x-2171	AF489774
<i>flavicaudus</i>			
French Polynesia	<i>flavicaudus</i> , Moorea 1	stri-x-2188	AF489775
	<i>flavicaudus</i> , Moorea 2	stri-x-2199	AF489776
	<i>flavicaudus</i> , Moorea 3	stri-x-2201	AF489777
	<i>flavicaudus</i> , Moorea 4	stri-x-2202	AF489778
Rangiroa	<i>flavicaudus</i> , Rangiroa 1	stri-x-2208	AF489779
	<i>flavicaudus</i> , Rangiroa 2	stri-x-2210	AF489780
	<i>flavicaudus</i> , Rangiroa 3	stri-x-2211	AF489781
<i>marginatus</i>			
Red Sea	<i>marginatus</i> , Red Sea 1	stri-x-2218	AF489782
	<i>marginatus</i> , Red Sea 2	stri-x-2219	AF489783
	<i>marginatus</i> , Red Sea 3	stri-x-2220	AF489784
<i>melanurus</i>			
Philippines	<i>melanurus</i> , Philippines 9	stri-x-2242	AF489788
	<i>melanurus</i> , Philippines 10	stri-x-2236	AF489787
New Guinea	<i>melanurus</i> , New Guinea 1	stri-x-2230	AF489785
	<i>melanurus</i> , New Guinea 3	stri-x-2232	AF489786
Palau	<i>melanurus</i> , Palau 3	stri-x-2227	AF489789

Table 1 Continued

Species–locale	Label on Figure 2	STRI ID	GenBank accession number	
<i>reticulatus</i>				
Japan	<i>reticulatus</i> A, Japan 1	stri-x-2257	AF489801	
	<i>reticulatus</i> A, Japan 3	stri-x-2259	AF489802	
	<i>reticulatus</i> A, Japan 5	stri-x-2261	AF489799	
Philippines	<i>reticulatus</i> A, Philippines 8	stri-x-2278	AF489791	
	<i>reticulatus</i> A, Philippines 9	stri-x-2279	AF489790	
Guam	<i>reticulatus</i> A, Guam 1	stri-x-2763	AF489795	
Palau	<i>reticulatus</i> B, Palau 1	stri-x-2262	AF489794	
	<i>reticulatus</i> A, Palau 2	stri-x-2263	AF489792	
	<i>reticulatus</i> B, Palau 3	stri-x-2264	AF489793	
	<i>reticulatus</i> A, Palau 2774	stri-x-2274	AY103260	
	<i>reticulatus</i> B, Palau 2775	stri-x-2275	AY103261	
	<i>reticulatus</i> B, Palau 2776	stri-x-2276	AY103262	
	<i>reticulatus</i> B, Palau 2777	stri-x-2277	AY103264	
	<i>reticulatus</i> B, Palau 2778	stri-x-2278	AY103263	
	New Guinea	<i>reticulatus</i> B, New Guinea 1	stri-x-2265	AF489803
		<i>reticulatus</i> B, New Guinea 2	stri-x-2266	AF489804
<i>reticulatus</i> B, New Guinea 3		stri-x-2267	AF489798	
<i>reticulatus</i> B, New Guinea 4		stri-x-2268	AF489797	
<i>reticulatus</i> B, New Guinea 5		stri-x-2269	AF489796	
Australia	<i>reticulatus</i> B, Heron Island 1	stri-x-2250	AF489739	
Indonesia	<i>reticulatus</i> B, Heron Island 5	stri-x-2254	AF489800	
	<i>reticulatus</i> B, Bali 1	stri-x-2154	AF489767	
	<i>reticulatus</i> B, Bali 2	stri-x-2155	AF489768	
	<i>reticulatus</i> B, Bali 3	stri-x-2156	AF489765	
	<i>reticulatus</i> B, Bali 4	stri-x-2157	AF489766	
<i>strasburgi</i>				
French Polynesia	<i>strasburgi</i> , Marquesas 1	stri-x-2290	AF489805	
	<i>strasburgi</i> , Marquesas 2	stri-x-2292	AF489806	
	<i>strasburgi</i> , Marquesas 3	stri-x-2293	AF489808	
	<i>strasburgi</i> , Marquesas 4	stri-x-2294	AF489807	
<i>trimaculatus</i>				
Red Sea	<i>trimaculatus</i> , Red Sea 3	stri-x-2377	AF489818	
	<i>trimaculatus</i> , Red Sea 4	stri-x-2378	AF489817	
Kenya	<i>trimaculatus</i> , Kenya 1	stri-x-2339	AF489809	
	<i>trimaculatus</i> , Kenya 2	stri-x-2340	AF489810	
Mozambique	<i>trimaculatus</i> , Mozambique 2	stri-x-2349	AF489819	
Reunion Island	<i>trimaculatus</i> , Reunion 1	stri-x-2380	AF489823	
Maldives	<i>trimaculatus</i> , Maldives 3	stri-x-2345	AF489820	
	<i>trimaculatus</i> , Maldives 4	stri-x-2346	AF489821	
Indonesia	<i>trimaculatus</i> , Bali 9	stri-x-2333	AY103266	
	<i>trimaculatus</i> , Bali 10	stri-x-2330	AY103265	
Japan	<i>trimaculatus</i> , Japan 1	stri-x-2334	AF489814	
	<i>trimaculatus</i> , Japan 2	stri-x-2335	AF489815	
	<i>trimaculatus</i> , Japan 4	stri-x-2337	AF489816	
New Guinea	<i>trimaculatus</i> , New Guinea 5	stri-x-2364	AF489822	
French Polynesia	<i>trimaculatus</i> , Moorea 1	stri-x-2300	AF489811	
	<i>trimaculatus</i> , Moorea 2	stri-x-2311	AF489812	
	<i>trimaculatus</i> , Moorea 10	stri-x-2301	AY103267	
<i>Chromis atrilobata</i>				
Costa Rica	<i>C. atrilobata</i> 1	stri-x-3498	AY103268	
Panama	<i>C. atrilobata</i> 2	stri-x-3499	AY103269	
<i>C. multilineata</i>				
Panama	<i>C. multilineata</i> 1	stri-x-3501	AY103272	
Venezuela	<i>C. multilineata</i> 2	stri-x-6091	AY103270	
	<i>C. multilineata</i> 3	stri-x-3500	AY103271	

ME/ML methods used the criterion of minimum evolution incorporating a maximum likelihood model of divergence to describe the relationship among the mitochondrial genotypes. A heuristic search algorithm was used to find the shortest tree based on the optimality criterion of minimum evolution. We used the ML model of Hasegawa *et al.* (1985) to estimate the transition/transversion ratio (TVR), nucleotide frequencies and the appropriate rate parameters from the data. Variation among sites was accounted for using a model that estimated separate rate categories for first, second and third codon positions for the two gene regions separately (for a total of six rate categories). This particular ML model was chosen because preliminary analyses using a codon position constrained permutation test showed evidence for nonhomogeneous rates of substitution between the two gene regions (unpublished results available from SSM upon request). In addition, we used a gamma approximation for rate variation among sites in an HKY85 model (HKY85 +  $\Gamma$ ) for comparison. ML model parameters were estimated iteratively. Branch support on the resulting tree was estimated via bootstrapping (1000 bootstrap randomizations using the Fast-Addition option in PAUP\*).

To verify that the particular analytical model employed did not bias our results, we also analysed the aligned sequences using other optimality criteria and search algorithms. Maximum likelihood analysis was performed using the program PUZZLE (Strimmer & von Haeseler 1996) with the HKY85 model estimating TVR, nucleotide composition and the gamma distribution parameter  $\alpha$  from the data, and estimating the branch support by 10 000 puzzling steps. Parsimony analysis was performed using three different weighting schemes: (1) equal weighting of all characters, (2) weighting transitions over transversions 7:1 and codon position with weights of 3:9:1 and (3) using Farris's (1969) successive approximations. A heuristic search was used for all weighting schemes (TBR, simple swapping), and branch support was inferred by bootstrapping (1000 randomizations using the Fast-Addition option). All parsimony analyses were performed using PAUP\*. Additional minimum evolution analyses were performed using the program METREE (Rzhetsky & Nei 1994a). The distance measure was a gamma Poisson approximation (using  $\alpha$  estimated by the ML analysis above), with standard errors and bootstrap support of the branch lengths estimated as described by Rzhetsky & Nei (1994b).

