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Author(s): W. Owen McMillan, Lee A. Weigt, Stephen R. Palumbi

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COLOR PATTERN EVOLUTION, ASSORTATIVE MATING, AND GENETIC DIFFERENTIATION IN BRIGHTLY COLORED BUTTERFLYFISHES (CHAETODONTIDAE)

W. OWEN McMILLAN,^{1,2} LEE A. WEIGT,³ AND STEPHEN R. PALUMBI^{2,4}

¹Departamento de Biología, Universidad de Puerto Rico, P.O. Box 23360, San Juan, Puerto Rico 00931-3360

²Department of Zoology and Kewalo Marine Laboratory, University of Hawaii, Honolulu, Hawaii 96822

³Field Museum of Natural History, Roosevelt Road at Lakeside Drive, Chicago, Illinois 60605

Abstract.—In butterflyfishes (Chaetodontidae), color pattern evolves rapidly and is often the only morphological trait separating closely related species. Vivid coloration is frequently assumed to provide critical signals for mate recognition and mate choice, but few direct experimental tests are available. Here we analyze the relationship between color pattern change, mate choice, and genetic differentiation in a group of three very closely related allopatric butterflyfishes. We found that in only one member of this group, *Chaetodon multicinctus*, is color pattern evolution associated with mate preference and genetic divergence. For its two sister species, *C. punctatofasciatus* and *C. pelewensis*, color pattern change has not resulted in assortative mating (based on laboratory pairing experiments and field observations) or in significant mtDNA or allozyme differentiation. In a contact zone on reefs in the Solomon Islands and Papua New Guinea, hybridization between the two forms has nearly homogenized color pattern differences. Outside these areas, however, color pattern remains distinct. Genetic variation is homogeneous over a much larger geographic scale. Sequence variation in the tRNA-proline end of the mitochondrial control region and allozyme variation was distributed widely within *C. punctatofasciatus* and *C. pelewensis*, which suggests few constraints to mitochondrial or nuclear gene flow across the color pattern boundary. These contrasting patterns strongly suggest that selection is maintaining color pattern differences in allopatry in the face of potentially homogenizing levels of gene flow. The mating pattern data show that this selection is not operating on mate recognition in the strictest sense, but probably on some other aspect of the social system of these territorial fish. In this case, divergence in mating preference can follow color pattern evolution, but is not contemporaneous with it.

Key words.—Hybrid zones, marine fishes, mate choice, mitochondrial control-region.

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The evolution of intraspecific differences that restrict gene flow and lead to speciation remains poorly understood (Andersson 1994). Divergence in specific mate recognition systems (sensu Paterson 1985) by changes in signals that coordinate mating can lead to evolution of assortative mating. Among taxa with complex behavior, these signals can be elaborate, involving both male and female elements, and even small changes may lead to strong mating barriers and rapid speciation. Conspicuous coloration has figured prominently in the development of theories of mate selection and has been implicated in both inter- and intrasexual selection in a large number of vertebrate and invertebrate taxa (reviewed in Andersson 1994). Empirical work on the role of conspicuous coloration in mating behavior has largely focused on sexually dimorphic species. In freshwater fishes, for example, bright male coloration can be important in male-male competition for breeding territories (Evans and Norris 1996) and in female-based mate choice (Baube et al. 1995; Endler and Houde 1995).

Here we investigate the association between color pattern evolution, assortative mating, and genetic differentiation in three incipient butterflyfishes (Chaetodontidae), *Chaetodon multicinctus*, *C. pelewensis*, and *C. punctatofasciatus*, using a combination of mating experiments, field observations, and molecular analysis. Like many coral reef fishes, butterflyfishes are spectacularly colored. These vivid patterns have intrigued evolutionary biologists for over a century (Wallace

1889); however, the sparse empirical work to date provides only a very rudimentary picture of the ecological and evolutionary significance of conspicuous coloration in coral reef fishes (Ehrlich et al. 1977; Andersson 1994). For example, among three species of damselfishes (Pomacentridae), male color pattern appears to be under both inter- and intra-sexual selection (Thresher and Moyer 1983). In contrast, Warner and co-workers concluded that the bright coloration of terminal-phase males of the blue-headed wrasse (*Thalassoma bifasciatum*) was relatively unimportant in determining mating success (Warner 1987; Warner and Schultz 1992). In many species of coral reef fishes, including all of the more than 125 species of butterflyfishes, both the male and the female are brightly patterned and coloration has been proposed as enhancing species and mate recognition (Lorenz 1966). Indeed, there is strong assortative mating among the 12 color morphospecies of the sexually monochromatic hamlet (Serranidae; Fischer 1980; Domeier 1994). In butterflyfishes, there also appears to be a consistent link between speciation (or at least taxonomic divisions) and color pattern change. Closely related butterflyfishes are morphologically similar but divergent in color pattern (Blum 1988). Although the existence of many closely related species that differ only in color pattern is consistent with a role of color pattern in mate recognition, the link between color pattern change and assortative mating in butterflyfishes remains untested.

Chaetodon multicinctus, *C. pelewensis*, and *C. punctatofasciatus* provide an excellent model system to examine the relationship between color pattern change, mate recognition, and speciation. All three are vividly patterned and possess

⁴ Present address: Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, Massachusetts 02138.

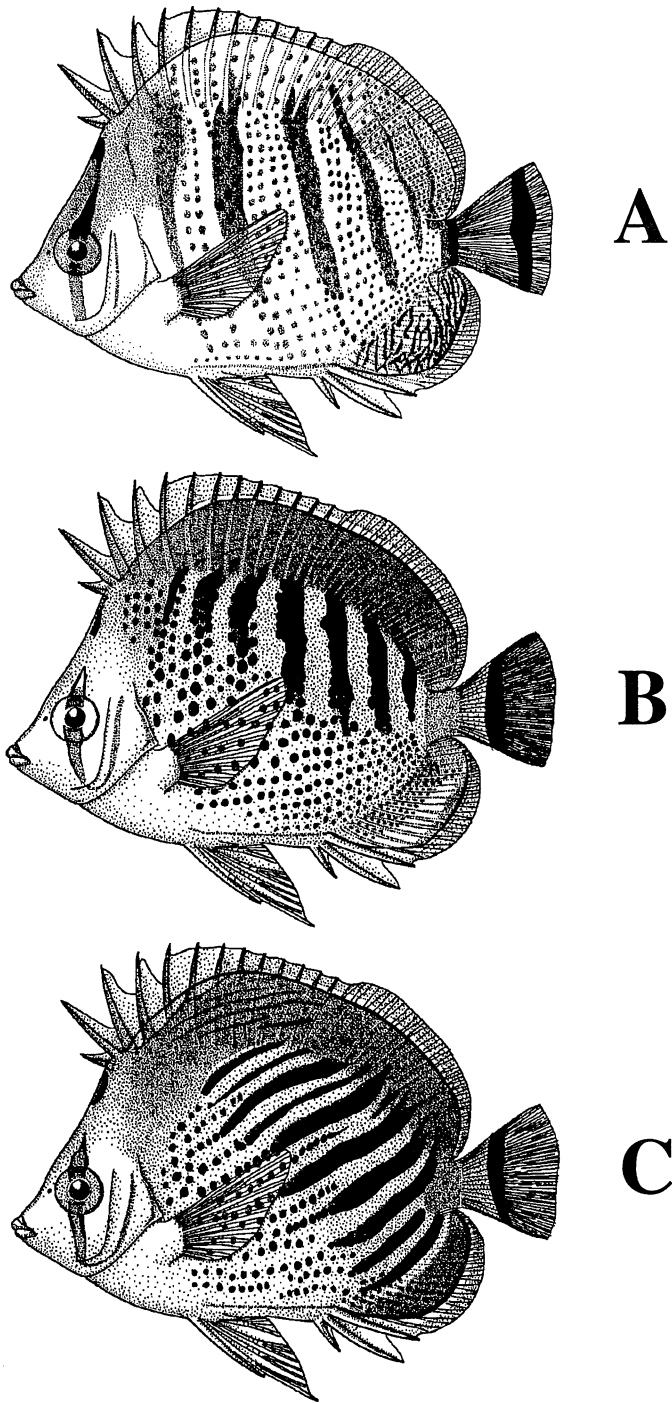


FIG. 1. *Chaetodon multicinctus* (A), *C. punctatofasciatus* (B), and *C. pelewensis* (C).

distinct black striations that run vertically along the body in *C. multicinctus* and *C. punctatofasciatus* and diagonally in *C. pelewensis* (Fig. 1). *Chaetodon pelewensis* and *C. punctatofasciatus* also share a yellow wash along the body, a bright orange splash on the caudal fin, and a yellow eye-band. Although only the ranges of *C. pelewensis* and *C. punctatofasciatus* overlap, all have been given species-level status because color pattern differences between them are as great or

greater than that observed between broadly sympatric sister species (Burgess 1978; Blum 1988). Molecular data indicate that the color pattern differences that define the three species has occurred very recently. Genetic differences in the mitochondrial cytochrome *b* gene (*cytb*) indicate that *C. punctatofasciatus*, *C. pelewensis*, and *C. multicinctus* diverged from their Indian Ocean sister species between one and two million years ago and from each other between 260,000 and 870,000 years ago (McMillan and Palumbi 1995). Rapid color pattern evolution within this time frame is also evident in at least two other monophyletic species complexes of Indo-west Pacific butterflyfishes (McMillan and Palumbi 1995).

Our laboratory experiments and field observations use pair formation as a meter of color pattern-based assortative mating. Pair bonding is the basis of reproduction in many butterflyfishes, including the three species examined here (Burgess 1978; Hourigan 1987, 1989; W. O. McMillan, pers. obs.). In the wild, male/female pairs are extremely long lasting, up to six years in *C. multicinctus*, and these pairs defend feeding territories (Hourigan 1987, 1989; pers. obs.). Although neighboring males have been observed to intrude on monthly spawning events (Lobel 1989), mating generally occurs between the male and the female of the pair in the water column above their territory. All three species are obligate coralivores and territory size is primarily determined by the amount of coral cover (Hourigan 1987). Although aggressive interactions among Chaetodontids are generally rare (Ehrlich et al. 1977), at least for *C. multicinctus*, both males and females have been observed to defend a territory. In this species, territorial defense tends to be sex specific; males defend against intrusion by other males and females defend against intrusion by other females (Hourigan 1987). In areas of coral and fish density, most available space on a reef is partitioned among male/female pairs, which creates a complex mosaic of contiguous territories (McMillan, pers. obs.).

To augment our behavioral data on color pattern-based assortative pairing, we sequenced a 200-base portion of the tRNA-proline (*tRNA^{Pro}*) end of the mitochondrial control-region from populations of *C. punctatofasciatus*, *C. pelewensis*, and *C. multicinctus* across the tropical west Pacific. In many vertebrates, this section of the control-region evolves much faster than other nuclear or mitochondrial regions (Meyer et al. 1990; Edwards 1993; Wakely 1993; Wenink et al. 1993; Lee et al. 1995; McMillan and Palumbi 1997). In these fishes, molecular evolution across this region is extremely rapid, between 33% and 100% divergence per million years (McMillan and Palumbi 1997), which permits fine-scale phylogenetic-based inferences into the history of female mediated gene flow. We augmented our genealogical perspective into the recent demographic history of this group with information on the structuring of allozyme variation between the three species. These two different types of genetic markers provide an excellent compliment to the historical pattern of gene flow inferred from the distribution of color pattern variation within this group.

MATERIALS AND METHODS

Assortative Pairing

In our laboratory experiments, pairing behavior was examined in adults collected from reefs across the tropical Pa-

cific. Fishes used in these experiments were obtained directly from collectors working in Palau (*C. punctatofasciatus*), the Philippines (*C. punctatofasciatus*), Fiji (*C. pelewensis*), the Cook Islands (*C. pelewensis*), and Molokai, Hawaii (*C. multicinctus*). Upon arrival in Honolulu, fishes were sexed by catheterization and males marked with small fin clips. Following this procedure, conspecifics were kept together in large holding tanks for a two-week quarantine and ectoparasite treatment period. Densities were high enough in our holding tank that we did not observe pairing behavior prior to our experiments. However, adults collected together may have been paired prior to our experiments. We could not control this type of experimental bias, which would tend to favor the formation of conspecific pairs.

In pair-bonding experiments, females of the three types were presented with two males, one conspecific and one heterospecific. The three adults used in each experiment were released together in a large (2500-gallon) donut-shaped tank at the Hawaii Institute of Marine Biology and allowed to acclimate overnight. A hidden observer (WOM) recorded (1) the length of time a female was associated with each male; (2) the time the two males were together; and (3) aggressive interactions among individuals during four or five 10-min observation periods (each separated by one hour) on a computer-based event recorder (Losey 1988). Pairing was defined as the association between individuals for more than 50% of the trial period. All possible combinations of a female and a conspecific and a heterospecific male were examined. These results were compared to conspecific controls ($n = 4$ for each of the three species) in which a female was present with two conspecific males. In these experiments, we attempted to use similar sized adults; however, *C. multicinctus* grew much larger than its two siblings and even the smallest adults were 15% larger than either *C. punctatofasciatus* or *C. pelewensis*. Following each trial, fishes were weighed and measured. Individuals in both the “choice” and control experiments were used only once.

Natural pairs of *C. pelewensis* and *C. punctatofasciatus* were examined in transect dives on reefs in the Solomon Islands and Papua New Guinea. The phenotype of all individuals encountered was scored on a scale of 1–5 (Fig. 2), where type 1 individuals possessed distinct and sharp diagonal bands characteristic of *C. pelewensis* and the black bands of type 5 individuals ran vertically, which is typical of *C. punctatofasciatus*. Type 4 individuals were clearly allied with *C. punctatofasciatus*, but some of the bands were Y-shaped rather than the typical nonbifurcating pattern. Type 2 individuals had a pattern similar to the *C. pelewensis*, but were less sharp and more wavelike (see Fig. 2). Type 3 individuals were intermediate and could not be reliably grouped with either *C. punctatofasciatus* or *C. pelewensis*.

We used a simple likelihood approach to estimate the conditional pairing probabilities between the five color pattern classes. Likelihood provides a powerful means of estimating parameters and support intervals and is particularly useful when comparing different models (Edwards 1972; McMillan et al. 1997). Under our model, pairing was envisioned to be a function of the probability that two individuals encounter each other and the probability that they pair when they encounter. For this model, we assumed that (1) $\Xi_{ij} = \Xi_{ji}$, where

Phenotypic score

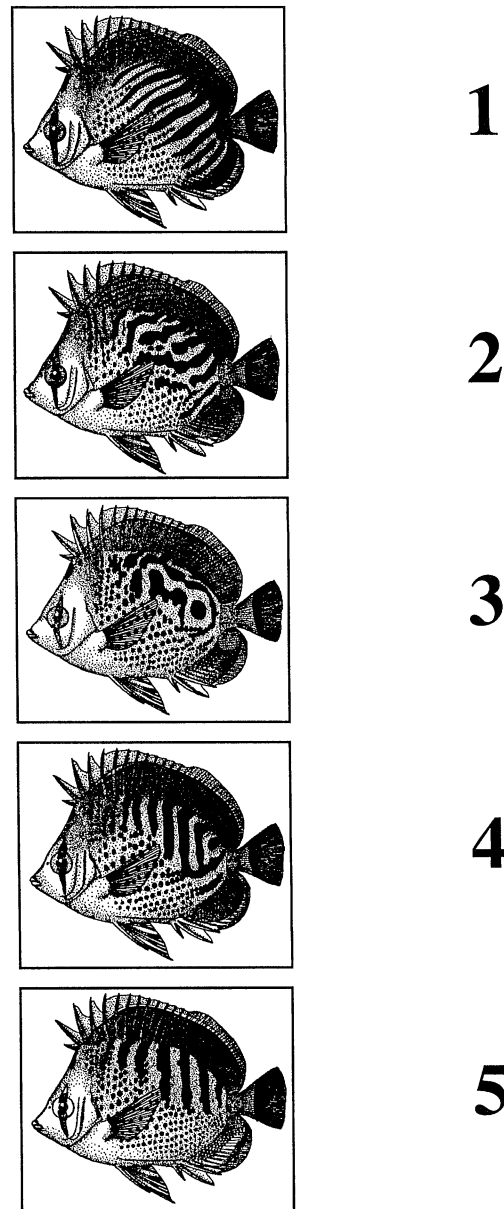


FIG. 2. The five phenotypic classes for individuals observed on reefs in the Solomon Islands and Papua New Guinea.