Our ATP8/6 *Dascyllus* phylogeny was compared to alternative trees using the parametric resampling/reanalysis approach ('SOWH test') developed by Goldman *et al.* (2000), following the instructions provided at <http://www.zoo.cam.ac.uk/zoostaff/goldman/tests>. For comparison with the Bernardi & Crane (1999) phylogeny, one representative individual per species was selected for the likelihood calculations (and *D. strasburgi* was

excluded in order to match their taxon sampling). Maximum likelihood values were calculated using the HKY85 +  $\Gamma$  model and the difference between these values was then tested against a null distribution based on 1000 simulated data sets generated using Seq-Gen (Rambaut & Grassly 1997).

To estimate the time of divergence among monophyletic mtDNA clades, we tested first whether ATP6/8 evolution in *Dascyllus* was consistent with a molecular clock hypothesis using the parametric bootstrap method (Huelsenbeck *et al.* 1996; Huelsenbeck & Crandall 1997). One hundred simulated data sets were generated using the program Seq-Gen (Rambaut & Grassly 1997). To simplify calculations, we used the HKY85 +  $\Gamma$  model with parameters estimated from the original data set and the tree presented in Fig. 2. Significance levels were tested as recommended by Huelsenbeck & Rannala (1997).

To overcome the known bias associated with using the average pairwise divergence among taxa as an estimate of time (Hillis *et al.* 1996), the time to the most recent common ancestor (MRCA) was determined from the clock-constrained ML tree using a method similar to Baldwin & Sanderson (1998). Parametric bootstrapping (100 simulated data sets) was used to estimate the (nodal) depth of the MRCA of the clades of interest. Parameters of the ML model (HKY85 +  $\Gamma$ ) were estimated for each simulated data set along the original ML tree and branch lengths were estimated with a molecular clock constraint. Node depths (MRCA) were calculated for each simulated data set by summing the branch lengths from the node of interest to the appropriate terminal taxa.

Branch lengths were converted to time by first calibrating an ATP6/8 specific molecular clock using the Pliocene geminates in the *Abudefduf saxatilis* group described in Bermingham *et al.* (1997a). Parametric bootstrapping was used to account for uncertainty in the calibration rates using the methods described above. The ML model used for the simulated data sets (100) was the HKY85 +  $\Gamma$  with parameters estimated from the original data set, which included various populations of *A. saxatilis*, *A. troschelli* and *A. abdominalis*, with the two *Chromis* species used as outgroups. The 100 simulated data sets were used to estimate 100 independent estimates of the MRCA of *A. saxatilis* (Caribbean) and *A. troschelli* (Eastern Pacific).

The mean and 95% confidence intervals for the estimates of time to MRCA for a particular clade in *Dascyllus* were then calculated by randomization. One thousand MRCA values were produced using the formula:  $(MRCAD_i * t_i) / MRCAG_i$ , where  $MRCAD_i$  is a randomly chosen (with replacement) parametric bootstrap estimate of the MRCA of a particular *Dascyllus* clade (with  $i = 1-1000$ ),  $MRCAG_i$  is a randomly chosen (with replacement) parametric bootstrap estimate of the MRCA of the geminate *Abudefduf* species and  $t$  is a randomly chosen time estimate which

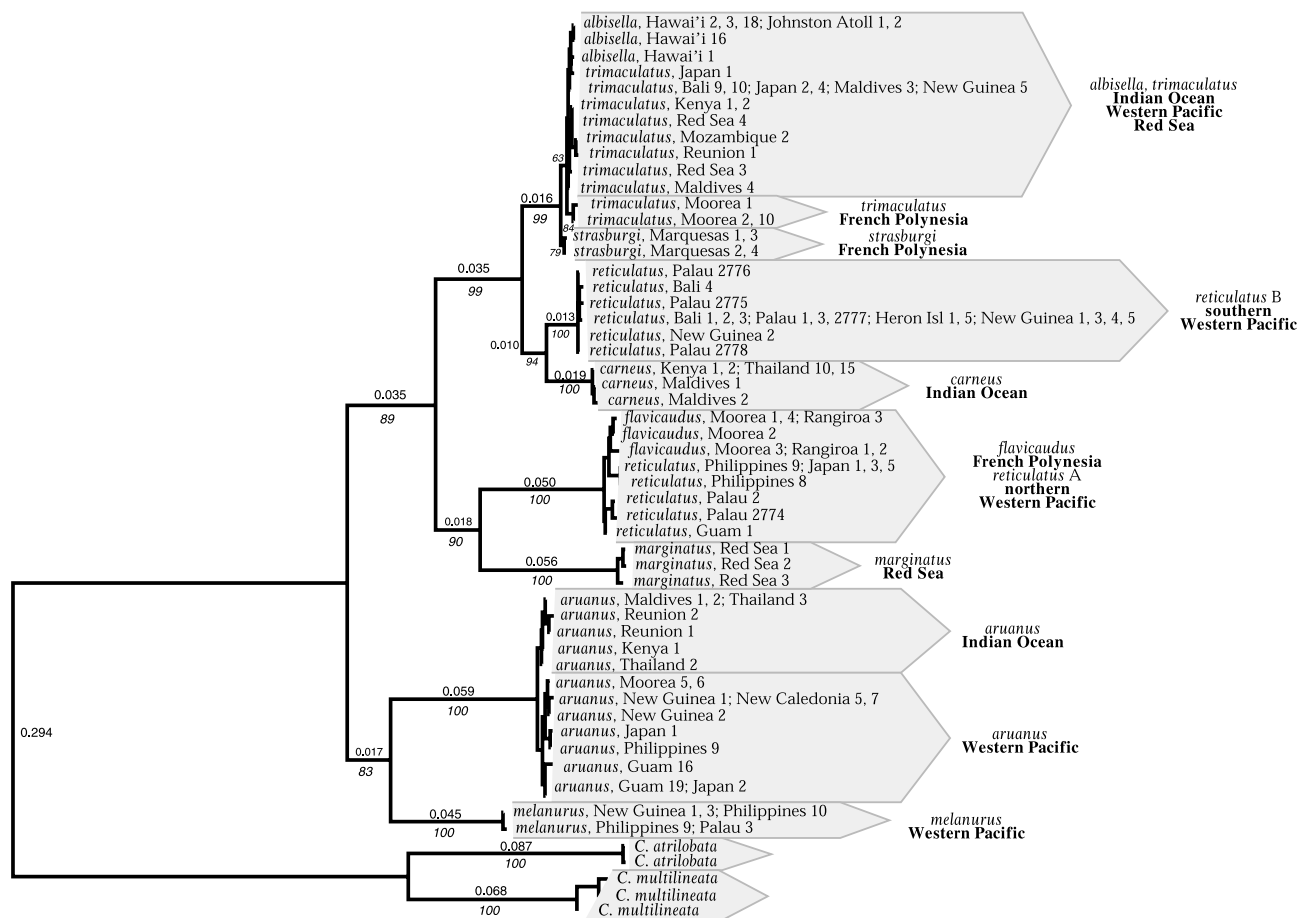


Fig. 2 *Dascyllus* phylogenetic relationships inferred from a ME/ML analysis of mtDNA ATPase 6/8 genes. Branch lengths are calculated using the ML model described in the text, and bootstrap values are given below branches. Except for terminal nodes, only branch lengths with bootstrap support values > 60% are given.

reflected an assumed date for the final closure of the Isthmus of Panama at between 2.9 mya to 3.5 mya (Coates & Obando 1996). The resulting distribution of MRCA estimates for a particular clade was used to estimate the mean and 95% confidence interval (CI). Using this method, the parametric bootstrap replicates thus provide an estimate of the error associated with any particular time to MRCA (or node depth). Significant differences among the average time to MRCA (i.e. clade depths) between different clades were tested using standard permutation tests. All randomizations and permutation tests were performed using the program Random Stats (<http://www.statistics.com>).

#### *Dascyllus* mating system and sexual pattern evolution

Evidence of protogyny in the various species of *Dascyllus* was mapped on the reduced phylogeny derived from the ME/ML analysis of the full data set using MacClade. Protogyny and average species' sizes were taken from

Godwin (1995) and Bernardi & Crane (1999). There is no evidence for protogynous hermaphroditism in the outgroup *Chromis*. No information is available concerning the presence of protogyny for *D. strasburgi*; *D. melanurus* and *D. carneus* are assumed to be protogynous based on observed social structure and unpublished histological analyses (K. Asoh, T. Yoshikawa, unpublished results).