Ξ_{ij} was the conditional probability of pairing between a female with an i -type color pattern and a male with a j -type color pattern; (2) the frequency of adults in the population was a good estimation of the probability of encounter, and (3) there was no sex bias in color pattern (i.e., females or males are not more likely to have a particular color pattern). Under these assumptions, the simplest conditional mating model was:

$$\Xi_{ij} = \frac{x_{ij}(Q_i)(Q_j)}{\sum x_{ij}(Q_i)(Q_j)}, \quad (1)$$

where Q_i was the proportion of females with an i -type color

pattern within a given area, Q_j was the proportion of males with a j -type color pattern within a given area, and x_{ij} was a mate choice parameter. The support (the natural logarithm of the likelihood, $\ln L$) for the different mating parameters is given by:

$$\ln L(x_{11}, x_{12}, x_{13}, \dots, x_{55}) = n_{11} \ln \bar{x}_{11} + n_{12} \ln \bar{x}_{12} + n_{13} \ln \bar{x}_{13} + \dots + n_{55} \ln \bar{x}_{55}, \quad (2)$$

where n_{ij} is observed numbers of pairs between individuals with an i and j color pattern phenotype (see Fig. 2). Likelihood values for the different mating parameters were estimated by maximizing the overall support.

The difference in support ($\Delta \ln L$) between hypotheses of different mating parameter values or between hypotheses that differed in the number of parameters was used as a measure of inference (Edwards 1972); $2\Delta \ln L$ is distributed approximately as a χ^2 under the null hypothesis, where the degrees of freedom are the numbers of parameters allowed to vary (Edwards 1972). The support limits for a particular parameter (asymptotically equivalent to 95% confidence limits [Edwards 1972]) are given by the values of that parameter for which the $\Delta \ln L$ drops below the 5% critical value of a χ^2 distribution (Edwards 1972; see also McMillan et al. 1997).

Phylogenetic and Population Analysis (mtDNA)

We sequenced the tRNA^{pro} end of the mitochondrial control region of 138 individuals of *C. multicinctus* ($n = 31$), *C. pelewensis* ($n = 51$), and *C. punctatofasciatus* ($n = 56$) from 10 localities across the tropical Pacific (see Fig. 5 for species ranges, collection localities, and sample sizes). All sequences were obtained from double-stranded templates amplified via PCR using a 12s rRNA primer (12sar) (5'-ATAGTGGGG-TATCTAATCCCAGTT-3') and a *Chaetodon* specific control-region (C.R.-1) (5'-ACCATATTATGTACTAGGCAC-3') under specifications described in McMillan and Palumbi (1997). All sequences were aligned by eye and phylogenetic trees were constructed with *C. guttatissimus* as an outgroup (McMillan and Palumbi 1995) using both maximum parsimony and neighbor-joining distance methods.

We explored the structuring of mitochondrial variation across the Pacific using analysis of molecular variation (AMOVA) and by examining the relationship between the age of a mtDNA lineage and its geographic distribution (Excoffier et al. 1992; Neigel and Avise 1993). For our AMOVA, we used the absolute number of sequence differences between mtDNA haplotypes as our Euclidean distance. Components of genetic variation were considered to be significantly different from random if the observed variance was greater than 95% of 500 values produced by randomly shuffling individuals among demes (program courtesy of L. Excoffier). MtDNA lineage age was estimated for a UPGMA tree constructed from a pairwise distance matrix corrected for multiple substitutions using a Kimura two-parameter model with transversions weighted 20 times transitions (Kimura 1980). The variance in the geographic distribution of mtDNA age classes was compared to 500 values obtained when geographic position was shuffled randomly onto the original UPGMA tree (see Neigel and Avise 1993; program courtesy of J. Neigel).

Genetic Differences at Allozyme Loci

To compliment our mtDNA dataset, we examined allozyme variation from approximately 30 individuals of each of the three species using standard starch gel electrophoresis (Murphy et al. 1990). All 30 individuals were taken from phenotypically homogeneous populations at the approximate center of each species' geographic distribution. The sampling locations were as follows: *C. multicinctus*, Molokai, Hawaii ($n = 32$); *C. punctatofasciatus*, the Philippines ($n = 30$); *C. pelewensis*, Viti-Levu, Fiji ($n = 27$).

The heart, liver, eyes, brain, and a small portion of skeletal muscle were homogenized in two volumes of grinding buffer (0.25M sucrose, 2% phenoxyethanol) and centrifuged at 12,000 rcf for 10 min at 4°C. The resulting supernatant was removed, absorbed onto filter wicks, and electrophoresed through slabs of 11% polymerized starch (Sigma) using four different running buffers (TC, CT, RW, and TVD). Thin slices of starch gels were stained for 19 different enzyme systems that yielded a total of 31 distinct loci. Following staining, gels were photographed and allelic variation was scored alphabetically in order of decreasing anodal mobility of the protein produced.

Allele frequencies at polymorphic loci were determined and genotype frequencies at polymorphic loci were tested for conformity to Hardy-Weinberg expectations. Nei's (1978) unbiased genetic distances (D) were calculated between all species. Computations were performed using BIOSYS-1 (Swofford and Selander 1981).

RESULTS

Laboratory Pairing Experiments

In the 55 experiments where a female was presented with two males, a single male/female pair was always formed. This pair spent the majority of the time swimming and feeding in close proximity to each other (Table 1).

In conspecific controls ($n = 4$ for each of the three species), there were no differences in the strength of pair bond or behavioral interactions among the three different species. Pairs spent on average 90% of the observational period feeding and swimming in close proximity to each other (Table 1, row A). In contrast, females associated with the other male less than 1% of the time in our control trials. The most spectacular result of our control experiments was the observation of very strong aggression among the conspecific males. On reefs, intra- and interspecific aggressive interactions among butterflyfishes are rare (Ehrlich et al. 1977); however, in our tank experiments, male-male aggression was violent in all trials where contact between the two males was observed. The "paired" male was clearly dominant and attacked the "secondary" male whenever he came within visual contact (Table 1, row A). Aggressive behavior included chases, bites, and spears with extended dorsal spines. Females in these trials exhibited very little aggression toward either male (Table 1, row A). Within the size range used, there was no indication that size, as measured in weight or standard length, was a good predictor of pairing ($P > 0.25$, paired t -test).

Trials with a single male *C. pelewensis* and male *C. punctatofasciatus* and either a female *C. punctatofasciatus* ($n =$

TABLE 1. Summary of the pair-bonding experiments with *Chaetodon multicinctus* (*multi*), *C. punctatofasciatus* (*pun*), and *C. pelewensis* (*pele*). Results from the four or five 10-minute observational periods were averaged to characterize a trial. Means with 95% confidence intervals based on a *t*-distribution for (1) the length of time pair was together; (2) levels of male-male aggression; and (3) levels of female aggression for the four different types of experiments were calculated using the mean values from separate trials.

Experiment ¹	Proportion of hetero-specific pairs	Time pair associated (%) ²	Time female associated w/secondary male (%) ²	Trials where contact b/w pair and secondary male observed (#)	Male-male aggression (%) ³	Female aggression toward secondary male (%) ³
A. Controls (12)	—	90.0 ± 7.2	0.4 ± 0.4	9	81.2 ± 11.2	0
B. <i>pun/pele</i> (18)	44.4	84.7 ± 8.1	6.5 ± 8.2	13	70.6 ± 15.8	8.8 ± 9.5
C. <i>pele/multi</i> (12)	8.3	86.6 ± 8.5	25.6 ± 16.4	11	29.0 ± 27.0	0
D. <i>pun/multi</i> (13)	23.1	73.6 ± 11.7	13.3 ± 13.1	10	30.0 ± 24.8	0

¹ Number of trials is given in parentheses.