#### Results

ATPase 6/8 sequences representing *Dascyllus* and *Chromis* outgroup species (Table 1 provides GenBank accession numbers) were aligned to homologous carp sequences (*Cyprinus carpio*; GenBank accession number: NC\_001606) and showed no insertions, deletions or unexpected stop codons (flanking tRNA sequences were excluded from analysis). A 10-bp overlap was found between the two gene regions as expected. The 842 bp *Dascyllus* ATPase 6/8 sequences comprised 205 variable sites, of which 197 are parsimony informative, 144 are fourfold degenerate sites and 135 are twofold degenerate sites. Most substitutions

occur at fourfold degenerate sites or as transitions at twofold degenerate sites and are therefore silent. No significant differences were found in nucleotide composition among mtDNA haplotypes at first, second or third codon position ( $P < 0.05$  for all comparisons). The frequencies of nucleotides at the different codon positions were consistent with current models of vertebrate mtDNA, i.e. a strong deficiency of Gs at second and third position sites and a relative paucity of Ts at first position sites (Perna & Kocher 1995).

Within the genus *Dascyllus* there were 20 detectable amino acid substitutions arrayed over 18 different codons. Six of the 18 replacement substitutions were found in the 58 codon positions making up the ATPase 8 gene, while 12 were found in the 228 codon sites comprising the ATPase 6 gene. This translates into a roughly twofold difference in the number of codon sites containing amino acid substitutions in the ATPase 8 vs. the ATPase 6 gene, indicating nonhomogeneous rates of amino acid substitutions in the two genes.

Within the recognized species, the level of HKY85 corrected sequence divergence was generally low ( $0.28\% \pm 0.05$ ) except in the case of *D. reticulatus*. Within *D. reticulatus*, the average level of sequence divergence was  $7.6\% \pm 6.7$  compared to an average within-species level of  $0.28\% \pm 0.05$  (range: 0.12–0.49%). The high level of sequence divergence within *D. reticulatus* is due to the presence of two very divergent mtDNA lineages described below. The level of divergence within each of the two divergent mtDNA lineages is 0.17% and 0.78%, which is comparable to the range of conspecific divergences observed in other species of *Dascyllus*.

There is no evidence for nucleotide saturation at any codon position for comparisons within the genus *Dascyllus*. There is also no evidence for saturation at first or second position sites in comparisons including the outgroup *Chromis* species, and only a minimal suggestion of saturation effects at third position sites.

The results of the ME/ML analysis are summarized in Fig. 2 (detailed trees describing the results from the various reconstruction methods are available from SSM upon request). The estimated ML model parameters are presented in Table 2. The estimates of the rate categories and TVR are consistent with a mitochondrial protein-coding gene region. Estimates of the gamma distribution parameter  $\alpha$  (0.21) are indicative of extensive site-to-site variation in nucleotide substitution patterns. The results from parsimony analyses (using three different weighting schemes), maximum likelihood (using the quartet puzzling method) and the various minimum evolution methods all yielded trees very similar to the one presented in Fig. 2. The differences among the various reconstruction methods involved the relative branching order of terminal branches within the principal mtDNA clades.

**Table 2** ML model parameter estimates used for the mtDNA-based phylogenetic analysis of *Dascyllus* relationships. Two model results are presented. The first is based on six rate category estimates, and the second is based on the gamma distribution approximation  $\alpha$ . TVR is the estimated transition/transversion ratio,  $k$  is the estimate of kappa,  $-\ln(\text{ML})$  is the model's negative log likelihood value. See text for details on models and estimation procedures

Parameter	ATP8	ATP6	Model
1st pos	0.371	0.351	
2nd pos	0.089	0.074	
3rd pos	1.685	2.785	
TVR			7.91 ( $k = 15.129$ )
$-\ln(\text{ML})$			3378.382
$\alpha$			0.204
TVR			10.241 ( $k = 19.60$ )
$-\ln(\text{ML})$			3563.567

There are seven well-supported clades within the genus *Dascyllus*. The branching order of all seven clades is completely resolved and well supported with bootstrap values  $> 85\%$  in the ME/ML analysis. The average genetic distance between the *Chromis* outgroup and *Dascyllus* ingroup mtDNA haplotypes was  $46.6\% \pm 6.8$ . In all treatments the *aruanus* complex (*D. aruanus* and *D. melanurus*) is upheld as a monophyletic group that diverges relatively early from all other *Dascyllus*. These two species are distinguished by  $10.9\% \pm 0.4$  sequence divergence from one another, and  $17.6\% \pm 1.2$  average distance from all other members of the genus. *D. marginatus* and *D. flavicaudus* also represent a sister pair in all analyses; however, the bootstrap support for this relationship was somewhat weaker and more variable across analytical methods than was the case for other sister pairs. *D. marginatus* and *D. flavicaudus* are separated by  $11.3\% \pm 0.2$  sequence divergence, compared to an average distance of  $18.7 \pm 1.1\%$  to *D. aruanus* and *D. melanurus*, and  $13.2\% \pm 0.6$  to their sister group, the *carneus/reticulatus* plus *trimaculatus* clade. The species composition and monophyly of the *trimaculatus* complex is also stable in all analyses. However, the ATPase data do not distinguish *D. albisella* from most *D. trimaculatus*. These two species are distinguished from the third member of the group, *D. strasburgi*, by three ATPase substitutions, a genetic distance of  $0.78\% \pm 0.14$ .

Based on our ATPase data set, *D. reticulatus* is composed of two extremely divergent mtDNA clades which are not sister lineages. *D. reticulatus* collected from Guam, Japan, Palau and the Philippines (= *reticulatus* A) carry mtDNA haplotypes almost identical to *D. flavicaudus*. Although the level of sequence divergence (0.78%) between *D. flavicaudus* and *reticulatus* A samples is somewhat larger than the range of divergences seen in comparisons within other species



of *Dascyllus* (range 0.12–0.49%), it is at least an order of magnitude less than the average among *Dascyllus* species ( $13.7\% \pm 4.7$ ).

*D. reticulatus* samples from Bali, New Guinea, the Great Barrier Reef and Palau (= *reticulatus* B) represent the sister group of *D. carneus*, from which they are separated by  $3.4\% \pm 0.1$  average sequence divergence. The *carneus/reticulatus* B clade is sister to the *trimaculatus* species complex. Both clades are supported by high bootstraps (100%) and an average sequence divergence of  $4.6\% \pm 0.38$ .

Although not the primary aim of our study, our geographical sampling was sufficient to provide a preliminary assessment of phylogeographical structure in *D. aruanus* and *D. trimaculatus*. Within *D. aruanus*, Indian Ocean (IO) individuals form a mtDNA lineage distinct from all Western Pacific (WP) *D. aruanus*. This relationship is supported by three character state changes ( $0.85\% \pm 0.16$  sequence divergence) and has strong bootstrap support (87%). Within *D. trimaculatus* there is evidence for a distinct French Polynesia lineage, separated by two character state changes ( $0.70\% \pm 0.17$  sequence divergence) from all other sampled populations, and a single synapomorphy ( $0.39\% \pm 0.12$  sequence divergence) unites all Indian Ocean samples of this species.

We used the SOWH test (Goldman *et al.* 2000) to compare the alternative *Dascyllus* phylogenies published by Godwin (1995) and Bernardi & Crane (1999). Our *Dascyllus* tree (Figs 2–4) had improved likelihood values compared to those for the phylogenies published by Godwin (1995) or Bernardi & Crane (1999) (2646.5127 vs. 2652.80317 using all individuals; 2174.61594 vs. 2181.05147 using one individual per species). The difference between the likelihood values was significant at  $P < 0.01$ .

No significant difference was found between the clock constrained and unconstrained ML estimates of the *Dascyllus* tree using the parametric bootstrap test ( $P > 0.05$ ). The estimated mean and 95% CI for the dates of divergence for the principle nodes in Fig. 2 are shown in Fig. 3. Although there was some overlap in the 95% CI around the time to MRCA to the seven principal clades, the average estimated time to the MRCA were significantly different for all pairwise comparisons ( $P < 0.01$  in all cases) except when comparing the time to MRCA for the *aruanus-melanurus* clade vs. the MRCA to *marginatus-flavicaudus-reticulatus* A ( $P > 0.05$ ).

The most parsimonious explanation for the evolution of protogyny indicates that the ability to change sex arose once in the ancestor to the entire genus, and was apparently lost only once in the ancestor to the *trimaculatus* complex. The reconstructed *Dascyllus* ancestor would have been small, suggesting that large body size has evolved twice, once in the *trimaculatus* lineage and once in *D. flavicaudus*. These results indicate that size and protogynous hermaphroditism are not necessarily linked in *Dascyllus*.

## Discussion

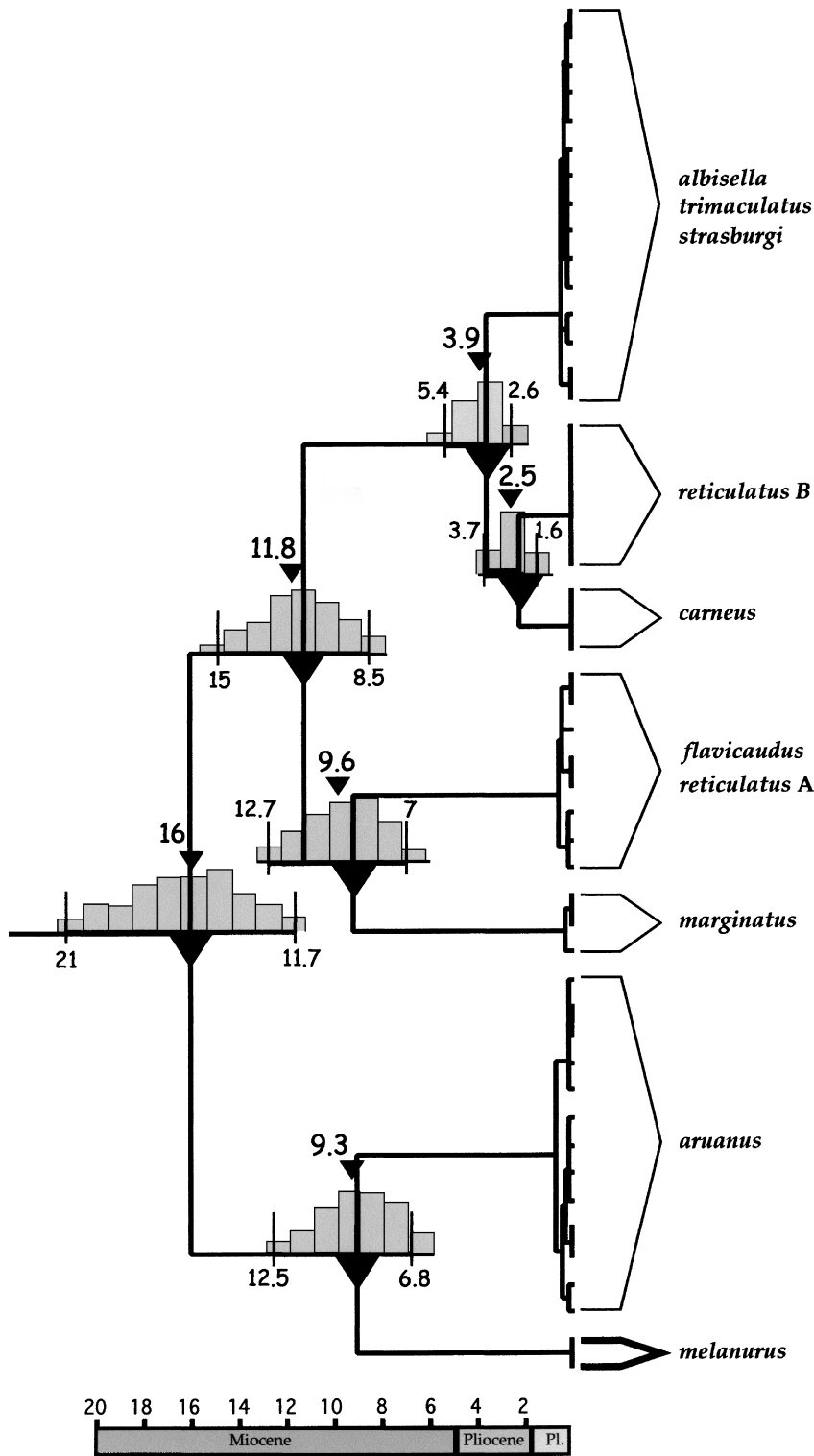
Our mtDNA-based phylogeny represents the second analysis of *Dascyllus* species-level relationships based on mitochondrial markers, but differs in several respects from the earlier, comparable research of Bernardi & Crane (1999). A trivial difference is our use of the mitochondrial ATP synthase genes vs. Bernardi & Crane's (1999) utilization of cytochrome b and 16 s rDNA sequences. Another was our inclusion of *D. strasburgi*, which was missing from their 1999 study. More importantly, our study included a considerably broader geographical and numerical sampling of the *Dascyllus* group as a whole, although Bernardi *et al.* (2001) have recently published a well-sampled phylogeographical study of the *trimaculatus* group. Our enhanced genus-wide sampling strategy revealed additional insights regarding the molecular systematics and historical biogeography of the genus and provided an improved evolutionary framework for considering the roles of earth history and life history in the diversification of *Dascyllus*. A new species endemic to atolls of the Central Pacific, *D. auripinnis*, was described while this manuscript was in review (Randall & Randall 2001); although not included in our study, *D. auripinnis* is a member of the *trimaculatus* group according to Randall & Randall (2001) and recent molecular systematic analysis (Bernardi *et al.* 2002).

### Molecule-based alpha taxonomy of the genus *Dascyllus*

We identified a one-to-one correspondence between five *Dascyllus* species and their associated monophyletic clusters of mtDNA haplotypes: *D. aruanus*, *D. melanurus*, *D. marginatus*, *D. carneus* and *D. strasburgi*. The lack of concordance between the remaining four nominal species and the mtDNA-based phylogeny indicates that the current taxonomy of *Dascyllus* provides an incomplete impression of evolutionary distinctiveness of the different species in this group (Fig. 2).

Randall & Allen (1977) identified three species in the *D. trimaculatus* complex: *D. trimaculatus*, *D. strasburgi*, and *D. albisella*. However, current taxonomy and the mtDNA results were in accord regarding only the phylogenetic uniqueness of *D. strasburgi*, the Marquesas endemic. For *D. trimaculatus*, the mtDNA ATPase data identified two distinct mtDNA clades, one apparently restricted to French Polynesia and the second with a widespread distribution extending from the Red Sea and Indian Ocean to the western Pacific. Finally, the Hawaiian endemic, *D. albisella*, could not be phylogenetically distinguished on the basis of its mtDNA ATPase genes from haplotypes representing the widespread *D. trimaculatus* clade (Fig. 2).

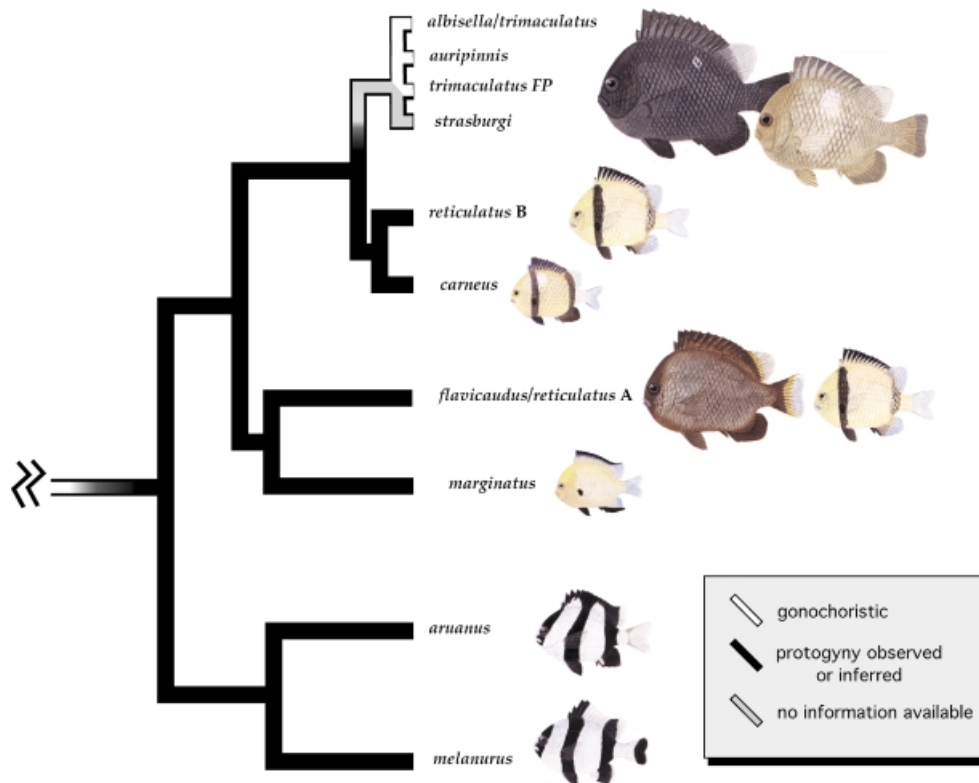
Randall & Allen (1977) presaged our mtDNA findings for the *D. trimaculatus* complex and had noted the 'difficult taxonomic decisions regarding the status of *D. albisella*