² Associated is defined as within 0.25 m to each other.

³ Levels of male-male aggression and female-male aggression were calculated as the proportion of time that individuals were in contact and involved in aggressive displays.

9) or a female *C. pelewensis* ($n = 9$) were qualitatively and quantitatively similar to controls (Table 1, row B). A single pair always formed and spent most of the observational period together and away from the “secondary” male (Table 1, row B). As in control trials, male-male aggression was intense and occurred in all 13 experiments where contact between males was observed. However, in these trials there was no evidence for assortative pairing based on color pattern: heterospecific pairs occurred in nearly half the 18 trials (Table 1, row B; $\chi^2 = 0.277$, $P = 0.27$, based on 500 randomizations [Roff and Bentzen 1989]). As in experiments with conspecifics, male dominance dictated pairing. Which male was dominant was apparently not mediated by female color pattern. In several trials ($n = 5$, data not presented), when the male *C. pelewensis* and *C. punctatofasciatus* were used in a second trial with a different female, the original dominance hierarchy was maintained and the new female paired with the dominant male. Weight or standard length was also not a good predictor of which male was paired ($P > 0.25$, paired *t*-test). However, six of eight heterospecific pairs involved a male *C. punctatofasciatus* and female *C. pelewensis* which possibly indicates some association between color pattern and aggressive superiority.

By contrast, the Hawaiian endemic, *C. multicinctus*, exhibited higher levels of behavioral isolation (Table 1, row C and D). In *multicinctus* × *pelewensis* trials (six using a female *C. multicinctus* and six using a female *C. pelewensis*) only one heterospecific pair formed ($\chi^2 = 7.543$; $P = 0.001$, based on 500 randomizations; Table 1, row C). Moreover, aggressive interactions among males in these trials were different from conspecific controls. In general, there was significantly less male-male aggression relative to controls with conspecifics (Mann-Whitney $U = 13$, $P = 0.005$, $df = 19$; Table 1, row C). In most of the trials where contact between males was observed, there was no, or only very slight, aggression. However, this response was not fixed and in four of the 11 trials where contact between males was observed, levels of aggression were high and identical to what was observed in conspecific trials.

A similar, but slightly more complex, pattern was evident

in our 13 (seven with a female *C. punctatofasciatus* and six with a female *C. multicinctus*) *multicinctus* × *punctatofasciatus* trials (Table 1, row D). Heterospecific pairs formed in three of these 13 trials, which indicates a slightly weaker association between color pattern and assortative pairing ($\chi^2 = 3.343$, $P = 0.06$, based on 500 randomizations; Table 1, row D). In every case, the heterospecific pair formed between a female *C. punctatofasciatus* and a much larger male *C. multicinctus*. As in the *pelewensis* × *multicinctus* trials, there were also significant differences in the levels of male-male aggression compared to conspecific controls (Mann-Whitney $U = 8.5$, $P = 0.003$, $df = 18$; Table 1, row D), although again, the response was bimodal; either aggression was very strong or nonexistent.

Field Observations

The small sample sizes and artificial conditions of our tank experiments makes conclusions about the association between color pattern and pair formation very tenuous. Fortunately, for *C. pelewensis* and *C. punctatofasciatus* it is possible to use natural pairs found in the contact zone to examine the association between color pattern and mate choice. One of the most striking features of the contact zone between the two was the extraordinary amount of previously unreported phenotypic variation. Nearly 70% of the 306 adults observed in the Solomon Islands (SI) had phenotypes intermediate between the two parental classes (Fig. 3). Roughly a third of these intermediate phenotypes could not easily be allied with either parental phenotype (type 3 in Fig. 2). Extensive phenotypic variation was also found in the northeast tip of Papua New Guinea (PNG). However, on these reefs, pure *C. punctatofasciatus* phenotypes dominated, but “hybrid” phenotypes still made up over a third of the individuals observed (Fig. 3).

The lack of color pattern-based mate selection between *C. pelewensis* and *C. punctatofasciatus* in our tank experiments was corroborated by observations on reefs in the SI and PNG. Eighty-five percent of the 404 adults observed were paired and there was no association between color pattern and

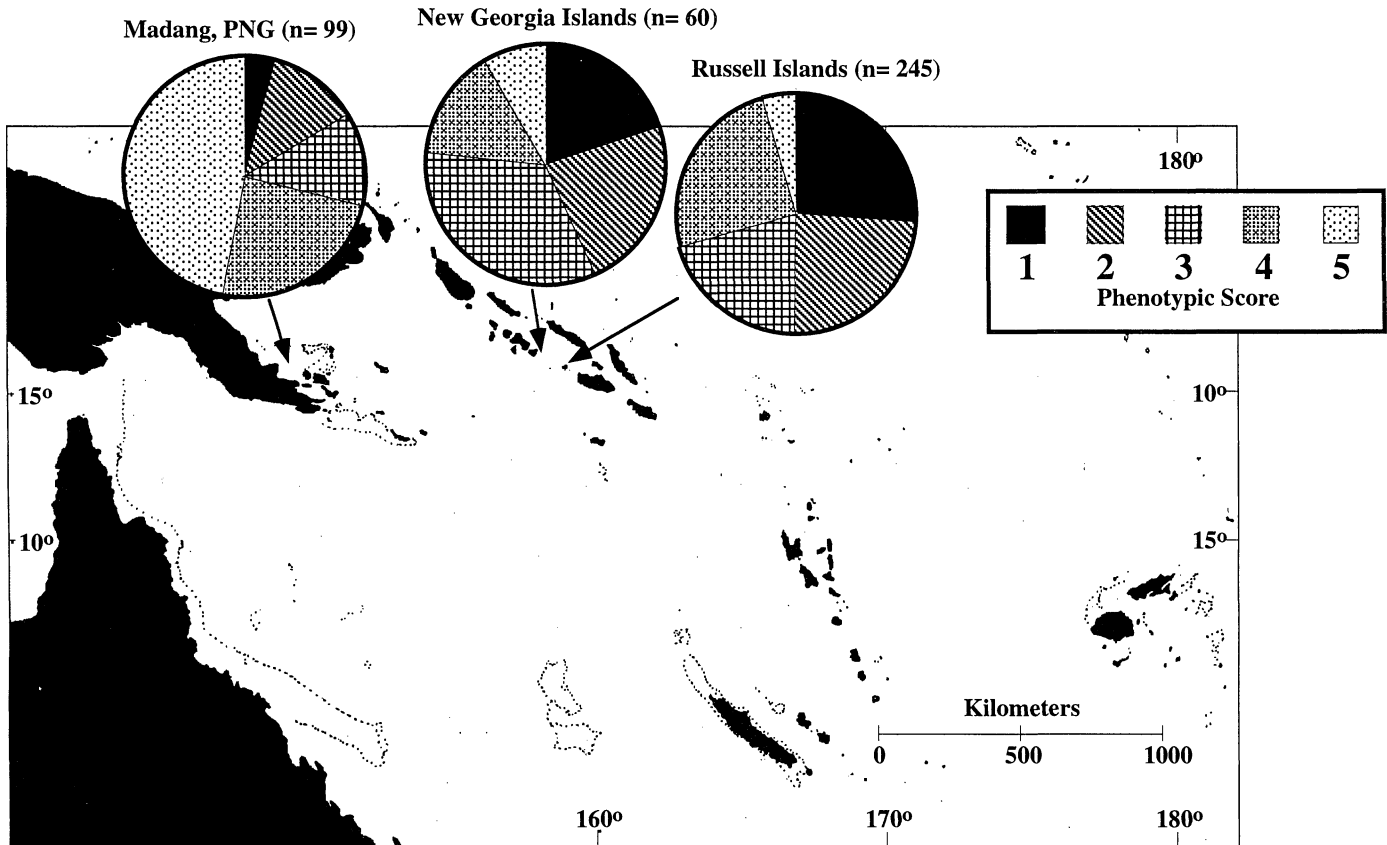


FIG. 3. Distribution of color pattern variation in areas of overlap between *Chaetodon punctatofasciatus* and *C. pelewensis*. See Figure 2 for phenotypic classes.

whether an individual was paired ($\chi^2 = 3.04$, $P > 0.50$, $df = 4$). Moreover, we found no compelling evidence for assortative pairing based on color pattern in either location. Many of the 171 pairs observed (128 in the SI and 43 in PNG) were between individuals with very different color pattern phenotypes. Table 2 shows the relative probability of pairing between the five phenotypic classifications on reefs within the Solomon Islands. The maximum likelihood estimates for all assortative mating parameters were similar and not significantly different from one, the expected value under a model of complete assortative mating. The lack of any color pattern-based assortative mating was also evident in the PNG population (data not presented). In the full model, in which all x_{ij} s were estimated separately in the two locations, the mating probability (x_{ij}) for all pairing combinations was high and similar to the probability of pairing between two "pure" *C. pelewensis* (x_{11}) or two "pure" *C. punctatofasciatus* color patterns (x_{55}). Indeed, our field observations were equally compatible with the simplest mating model in which individuals paired randomly with respect to color pattern ($2\Delta\ln L = 14.6072$; $df = 27$; $P > 0.5$).

We used a similar likelihood approach to test if individuals with similar color patterns (i.e., 1-1, 1-2, 2-2, 2-3, 3-3, 3-4, 4-4, 4-5, 5-5 pairs) were more likely to be paired than individuals with different color patterns (i.e., 1-3, 1-4, 1-5, 2-4, 2-5, 3-5 pairs). Again, there was no evidence for color pattern-based assortative mating. The probability of pairings

between unlike phenotypes was identical relative to those between similar patterned fishes. In this case, the maximum likelihood estimation of the pairing parameter between unlike patterned fishes was 1.02 relative to the probability of pairing between similar patterned fishes, with support limits of 1.38 and 0.74.

Phylogenetic and Geographic Patterning of mtDNA Variation

Sequence variation within the tRNA-proline end of the mitochondrial control-region was high. Nearly all of the 138 individuals examined possessed a unique and, often quite distinct, DNA sequence. However, the majority of differences across this 195-base region were single-base transitions and sequences could be readily aligned by eye (see McMillan and Palumbi 1997).

In both parsimony and neighbor-joining trees, control-region variation clustered into three major phylogenetic groupings (A, B, and C) that were defined by five to 15 changes (Fig. 4). Of the three clades, bootstrap support for clade C was the most robust. Branches leading to clade A and B were supported in 70% or fewer bootstrap replications of neighbor-joining trees constructed from a Kimura-corrected distance matrix (Fig. 4). However, sites free to vary across this region saturate very quickly and multiple changes at the same nucleotide position probably eroded bootstrap

TABLE 2. Relative probability of pairing between the five phenotypic classifications on reefs within the Solomon Islands. To calculate the different mating parameters, x_{11} was set to one and the 14 remaining x_{ij} s estimated so that the overall $\ln L$ was maximized (see Methods). Pair data from the New Georgia and the Russell Islands were combined because there were no significant differences in the distribution of phenotypes between reefs in the two areas ($\chi^2 = 6.359$, $P > 0.15$, $df = 4$).

Pair	Observed pairings	Maximum likelihood estimation of x_{ij} ¹	Expected pairings (full model) ²	Expected pairings (random mating) ³
1-1	10	1	9.7	8.0
1-2	13	0.70 (1.19, 0.37)	13.1	15.3
1-3	12	0.67 (1.16, 0.35)	12.0	14.6
1-4	13	0.73 (1.25, 0.39)	13.1	14.6
1-5	3	0.73 (1.95, 0.18)	3.0	3.4
2-2	7	0.78 (1.56, 0.32)	7.0	7.3
2-3	12	0.70 (1.22, 0.36)	12.0	14.1
2-4	17	0.99 (1.60, 0.57)	17.0	14.1
2-5	4	1.02 (2.45, 0.31)	4.0	3.2
3-3	11	1.33 (2.37, 0.67)	11.0	6.7
3-4	13	0.79 (1.35, 0.42)	13.0	13.5
3-5	3	0.80 (2.11, 0.19)	3.0	3.1
4-4	5	0.61 (1.35, 0.21)	5.0	6.7
4-5	4	1.06 (2.53, 0.32)	4.0	3.1
5-5	1	2.32 (10.46, 0.13)	1.0	0.4

¹ Upper and lower support limits, in parentheses, were values of x_{ij} that caused $2\Delta \ln L$ to fall below the 0.05 critical value of a χ^2 distribution with one degree of freedom (see Edwards 1972).

² Expected values were calculated using the maximum likelihood estimation of the different mating parameters.

³ Expected values were calculated assuming that all x_{ij} were equal.

confidence for these clades (McMillan and Palumbi 1997). Both of these lineages were extremely diverse (average corrected pairwise divergence was 7.8% within clade A and 9.6% within clade B; Table 3) and multiple substitutions at the same nucleotide position clearly occurred. For example, within clade B, variable nucleotide positions changed on average 2.8 times in the most parsimonious arrangements of individuals. Nonetheless, average genetic differences within these clades were 2.3–3.1 times smaller than average differences between clades, which reinforces the impression that mtDNA variation was grouped into three distinctive evolutionary clusters. The very rapid rate of control-region evolution also made determining the evolutionary relationships among these three clades impossible (McMillan and Palumbi 1997).

Other than a fivefold reduction in genetic variation within clade C relative to clade A and B (Table 3), the pattern of branching events within the three clades was similar. There was no evidence for further clade structure within the three lineages. Even within the highly diverse clades, A and B, there was little phylogenetic resolution. Indeed, in these lineages, as in lineage C, the internal branches were shorter than terminal branches generating a distinctive star-shaped phylogenetic pattern. As an example, in clade B there was on average 1.3 changes along any of the $(n - 2)$ -terminal nodes relative to an average of over three changes occurring along the n -terminal branches. Consistent with a star phylogeny, the frequency distribution of all pairwise comparisons within each clade was clearly “unimodal” and plots of the number

of lineages through time were linearized after “epidemic” transformations (data not shown; see Slatkin and Hudson 1991; Rogers and Harpending 1993; Nee et. al 1995; Rogers 1995).

The degree of behavioral isolation among the three species evident in our pairing experiments and observations of contact zones between *C. punctatofasciatus* and *C. pelewensis* was matched by the history of female gene flow deduced from our mtDNA phylogeny. Only *C. multicinctus* showed a phylogenetic grouping consistent with its species-level status. Twenty-six of 31 *C. multicinctus* possessed control-region sequences that fell within clade C, which makes this clade the only “species-specific” lineage. The remaining five *C. multicinctus* fell along three terminal branches in clade B (Fig. 4).

By contrast, individuals of *C. pelewensis* and *C. punctatofasciatus* fell randomly within the remaining two clades. The complete mixing of *C. pelewensis* and *C. punctatofasciatus* color patterns on the mtDNA tree was evident both between and within clades. Thus, whether a fish possessed a control-region sequence that fell within clade A or B was not a good predictor of an individual’s color pattern ($\chi^2 = 1.5$; $P > 0.20$, $df = 1$). Even more noteworthy was the observation that within the two clades very similar control-region sequences were shared between the color forms. For example, within clade B, there are 12 instances where a *C. pelewensis* and *C. punctatofasciatus* fell together at the terminal node of our neighbor-joining tree (* in Fig. 4). Across the entire tree, branch lengths of these terminal “heterospecific” nodes were identical to lengths of terminal branches containing individuals of the same color pattern phenotype ($t = 0.922$; $P = 0.36$, $df = 30$).