**Fig. 3** The estimated average time of divergence and 95% CI for the principal *Dascyllus* clades. We estimated the 95% CI for each node by parametric bootstrapping. Calibrations are based on a geminate pomacentrid species pair (*Abudefduf*) formed putatively by the Pliocene rise of the Isthmus of Panama (see text and Bermingham *et al.* 1997a).

and *D. strasburgi*, which are obvious offshoots of the *D. trimaculatus* ancestral stock'. Bernardi & Crane (1999) provided mtDNA-based evidence for the reciprocal monophyly of *D. albisella* and *D. trimaculatus*. However, this result was an artefact of limited geographical sampling

and was incorrect. Although more focused mtDNA control region-based analysis supported the phylogenetic distinctiveness of *D. albisella*, this species nests within *D. trimaculatus* (Bernardi *et al.* 2001). Thus, from both an ATPase and d-loop perspective, *D. trimaculatus* mtDNAs



**Fig. 4** Mapping of protogynous hermaphroditism on a generalized *Dascyllus* phylogeny derived from Fig. 2. Images (Lieske & Myers 1996) of the various *Dascyllus* species are scaled (relative to *D. trimaculatus*) to represent the maximum female body size in each species as described in Randall & Allen (1977). Only two of the four putative species in the *trimaculatus* complex are shown: *D. trimaculatus* (left) and *D. albisella* (right). Sexual pattern has not been described for *D. strasburgi*; *D. melanurus* and *D. carneus* are inferred to be protogynous based on social structure and histological analysis (K. Asoh, T. Yoshikawa, unpublished results).

are paraphyletic with respect to *D. albisella* mtDNAs, and although *D. strasburgi* appears phylogenetically distinct it is probably more informative to consider *D. trimaculatus* a species group. This concept permits one to appreciate more the recent isolation of *Dascyllus* evolutionary lineages in French Polynesia and in the Marquesas in contrast to the geographically extensive distribution of the third *D. trimaculatus* mtDNA ATPase clade (Figs 1 and 2).

A second apparent note of discord between the alpha taxonomy of *Dascyllus* and our results concerns *D. reticulatus*. This species is characterized by two very different mtDNA lineages, one of which (*reticulatus* B) is sister to *D. carneus*, while the other (*reticulatus* A) is extremely similar to the mtDNAs carried by *D. flavicaudus* (Fig. 2). *Dascyllus reticulatus* is distinguished by its mtDNA (*reticulatus* B) in the southern part of its distribution only, represented in our study by samples from Bali, New Guinea and the Great Barrier Reef. In the northern part of its distribution, represented in our study by samples from Guam, Japan and the Philippines, *D. reticulatus* (*reticulatus* A) cannot be distinguished confidently from *D. flavicaudus* on the basis of mtDNA. Both *reticulatus* A and B lineages are represented in the population from Palau. This island group appears to

lie on the boundary between the north and south regions occupied by the *reticulatus* A and B lineages, respectively. The *D. reticulatus* sample used by Bernardi & Crane (1999) is of unknown provenance, but is phylogenetically equivalent to our *reticulatus* B. Their phylogeny does not include a representative of *reticulatus* A.

The general lack of sequence divergence between *D. flavicaudus* and *reticulatus* A mtDNAs contrasts extreme differences in size and colour between these two lineages (Fig. 4). *Dascyllus reticulatus* A and B, on the other hand, have no documented phenotypical differences, yet are characterized by approximately 13% in mtDNA sequence divergence. Two scenarios, neither simple to accept, might account for the data. In the first, one could argue that we have recovered the two mtDNA lineages representing the nominal species, *D. flavicaudus* and *D. reticulatus*, which suggests in turn that *D. flavicaudus* mtDNA has introgressed into *D. reticulatus*, spreading subsequently through northern Pacific Plate *D. reticulatus* populations. This model argues for hybridization between two species that are not typically syntopic, followed by the relatively rapid replacement of the ancestral *D. reticulatus* mtDNA lineage in the northern part of the *D. reticulatus* species

range, but not in the southern part. The distribution of *D. flavicaudus* is limited to the Society and Tuamotu Islands (Randall & Allen 1977), and it is difficult although not impossible to imagine the introgression of *D. flavicaudus* mtDNA through the larger and geographically more extensive population(s) of northern Pacific Plate *D. reticulatus*, or to imagine that once widespread *D. flavicaudus* populations have collapsed to a French Polynesian endemic. Although we are not aware of reported hybridization between *Dascyllus* species, mixed schools of *D. aruanus* and *D. marginatus* in the Red Sea exert heterospecific influence on reproduction (Shpigel & Fishelson 1986), and suggest that hybridization might be possible between more closely related species. Finally, it is worth noting that the lack of shared mtDNA haplotypes between *D. reticulatus* and *D. flavicaudus* argues against any ongoing hybridization between these two species.

It could also be argued that *D. flavicaudus* is simply a recent isolate of *reticulatus* A in French Polynesia, similar to the recently isolated lineages of the *D. trimaculatus* complex in French Polynesia and the Indian Ocean (Bernardi *et al.* 2001), and including *D. strasburgi* in the Marquesas, *D. albisella* in Hawaii and *D. auripinnis* in Fiji, the Line Islands, Phoenix Islands and the northern Cook Islands. If this model is correct it would be worthwhile investigating the evolutionary forces that have led to the rapid and pronounced morphological divergence between *D. flavicaudus* and *reticulatus* A, as well as those responsible for the long-term maintenance or convergent evolution of size and colour pattern, which have veiled the phylogenetic separation of *reticulatus* A and B until now. Nuclear markers should shed more light on the history of *D. flavicaudus* and *D. reticulatus*, but an analysis of the slowly evolving creatine kinase gene (1900 bp) and recombination activating gene (1500 bp) provided no phylogenetic resolution among *reticulatus* A, *reticulatus* B and *D. flavicaudus*. In fact these nuclear genes phylogenetically separate only *D. aruanus*/*D. melanurus* from the remainder of the species in the genus (Quenouille & Bermingham, unpublished results).

#### *Intraspecific phylogeography of Dascyllus*

Two *Dascyllus* species, *D. aruanus* and *D. trimaculatus*, are geographically widespread and include both Indian and Pacific Ocean populations. Our analysis reveals phylogeographical structure in both. In *D. aruanus*, samples from the IO ranging from Kenya to Thailand form a monophyletic lineage separate from the remaining WP samples. In contrast, the phylogeographical break between *D. trimaculatus* populations lies much further to the east and separates the French Polynesian collection of this species from all others. With regard to *D. trimaculatus* phylogeography, we direct the reader to a recent study of this species by Bernardi *et al.* (2001), which evidences additional phylo-

geographical structure based on the faster evolving mtDNA control region.

Notwithstanding the contemporary sympatry of the two species, there is no phylogeographical signal of shared history. There is, however, a temporal signal suggesting that the phylogeographical structure in both species is recent and roughly contemporaneous. The mtDNA ATPase lineages representing *D. trimaculatus* are each separated by a minimum of two nucleotide substitutions, whereas the IO and WP *D. aruanus* lineages are separated by three nucleotide changes. It is worth noting that two ATPase substitutions also separate the most closely related *D. strasburgi* and *D. trimaculatus* mtDNA haplotypes. All substitutions are silent, suggesting roughly similar separation times for each of these phylogenetic lineages under the assumption of a molecular clock. Our phylogeographical analyses are based on small sample sizes and thus must be considered preliminary.

#### *Molecular systematics of Dascyllus*

Our phylogenetic analysis of *Dascyllus* mtDNA ATPase reveals relationships between species and species groups that differ somewhat from earlier ideas based on morphology (Randall & Allen 1977; Godwin 1995), but are largely concordant with the mtDNA-based *Dascyllus* phylogeny published by Bernardi & Crane (1999). The principal difference between the morphology and molecule-based *Dascyllus* hypotheses concerns the evolutionary significance of species' groups implied by Godwin's (1995) designation of three named complexes: *aruanus* (*D. aruanus* and *D. melanurus*), *reticulatus* (*D. reticulatus*, *D. carneus*, *D. flavicaudus* and *D. marginatus*) and *trimaculatus* (*D. trimaculatus*, *D. albisella* and *D. strasburgi*). The mtDNA data clearly reject the reciprocal monophyly of the three named species complexes, and support only the species composition of the *aruanus* complex. This complex, comprised of *D. aruanus* and *D. melanurus* mtDNA haplotypes, is sister to a clade that includes all remaining *Dascyllus* species (Figs 2–4).