In light of the complete mixing of *C. pelewensis* and *C. punctatofasciatus* color patterns within the mtDNA clades A and B, the geographic patterning of these clades across the west and south Pacific was curious. At one level, there was no relationship between the age of a mtDNA lineage and its geographic distribution within either clade A or B ($P > 0.25$ for both longitude and latitude). Thus, even the youngest mtDNA lineage classes were broadly distributed across the tropical west Pacific and generated a pattern in which only 3.0% of mitochondrial variation was partitioned among populations across this vast area. Despite this pattern, the two mitochondrial lineages were not distributed randomly ($\chi^2 = 13.8$, $P = 0.035$, $df = 6$). This difference was due to the higher than expected proportion of clade B in the Tahitian samples ($n = 11$) and clade A in the Indonesian samples ($n = 6$). When these two peripheral populations were eliminated, there was no evidence for genetic structuring of within or between west and south Pacific populations of *C. pelewensis* and *C. punctatofasciatus* ($\chi^2 = 4.72$, $P = 0.325$, $df = 4$).

Distribution of Allozyme Variation

The distribution of genetic variation among the three species was similar at allozyme loci. Genetic differences between the three were slight. Of the 31 loci examined, 12 were monomorphic across all three species and nine showed only slight variation. Significant polymorphism (major allele at less than

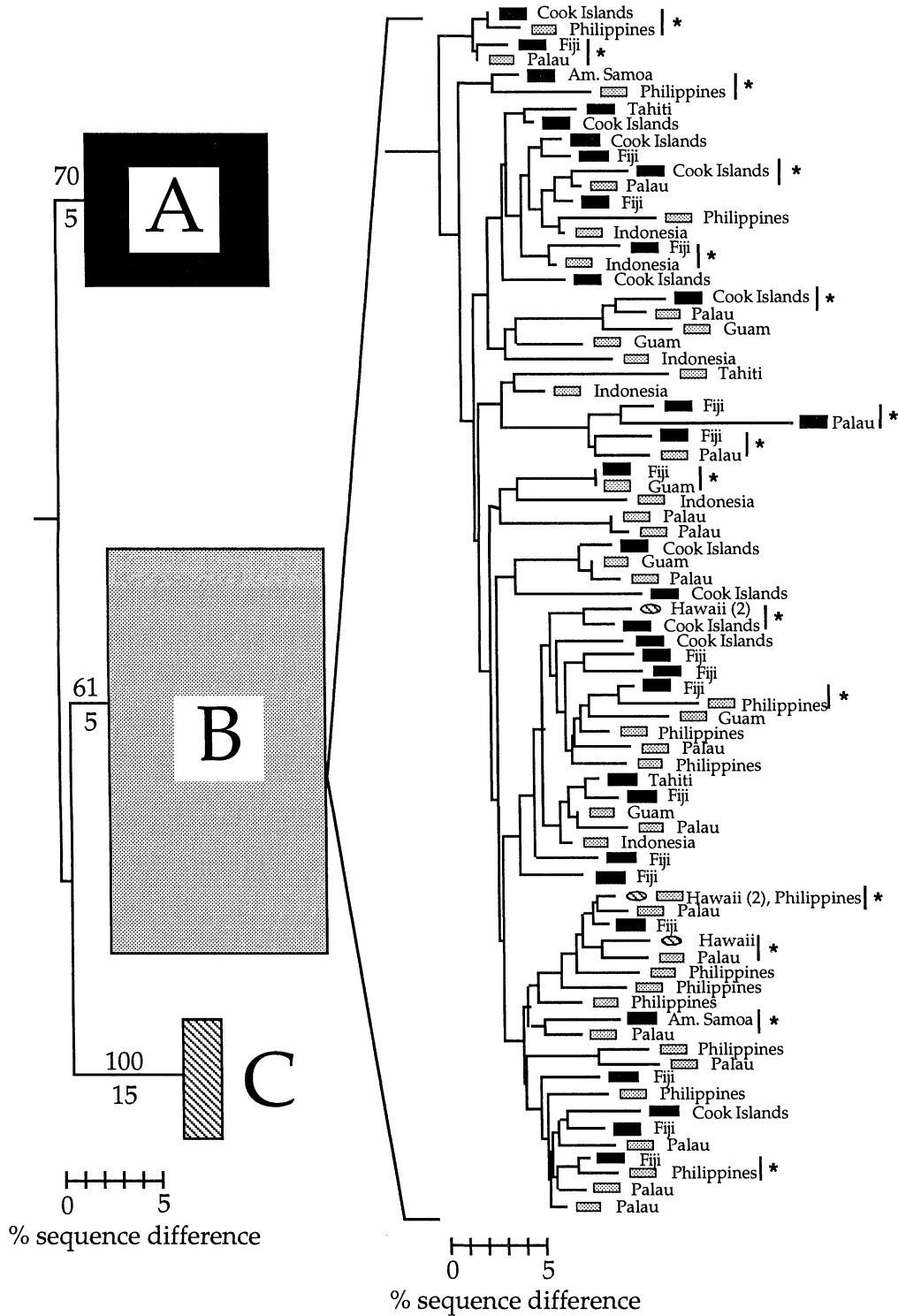


FIG. 4. Neighbor-joining illustration of the three major mtDNA lineages. The size of each shaded box is proportional to the average pairwise genetic distances (width) and the number of individuals (height) within each of the three major lineages. Genetic distances between individuals used in the construction of this tree were corrected for multiple hits using a Kimura two-parameter model (Kimura 1980). In this tree, transitions were weighted 20 times transversions. Support for each clade is given by the percentage agreement in a consensus of trees produced from 100 bootstrap simulations of the dataset (above branch) and the number of informative nucleotide changes (below branch). *Chaetodon guttatissimus* was used as the outgroup to root this tree. The telescoped portion represents the relationships among the 81 individuals of *C. punctatofasciatus* (shaded boxes), *C. pelewensis* (black boxes), and *C. multinctus* (shaded circles) within clade B. Terminal nodes highlighted by an asterisk unite fishes with different color patterns. Clade A showed a similar mixture of *C. punctatofasciatus* and *C. pelewensis*, while Clade C contained only *C. multinctus*. Neighbor-joining trees constructed using distances estimated by Jukes and Cantor (1969), maximum likelihood by Felsenstein (1995), and Tamura and Nei (1993) methods yielded

0.95 frequency) was evident at the remaining 10 loci. The only significant deviation ($P < 0.05$) of genotype frequencies from Hardy-Weinberg equilibrium occurred at the PEP A locus in Fijian populations of *C. pelewensis*. However, because at least one deviation was expected to occur by chance in our 39 χ^2 tests, we attributed this observation to sampling artifact rather than a biologically meaningful pattern.

There were no fixed allelic differences among the three species at the polymorphic allozyme loci (Table 4). However, *C. multinctus* was clearly genetically distinct from its two sisters and showed a Nei's D of 0.040 and 0.046 from *C. punctatofasciatus* and *C. pelewensis*, respectively. At two loci, *Gpi-2* and *Mdh-2*, the dominant allele in Hawaiian populations of *C. multinctus* was found in very low frequency within *C. punctatofasciatus* and *C. pelewensis*. At other polymorphic loci, *C. multinctus* showed much smaller levels of variation and had significantly lower levels of heterozygosity relative to its two siblings.

By contrast, *C. punctatofasciatus* and *C. pelewensis* were nearly identical at allozyme loci. Nei's D between these two species was very slight (about 0.002). The largest difference in the allele frequency occurred at the *Mdh-2* locus. Within *C. pelewensis*, the "A" allele occurred at a frequency of nearly 34% within Fijian populations. In contrast, in Philippine populations of *C. punctatofasciatus* that same allele reached a frequency of less than 10% (Table 4). However, at other polymorphic loci, allele frequencies were nearly identical despite the nearly 7500 km separating populations of the two species.