In agreement with Bernardi & Crane (1999), our mtDNA ATPase-based phylogeny identifies a *flavicaudus*/*marginatus* clade that is sister to a clade uniting the *D. trimaculatus* group, *D. carneus* and *D. reticulatus*. The monophyly of the *carneus*/*reticulatus*/*trimaculatus* clade is supported strongly; however, the Bernardi & Crane (1999) phylogeny and the one presented here differ significantly with respect to the relationship of the species comprising this clade. Bernardi & Crane (1999) suggest that *D. carneus* is basal to *D. reticulatus*, which in turn is the sister of the *trimaculatus* group. In contrast, our phylogeny shows high bootstrap support for the reciprocal monophyly of the *carneus*/*reticulatus* B clade and the *trimaculatus* group (Figs 2–4), and is the most parsimonious reconstruction using any of the different weighting schemes. Our *Dascyllus* tree also had an

improved likelihood value compared to the Bernardi & Crane phylogeny, and the difference was significant at  $P < 0.01$  using the parametric bootstrap approach developed by Goldman *et al.* (2000). Furthermore, analysis of the complete mtDNA cytb gene representing the *Dascyllus* samples used in our study provides further support of the phylogeny presented in Figs 2–4 (Quenouille & Bermingham, in preparation). The difference in results between the Bernardi & Crane (1999) study and ours probably owes to the increased lineage sampling in our investigation.

#### Historical biogeography of *Dascyllus*

Our estimates of the mean and standard errors of dates to the MRCA for the various clades of *Dascyllus* (Fig. 3) are characterized by relatively large confidence intervals because we have explicitly accounted for two sources of error: our estimate of mtDNA divergence and our molecular-clock calibration. Large confidence intervals notwithstanding, we can make some reasonable chronological estimates regarding the origins of the different clades.

We estimate that *Dascyllus* diversification began some time in the mid-Miocene, with the MRCA of all extant species dating around 16 Ma (21–11.7 Ma). Given that the earliest pomacentrid fossils are recorded from the Eocene Monte Bloca deposits in Northern Italy (Blot 1980; Bellwood & Sorbini 1996), a Miocene origin for the genus *Dascyllus* would seem reasonable. It appears that the early diversification in the genus coincides with a period of profound tectonic change in the Indo-Pacific region, suggesting that vicariance may have played a causal role in the initial diversification of *Dascyllus*. The middle to late Miocene marks the northward migration of Australia with the subsequent tectonic uplift of the Indian Archipelago, and foraminifera data indicate very limited surface water exchange between the Indian and Pacific basins at this time (Kennett *et al.* 1985; Hodell & Vayavananda 1993). In addition, continental shelf area was reduced dramatically at this time owing to a major sea level regression (Haq *et al.* 1987). The biogeographical importance of these events is highlighted by a shift in the species composition of coral communities before and after this general time period (Choat & Bellwood 1991).

Whether one recognizes two or three ancestral lineages forming during the early diversification of the genus, it is clear that the descendants of each lineage have at some point covered the entire IWP distribution (Fig. 2). The sister group relationship of *D. marginatus* and *D. flavicaudus* is particularly interesting in this regard, given the contemporary distribution of these two species in the Red Sea and French Polynesia. If support for the sister status of *D. marginatus* and *D. flavicaudus* holds, then one or both of these species, or their common ancestor, must have gone extinct over large portions of the ancestral range to yield the

modern biogeographical pattern. The inferred reduction in range would be appreciably less if *reticulatus* A turns out to be the cryptic sister species of *D. flavicaudus*. Extinction or changes in distribution patterns are difficult to demonstrate in the absence of a fossil record. However, a decrease in the range of *D. flavicaudus* is supported indirectly if it turns out that introgressive hybridization, rather than recent speciation, explains the mtDNA similarity between *D. flavicaudus* and *reticulatus* A.

Excepting the *D. marginatus* and *D. flavicaudus* clade, our molecular systematic analysis of *Dascyllus* provides overwhelming evidence of secondary sympatry under a vicariant model of speciation. Not only do the other two lineages (*aruanus/melanurus* and *carneus/reticulatus/trimaculatus*) persist in sympatry across the breadth of the IWP, but each lineage also includes sister taxa that coexist in sympatry: *D. aruanus* and *D. melanurus* in central IWP and *D. trimaculatus* with the *carneus/reticulatus* B clade throughout most of the IWP (Fig. 1).

Given that the most closely related species pairs have largely nonoverlapping distributions (*carneus/reticulatus* B and the members of the *trimaculatus* species group), it seems reasonable to speculate on possible causes of vicariance-based speciation in the genus. Our estimated date of 9.3 Ma (6.8–12.5 Ma) for the origin of *D. marginatus* suggests that this species was isolated geographically by the Miocene uplifting of the Red Sea region roughly 9 Ma, rather than by restricted surface water exchange during Plio–Pleistocene sea level reductions (Rohling 1994). The split between the ancestors of *carneus/reticulatus* and the *trimaculatus* species group dates to 3.9 Ma (5.4–2.6 Ma) and corresponds to foraminifera data, suggesting that surface water connections between the Indian and Pacific basins were severed completely by the late Miocene to early Pliocene, due probably to the ongoing sea level regression (Haq *et al.* 1987; Hodell & Vayavananda 1993). Under this model we presume the *trimaculatus* ancestor was geographically isolated in the Pacific (given this clade's more extreme distribution and differentiation to the east), with the *carneus/reticulatus* ancestor isolated in the Indian Ocean. It is very probable that IO/WP barrier also played a role in the subsequent formation of *D. carneus* and *D. reticulatus* roughly 2.5 Ma (3.7–1.6 Ma), and caused the phylogeographical break reported here for *D. aruanus* and by Bernardi *et al.* (2001) for *D. trimaculatus*.

The role of the IO/WP barrier in marine diversification has long been noted (Valentine & Jablonski 1983), and is emphasized frequently in phylogeographical studies of IWP taxa (review in Benzie 1999). Studies of butterfly fish (McMillan & Palumbi 1995), barramundi (Chenoweth *et al.* 1998), sea urchins (Lessios *et al.* 2001), starfish (Williams & Benzie 1998) and coconut crabs (Lavery *et al.* 1996) have each documented a mtDNA-based phylogeographical break between populations or species found in the two

oceans, with divergence values ranging from 0.47 to 2%, and estimated dates of divergence from 335 000 years to 2 Ma. The staggered temporal pattern of speciation and geographical differentiation across the IO/WP barrier matches what we have observed in *Dascyllus*, and such staggered differentiation will often be produced if fluctuating sea levels cause cyclical alterations in the permeability of barriers to gene flow (Bermingham & Avise 1986). Whether or not speciation occurs across such an ephemeral barrier probably depends on the length of time that the barrier is impermeable to gene flow, and whether evolutionary changes in the allopatrically separated populations result from differential selection or drift alone.

#### *Dascyllus* mating system and sexual pattern evolution

Variation in body size and sexual pattern (i.e. protogyny or gonochorism) renders *Dascyllus* a useful group for investigating life-history evolution. Godwin (1995) proposed that the presence or absence of protogynous sex change in *Dascyllus* might result from body-size evolution or phylogenetic inertia. The body-size model posits that an evolutionary increase in *Dascyllus* body size would have been associated with increased female mobility, thus decreasing a large males ability to monopolize females and, in turn, to a loss of protogyny. The phylogenetic inertia model applied to *Dascyllus* posits that descendant species will retain the sexual pattern of their ancestors, regardless of body size. *D. flavicaudus* is a large-bodied species in an evolutionary lineage that otherwise includes only small-bodied protogynous species (Fig. 4), and thus provides a test of the alternative models. The body-size hypothesis predicts *D. flavicaudus* to be gonochoristic, whereas the phylogenetic inertia model applied to the hypothesis of *Dascyllus* relationship presented here (Figs 2–4) predicts protogynous hermaphroditism for the species. The observation of protogyny in *D. flavicaudus* supports the phylogenetic inertia model over the body-size model.

The mtDNA-based reconstruction of *Dascyllus* phylogeny suggests that the ability to change sex arose once in the ancestor to the entire genus, and was apparently lost only once in the ancestor to the *trimaculatus* complex (Fig. 4). The reconstructed *Dascyllus* ancestor would have been small, suggesting that large body size has evolved twice, once in the *trimaculatus* lineage and once in *D. flavicaudus*. Thus, under the phylogenetic hypothesis presented here, size and protogynous hermaphroditism are not necessarily linked in *Dascyllus*.