DISCUSSION

Of three incipient allopatric/parapatric color-pattern species, only *C. multinctus* shows concordant differences in morphology, genetics, and pairing behavior. Although some of the mtDNA distinctiveness of this species may be explained by its geographic isolation, color pattern change is associated with differences in pairing behavior and interspecific aggression (Table 1, row C and D). For the comparably distinctive *C. punctatofasciatus* and *C. pelewensis*, however, color pattern changes are neither associated with divergence in mate recognition signals nor genetic divergence at mtDNA or allozyme loci. These results make it unlikely that color pattern change in this group has been mediated by signals that specifically promote mate recognition. Instead, the increased reproductive isolation we observe in *C. multinctus* probably evolved as an incidental consequence of color pattern divergence, which in turn led to increased genetic divergence at neutral genetic loci. This sequence of events would be impossible to document without comparisons among all three species pairs.

Despite the absence of color pattern-based mate choice or mtDNA and allozyme differentiation, color pattern differences between *C. pelewensis* and *C. punctatofasciatus* remain

TABLE 3. Average percent genetic difference of all pairwise comparisons of individuals (1) within clades (along diagonal); (2) between clades uncorrected (below diagonal); and (3) between clades corrected (above diagonal). Corrected differences were calculated by subtracting the average within-clade variation from the average between-clade variation (Wilson et al. 1985). Genetic distances between individuals were corrected for multiple hits at the same nucleotide position using a Kimura two-parameter distance correction with transversions weighted 20 times transitions (Kimura 1980).

	A	B	C
Clade A	7.8	9.4	20.4
Clade B	17.6	8.6	16.4
Clade C	25.1	21.5	1.6

true across most of the south and west Pacific (Fig. 5). For example, Philippine populations of *C. punctatofasciatus* and Fijian populations of *C. pelewensis* show no differences in the distribution of mtDNA or allozyme variation, but have completely different color patterns (Fig. 5). Two possible scenarios could explain this pattern. First, this pattern could be explained by differences in fixation times among loci that are under different selective pressures. Thus, rapid directional selection on color pattern could have fixed color pattern differences while retaining much of the mtDNA and allozyme variation present in the ancestral population (Neigel and Avise 1986; Moran and Kornfield 1993; McMillan and Palumbi 1995, 1997). Second, it is possible the two distinctive color patterns emerged in the more distant past and that hybridization has permitted mtDNA and allozyme loci to move across the color pattern boundary. In this case, the discrepancy between mitochondrial and allozyme markers and color pattern indicates that selection is preventing the erosion of color pattern differences outside the region of contact in the face of homogenizing levels of gene flow.

These two competing evolutionary scenarios are extremely difficult to distinguish when differentiation has occurred very recently. At the latest, the color pattern differences that define *C. pelewensis* and *C. punctatofasciatus* must have evolved within the last one to two million years because the two diverged from their Indian Ocean sister (McMillan and Palumbi 1995). However, the evolution of these two forms is probably far more recent. For example, assuming the mtDNA control-region evolves at 33–100% per million years, then the observed color pattern differences must have evolved within the last 20,000–50,000 years, under the ancestral variation scenario, or within the last 80,000–270,000, under the hybridization scenario (McMillan and Palumbi 1997). Indeed, much of the similarity at allozyme loci between *C. pelewensis* and *C. punctatofasciatus* may be due to retained ancestral variation. Even in *C. multinctus*, which shows strong mtDNA differentiation, there are only slight frequency differences at allozyme loci, which generate a small, but sig-

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similar topologies. Trees using parsimony under various weighting schemes were likewise similar with one notable exception. In trees constructed with transversions weighted 20 times transitions, individuals in clade A clustered together at the base of the suite of most parsimonious trees, but failed to group within a defined lineage. Consistency and retention indexes for parsimony trees were 0.25 and 0.77, respectively.

TABLE 4. Allele frequencies for the 19 variable loci in *Chaetodon multincinctus*, *C. punctatofasciatus*, and *C. pelewensis*.

Locus	Allele	<i>C. multincinctus</i>	<i>C. punctatofasciatus</i>	<i>C. pelewensis</i>
<i>Aat-1</i>	A			0.043
	B	0.020		
	C	0.980	1.000	0.957
<i>Aat-2</i>	A		0.018	
	B	1.000	0.982	1.000
<i>Dia</i>	A	0.909	1.000	0.978
	B	0.091		0.022
<i>Est-1</i>	A	0.045	0.161	0.177
	B	0.955	0.732	0.694
	C		0.089	0.097
	D		0.018	0.016
	E			0.016
<i>Est-2</i>	A	0.076		
	B	0.924	0.982	1.000
	C		0.018	
<i>Est-3</i>	A	1.000	0.963	0.968
	B		0.037	0.032
<i>G6pd</i>	A	0.280	0.278	0.261
	B	0.700	0.667	0.696
	C	0.020	0.056	0.043
<i>Gpi-1</i>	A	0.985	0.946	0.935
	B	0.015		0.032
	C			0.016
	D		0.054	0.016
<i>Gpi-2</i>	A		0.036	
	B		0.607	0.758
	C			0.048
	D	1.000	0.357	0.194
<i>Icd-2</i>	A	0.879	1.000	1.000
	B	0.121		
<i>Me-1</i>	A	0.020		
	B	0.980	0.875	0.957
	C		0.100	0.043
	D		0.025	
<i>Me-2</i>	A	0.980	0.975	0.978
	B	0.020	0.025	0.022
<i>Mdh-1</i>	A			0.016
	B	1.000	1.000	0.984
<i>Mdh-2</i>	A	0.985	0.089	0.339
	B	0.015	0.911	0.661
<i>Pepb-2</i>	A	1.000	0.964	1.000
	B		0.018	
	C		0.018	
<i>Peps</i>	A	0.030	0.036	0.016
	B	0.970	0.964	0.984
<i>Pepa</i>	A		0.271	0.273
	B	1.000	0.708	0.727
	C		0.021	
<i>Pgm-3</i>	A			0.016
	B	1.000	0.982	0.984
	C		0.018	
<i>Sod</i>	A		0.018	1.000
	B	1.000	0.982	

nificant, Nei's *D* of 0.04. It is important to note that the overall level of allozyme variation we observe within this complex is similar to levels reported among conspecific populations

of marine fishes across the Pacific (Winans 1980; Rosenblatt and Waples 1986; Planes 1993).

With this caveat in mind, we argue that the phylogenetic and geographic patterning of mtDNA variation in *C. pelewensis* and *C. punctatofasciatus* is most consistent with a recent history marked by hybridization and extensive gene flow. First, the presence of three major mtDNA clades distinguished by many mutations suggests that color pattern differences and the major breaks in the mitochondrial phylogeny probably evolved together. The historical association between mtDNA clade and color pattern type is still evident in the strong geographic and species specificity of clade C with *C. multincinctus*. Second, the geographic pattern of mtDNA variation in *C. punctatofasciatus* and *C. pelewensis* is most easily understood under a scenario of hybridization and introgression. Although mtDNA lineages A and B are distributed randomly in much of the south and west Pacific, at the extreme of the range of *C. punctatofasciatus* and *C. pelewensis* there is a shift favoring one clade over another. Nearly 70% of our Tahitian *C. pelewensis* had a clade A mtDNA haplotype, whereas all of Indonesian *C. punctatofasciatus* had a clade B haplotype (Fig. 5). This pattern could have occurred by chance, but it more likely reflects the original association of the *C. pelewensis* and *C. punctatofasciatus* phenotypes with clade A and B, respectively. Third, the pattern of branching events within the two clades suggests a recent demographic history characterized by explosive population growth. In both clades, most of the branching events occurred toward the root of the lineage, which is similar to the pattern of human mtDNA variation and some viral genome sequences (Di Rienzo and Wilson 1991; Slatkin and Hudson 1991; Harpending et al. 1993; Rogers and Harpending 1993; Holmes et al. 1995; Rogers 1995). This demographic setting, coupled with the high rates of gene flow necessary to explain the nearly random distribution of mtDNA variation in the south and west Pacific, would likely inhibit morphological differentiation rather than promote it.