The hypothesis accounting for the adaptive significance of protogynous sex change (the size-advantage model) states that an individual is expected to change sex from female to male when its reproductive success is a function of age or size. Individuals attain higher reproductive success as a female when young or small, but as a male

when older and larger (Ghiselin 1969; Warner 1975; Warner *et al.* 1975). This condition occurs frequently under a single-male polygynous mating system, where a single large male defends resources such as food and sheltering sites required by females and mates with all the resident females, or in more open systems in which a few large males defend limited spawning sites and mate with a large number of females (Warner 1984, 1988a). There is a good correspondence between protogyny and a polygynous mating system in fishes, and the large-male mating advantage has been considered the major factor leading to the evolution of protogyny (Warner 1988a,b).

Although there has been some confusion in the past, Godwin's body-size model is not equivalent to the original size-advantage model. The body-size model is one of many alternative hypotheses regarding factors that could potentially lead to a decrease in large-male mating advantage and hence the absence of protogyny. Rejection of the body-size model in the case of *D. flavicaudus* indicated that body size *per se* is not sufficient to explain the presence or absence of protogyny in the genus, but it does not necessarily follow that body size is not an important factor in the evolution of protogynous hermaphroditism. The absence of protogyny in the large-bodied *trimaculatus* complex certainly suggests the importance of body size. The mating system and variation in male reproductive success is determined primarily by spatial as well as the temporal dispersion of females or of resources critical for females (Emlen & Oring 1977; Vehrencamp & Bradbury 1984; Davies 1991). How body size and the spatial and temporal distribution of females and those resources critical for females have interacted to lead to the loss of protogyny in the *trimaculatus* complex, and its retention in the other *Dascyllus* species, needs to be determined in future studies.

#### Conclusion

This study highlights the importance of adequate taxonomic and geographical sampling when inferring the phylogenetic relationships among closely related species. Incomplete sorting of ancestral polymorphisms and introgression can easily be overlooked when using insufficient taxonomic or geographical sampling, limiting severely the accuracy of evolutionary inferences (Funk 1999). In this particular case study, the separation of *D. reticulatus* into two long-isolated mtDNA lineages, the very close phylogenetic relationship of *D. flavicaudus* with one of these *D. reticulatus* lineages and the sister relationship of *D. carneus* to the other *D. reticulatus* lineage, underscore the importance of adequate geographical sampling. Enriched geographical sampling has led to a refined understanding of *Dascyllus* diversification history, as well as strong inference regarding changes in species' ranges over time, and body size and sexual pattern evolution in the genus.

## Acknowledgements

We thank Guy Reeves, Ross Robertson, Alisdair Edwards and Denis Goulet for the generous gift of *Dascyllus* tissue samples and specimens. We are grateful for the superb laboratory assistance of Nimiadina Gomez, Gustavo Ybazeta and Maribel Gonzalez. We thank Gustav Paulay and Chris Meyer for helpful comments on the manuscript, and Bob Richmond for support of S.M. while he was exiled on Guam. Anonymous reviewers provided constructive criticism that improved the quality of the manuscript, and we gratefully acknowledge one of these reviewers for suggesting that we incorporate discussion of convergence as an alternative to introgressive hybridization to explain the phenotypical similarity of *D. reticulatus* A and B, which contrasts the high level of divergence between their mtDNAs. E. Lieske, R. Myers and Princeton University Press are gratefully acknowledged for permission to utilize the images of *Dascyllus* presented in Fig. 4. The Smithsonian Institution Molecular Systematics program provided funding for our study.

## References

- Allen GR (1991) *Damselfishes of the World*. Mergus Publishers, Germany.
- Asoh K, Kasuya M (2002) Gonadal development and mode of sexuality in a coral reef damselfish, *Dascyllus trimaculatus*. *Zoological Journal*, **256**, 301–310.
- Asoh K, Yoshikawa T, Kasuya M (2001) Gonadal development and non-functional protogyny in a coral reef damselfish, *Dascyllus albisella*. *Journal of Fish Biology*, **58**, 1601–1616.
- Baldwin BG, Sanderson MJ (1998) Age and rate of diversification of the Hawaiian silversword alliance (Compositae). *Proceedings of the National Academy of Sciences USA*, **95**, 9402–9406.
- Banford HM, Bermingham E, Collette BB, McCafferty SS (1999) Phylogenetic systematics of the *Scomberomorus regalis* (Teleostei: Scombridae) species group: molecules, morphology, and biogeography of Spanish mackerels. *Copeia*, **3**, 596–613.
- Bellwood D, Sorbini L (1996) A review of the fossil record of the Pomacentridae (Teleostei: Labroidae) with a description of a new genus and species from the Eocene of Monte Bolca, Italy. *Zoological Journal of the Linnean Society*, **117**, 159–174.
- Benzie JAH (1999) Genetic structure of coral reef organisms: ghosts of dispersal past. *American Zoologist*, **39**, 131–145.
- Bermingham E, Avise JC (1986) Molecular zoogeography of freshwater fishes in the southeastern United States. *Genetics*, **113**, 939–965.
- Bermingham E, Banford H, Martin AP, Aswani V (1997b) Smithsonian Tropical Research Institute neotropical fish collections. In: *Neotropical Fish Collections* (ed. Malabarba L), pp. 37–38. Museo de Ciencias E Tecnologia, PUCRS, Puerto Alegre, Brazil.
- Bermingham E, McCafferty S, Martin A (1997a) Fish biogeography and molecular clocks: perspectives from the Panamanian Isthmus. In: *Molecular Systematics of Fishes* (eds Kocher TD, Stepien C), pp. 113–138. Academic Press, San Diego, CA.
- Bernardi G, Crane N (1999) Molecular phylogeny of the humbug damselfishes inferred from mtDNA sequences. *Journal of Fish Biology*, **54**, 1210–1217.
- Bernardi G, Holbrook SJ, Schmitt RJ (2001) Dispersal of the coral reef three-spot damselfish, *Dascyllus trimaculatus*, at three spatial scales. *Marine Biology*, **138**, 457–465.
- Bernardi G, Holbrook SJ, Schmitt RJ, Crane NL, DeMartini E (2002) Species boundaries, populations, and color morphs in the coral reef three-spot damselfish (*Dascyllus trimaculatus*) species-complex. *Proceedings of the Royal Society of London, Biological Sciences*, **269**, 599–605.
- Blot J (1980) La faune ichthyologique des gisements du Monte Bolca (Province de Vérone, Italy). Catalogue systématique présentant l'état actual des recherches concernant cette faune. *Bulletin Du Museum d'Histoire Naturelle*, **2**, 339–396.
- Bowen BW, Bass AL, Rocha LA, Grant WS, Robertson DR (2001) Phylogeography of the trumpetfish (*Aulostomus*) ring species complex on a global scale. *Evolution*, **53**, 1029–1039.
- Chenoweth SF, Hughes JM, Keenan CP, Lavery S (1998) Concordance between dispersal and mitochondrial gene flow: isolation by distance in a tropical teleost, *Lates calcarifer* (Australian barramundi). *Heredity*, **80**, 187–193.
- Choat JH, Bellwood DR (1991) Reef fishes: their history and evolution. In: *The Ecology of Fishes on Coral Reefs* (ed. Sale PF), pp. 39–66. Academic Press, San Diego, CA.
- Coates D (1982) Some observations on the sexuality of humbug damselfish, *Dascyllus aruanus* (Pisces, Pomacentridae) in the field. *Zish Tierpsychology*, **59**, 7–18.
- Coates AB, Obando J (1996) The geologic evolution of the Central American isthmus. *Evolution and Environment in Tropical America* (eds Jackson J, Budd AF, Coates AG), pp. 21–56. Chicago University Press, Chicago, IL.
- Cole KS (2002) Gonadal morphology, sexual development, and colony composition in the obligate coral-dwelling damselfish *Dascyllus aruanus*. *Marine Biology*, **140**, 165–173.
- Davies NB (1991) Mating systems. In: *Behavioural Ecology, an Evolutionary Approach*, 3rd edn (eds Krebs JR, Davies NB), pp. 263–294. Blackwell Scientific Publications, Oxford.
- Emlen ST, Oring LW (1977) Ecology, sexual selection, and the evolution of mating systems. *Science*, **197**, 215–223.
- Farris JS (1969) A successive approximations approach to character weighting. *Systematic Zoology*, **18**, 374–385.
- Fricke HW (1977) Community structure, social organization, and ecological requirements of coral reef fish (Pomacentridae). *Helgolander Wissenschaften Meeresunters*, **30**, 412–426.
- Fricke HW (1980) Control of different mating systems in a coral reef fish by one environmental factor. *Animal Behavior*, **28**, 561–569.
- Fricke HW, Holzberg S (1974) Social units and hermaphroditism in a pomacentrid fish. *Naturwissenschaften*, **61**, 367–368.
- Funk DJ (1999) Molecular systematics of cytochrome oxidase I and 16S from *Neochlamisus* leaf beetles and the importance of sampling. *Molecular Biology and Evolution*, **16**, 67–82.
- Ghiselin MT (1969) The evolution of hermaphroditism among animals. *Quarterly Review of Biology*, **44**, 189–208.
- Godwin J (1995) Phylogenetic and habitat influences on mating system structure in the humbug damselfishes (*Dascyllus*, Pomacentridae). *Bulletin of Marine Science*, **57**, 637–652.
- Goldman N, Anderson JP, Rodrigo AG (2000) Likelihood-based tests of topologies in phylogenetics. *Systematic Biology*, **49**, 652–670.
- Haq BU, Harbenbol J, Vail PR (1987) Chronology of fluctuating sea levels since the Triassic. *Science*, **235**, 1156–1166.
- Hasegawa M, Kishino H, Yano T (1985) Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution*, **22**, 160–174.
- Hillis DM, Mable BK, Moritz C (1996) Applications of molecular systematics: the state of the field and a look to the future. In: *Molecular Systematics* (eds Hillis DM, Moritz C, Mable BK), pp. 515–543. Sinauer Associates Inc., Sunderland, MA.

- Hodell DA, Vayavananda A (1993) Middle Miocene paleoceanography of the western equatorial Pacific (DSDP site 289) and the evolution of *Globorotalia* (Fohsella). *Marine Micropaleontology*, **22**, 279–310.
- Huelsenbeck JP, Crandall KA (1997) Phylogeny estimation and hypothesis testing using maximum likelihood. *Annual Review of Ecology and Systematics*, **28**, 437–466.
- Huelsenbeck JP, Hillis DM, Jones R (1996) Parametric bootstrapping in molecular phylogenetics: applications and performance. In: *Molecular Zoology: Advances, Strategies, and Protocols* (eds Ferraris JD, Palumbi SR), pp. 19–45. Wiley-Liss, New York.
- Huelsenbeck JP, Rannala B (1997) Phylogenetic methods come of age: testing hypotheses in an evolutionary context. *Science*, **276**, 227–232.
- Kennett JP, Keller G, Srinivasan MS (1985) Miocene planktonic foraminiferal biogeography and paleoceanographic development of the Indo-Pacific region. *Geological Society of America Memoir*, **163**, 197–236.
- Lavery S, Moritz C, Fielder DR (1996) Indo-Pacific population structure and evolutionary history of the coconut crab *Birgus latro*. *Molecular Ecology*, **5**, 557–570.
- Lessios HA, Kessing BD, Pearse JS (2001) Population structure and speciation in tropical seas: global phylogeography of the sea urchin *Diadema*. *Evolution*, **55**, 955–975.
- Lessios HA, Kessing BD, Robertson DR, Paulay G (1998) Phylogeography of the pantropical sea urchin *Eucidaris* in relation to land barriers and ocean currents. *Evolution*, **53**, 806–817.
- Lieske E, Myers R (1996) *Coral Reef Fishes*. Princeton University Press, Princeton, NJ.
- Maddison W, Maddison D (1992) *Macclade, Analysis of Phylogeny and Character Evolution, Version 3*. Sinauer Associates, Sunderland, MA.
- McMillan WO, Palumbi SR (1995) Concordant evolutionary patterns among Indo-West Pacific butterflyfishes. *Proceedings of the Royal Society of London Series B — Biological Sciences*, **260**, 229–236.
- Palumbi SR (1992) Marine speciation on a small planet. *Trends in Ecology and Evolution*, **7**, 114–118.
- Palumbi SR, Grabowsky G, Duda T, Geyer L, Tachino N (1997) Speciation and population genetic structure in tropical Pacific sea urchins. *Evolution*, **51**, 1506–1517.
- Paulay G (1997) Diversity and distribution of reef organisms. In: *Life and Death of Coral Reefs* (ed. Birkeland C), pp. 298–373. Chapman & Hall, New York.
- Perna NT, Kocher TD (1995) Unequal base frequencies and the estimation of substitution rates. *Molecular Biology and Evolution*, **12**, 359–361.
- Rambaut A, Grassly NC (1997) Seq-Gen: an application for the Monte Carlo simulation of DNA sequence evolution along phylogenetic trees. *Computer Applied Biosciences*, **13**, 235–238.
- Randall HA, Allen GR (1977) A revision of the damselfish genus *Dascyllus* (Pomacentridae) with the description of a new species. *Records of the Australian Museum*, **31**, 349–385.
- Randall JE, Randall HA (2001) *Dascyllus auripinnis*, a new pomacentrid fish from atolls of the Central Pacific Ocean. *Zoological Studies*, **40**, 61–67.
- Rohling EJ (1994) Glacial conditions in the Red Sea. *Paleoceanography*, **9**, 653–660.
- Rosen BR (1988) Progress, problems, and patterns in the biogeography of reef corals and other tropical marine organisms. *Helgoländer Meeresuntersuchungen*, **42**, 269–301.
- Rzhetsky A, Nei M (1994a) METREE: a program package for inferring and testing minimum-evolution trees. *Cabios*, **10**, 409–412.
- Rzhetsky A, Nei M (1994b) A simple method for estimating and testing minimum evolution trees. *Molecular Biology and Evolution*, **9**, 945–967.
- Sale PF (1970) Behaviour of the humbug fish. *Australian Natural History*, **16**, 362–366.
- Schwarz AL, Smith CL (1990) Sex change in the damselfish *Dascyllus reticulatus* (Richardson) (Perciformes: Pomacentridae). *Bulletin of Marine Science*, **46**, 790–798.
- Seutin G, White BN, Boag PT (1991) Preservation of avian blood and tissue samples for DNA analyses. *Canadian Journal of Zoology*, **69**, 82–90.
- Shpigel M, Fishelson L (1986) Behavior and physiology of coexistence in two species of *Dascyllus* (Pomacentridae, Teleostei). *Environmental Biology of Fishes*, **17**, 253–265.
- Strimmer K, Von Haeseler A (1996) Quartet puzzling: a quartet maximum likelihood method for reconstructing tree topologies. *Molecular Evolution and Biology*, **13**, 964–969.
- Swofford DL (2001) *PAUP\*. Phylogenetic Analysis Using Parsimony (\*and Other Methods), Version 4.0*. Sinauer Associates, Sunderland, MA.
- Thresher RE (1984) *Reproduction in Reef Fishes*. TFH Publications, Neptune City, New Jersey.
- Valentine JW, Jablonski D (1983) Speciation in the shallow sea: General patterns and biogeographic controls. In: *Evolution, Time, and Space: the Emergence of the Biosphere* (eds Sims RW, Price JH, Whalley PES), pp. 201–226. Academic Press, London.
- Vehrencamp SL, Bradbury JW (1984) Mating systems and ecology. In: *Behavioural Ecology, an Evolutionary Approach*, 2nd edn (eds Krebs JR, Davies NB), pp. 251–278. Sinauer Associates, Sunderland, MA.
- Warner RR (1975) The adaptive significance of sequential hermaphroditism in animals. *American Naturalist*, **109**, 61–62.
- Warner RR (1984) Mating behavior and hermaphroditism in coral reef fishes. *American Scientist*, **72**, 128–136.
- Warner RR (1988a) Sex change in fishes: hypotheses, evidence, and objections. *Environmental Biology of Fishes*, **22**, 81–90.
- Warner RR (1988b) Sex change and the size-advantage model. *Trends in Ecology and Evolution*, **3**, 133–136.
- Warner RR, Robertson DR, Leigh EG Jr (1975) Sex change and sexual selection. *Science*, **190**, 633–638.
- Williams ST, Benzie JAH (1998) Evidence of a biogeographic break between populations of a high dispersal starfish: congruent regions within the Indo-West Pacific defined by color morphs, mtDNA, and allozyme data. *Evolution*, **52**, 87–99.

---

The authors are united by their interest in the ecology and evolution of coral reef fishes. Shawn McCafferty, Brice Quenouille and Eldredge Bermingham carry forward research on the evolutionary genetics of fishes and other organisms from their STRI laboratory at the Pacific mouth of the Panama Canal. Serge Planes divides his time between the Mediterranean and French Polynesia and has a long-standing interest in the ecological genetics of *Dascyllus aruanus*. Kazue Asoh studies coral-reef fish reproductive ecology and is currently a postdoctoral fellow at Ohio State University. Guy Hoelzer, perhaps blown away by the amazing diversity of coral reef fishes, has turned to chaos theory for the answers.

---