Butterflyfishes and Butterflies

The behavioral, genetic, and phenotypic patterns between *C. pelewensis* and *C. punctatofasciatus* make it impossible to justify their continued species-level status under most current species definitions. There is no evidence for reproductive isolation (Mayr 1963), divergence in mate recognition signals (Paterson 1985), genetic cohesion (Templeton 1989), or multilocus genotypic differences (Mallet 1995). Indeed, these patterns most closely parallel those reported in hybrid zones among races of neotropical butterflies and North American birds (Mallet 1993; Moore and Price 1993). In zones of sympatry among color pattern races of *Heliconius* butterflies and between the red- and yellow-shafted flickers, *Colaptes auratus*, hybrid phenotypes are common and there is no evidence of mate choice based on color pattern. However, the transition between color forms, which varies from 10 to 100 km in butterflies and up to 300 km in flickers, is much narrower than expected given estimates of per-generation dispersal. In both cases, strong frequency-dependent selection, which is not directly related to mate choice, acts at the edges of the hybrid zone to maintain color pattern distinctions in

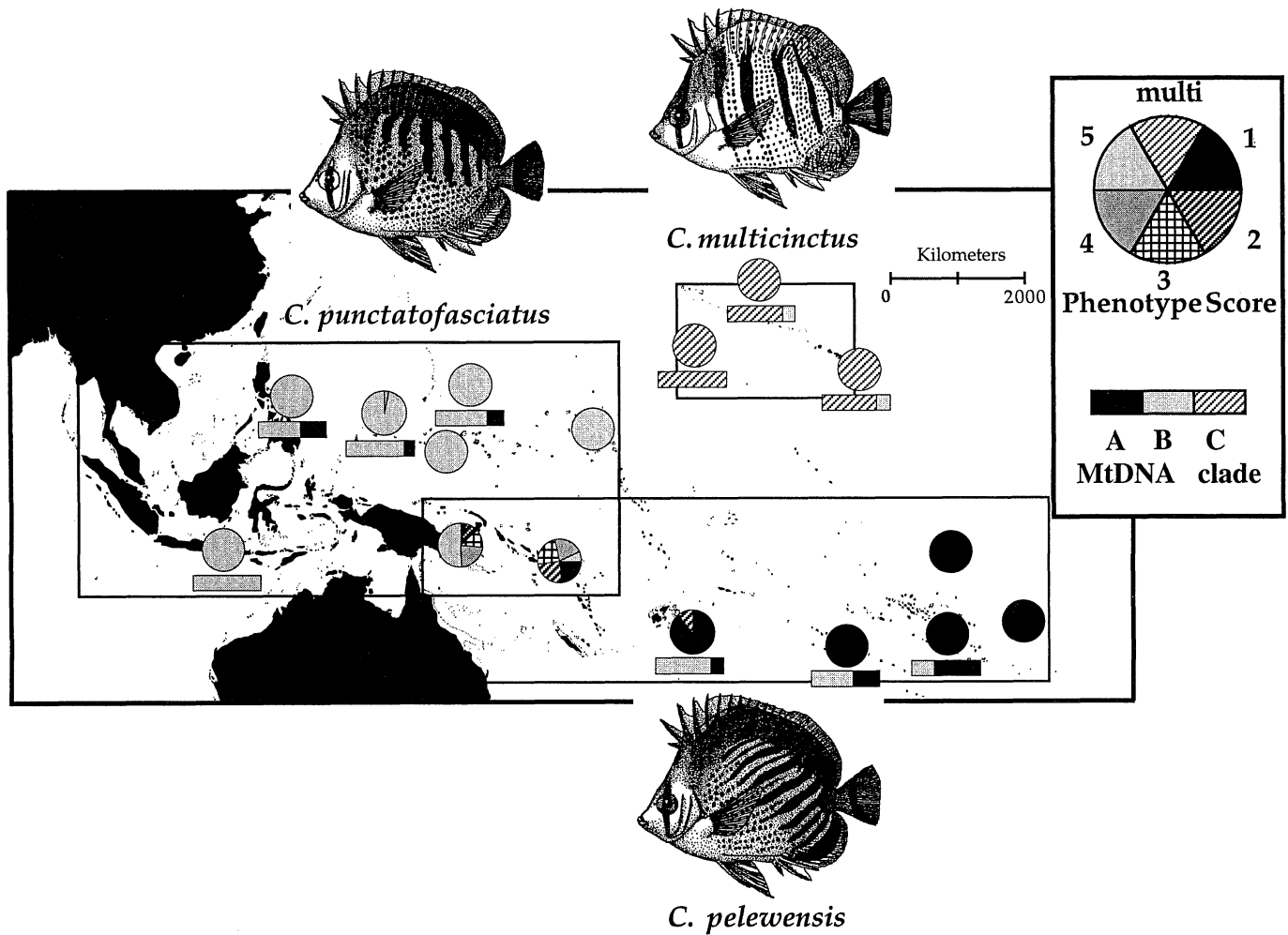


FIG. 5. Geographic distribution of phenotypic (circles) and mtDNA (rectangles) variation for *Chaetodon multicinctus*, *C. pelewensis*, and *C. punctatofasciatus*. The larger rectangles represent the approximate geographic range of each species (Blum 1988, 1989). For phenotypic variation, individuals were given a score based on deviations from the "pure" parental phenotypes (see Fig. 1). Pie diagrams for mtDNA represent the number of individuals from each sampling location that fell within each of the three major mtDNA lineages (Fig. 2): ■, clade A; □, clade B; ▨, clade C. Sampling location and sizes are as follows. For phenotypic variation: Midway Island ($n = 14$); Oahu, Hawaii ($n = 20$); Molokai, Hawaii ($n = 70$); Johnston Atoll ($n = 25$); Pitcairn Island ($n = 8$); the Marquesas ($n = 3$); Society Islands and Tuamotus archipelago ($n = 44$); Cook Islands ($n = 21$); Fiji ($n = 87$); Indonesia ($n = 12$); the Philippines ($n = 35$); Caroline Islands ($n = 5$); Palau ($n = 88$); Guam ($n = 11$); Marshall Islands ($n = 21$); the Solomon Islands ($n = 314$); and Papua New Guinea ($n = 90$). For mtDNA variation: Oahu, Hawaii ($n = 15$); Midway Island ($n = 11$); Johnston Atoll ($n = 6$); Society Islands ($n = 9$); Cook Islands ($n = 18$); American Samoa ($n = 2$); Fiji ($n = 21$); Guam ($n = 8$); Palau ($n = 20$); the Philippines ($n = 21$); and Indonesia ($n = 7$).

the face of extensive hybridization and gene flow (Mallet 1993; Moore and Price 1993).

A similar selection regime may operate on color pattern in these Pacific butterflyfishes. Under this model, the ability of a fish to survive and reproduce must depend on its color pattern relative to the color pattern of other fishes on the reef where it settles. For a hybrid zone maintained by frequency-dependent selection, selection acting on a gene involved in the determination of a polygenic trait is equivalent to $(2.8d/w)^2$, where d is the average dispersal, defined as the standard deviation in parent to offspring distance, and w is the width of the cline (Mallet and Barton 1989a). The transition between *C. pelewensis* and *C. punctatofasciatus* occurs over approximately 2400 km. This estimate is an order of mag-

nitude broader than either terrestrial example, but is similar to other marine hybrid zones (Bert and Harrison 1988; Quezada et al. 1995).

The much broader transition between the two color forms is probably the result of a much higher per-generation dispersal distance rather than weaker selection on color pattern. Estimates of per-generation dispersal in *Heliconius erato* are on the order of one kilometer (Mallet 1993). Even in flickers, which are migratory, most fledglings nest near the parents and adults show strong breeding site fidelity. These birds, likewise, show marked population structuring (Moore and Price 1993). In contrast, butterflyfish have larvae that drift in the plankton for 40–60 days before settling (Hourigan and Reese 1987). Pacific sea urchins with similar planktonic pe-

riods have genetic structure only over 1000–2000 km (Palumbi et al. 1997) and most fish with such larval periods exhibit similarly low levels of genetic structure (Winans 1980; Rosenblatt and Waples 1986). In this case, even the youngest mtDNA lineages are distributed randomly across the entire tropical west Pacific, which makes phylogenetic estimates of per-generation dispersal impossible (Neigel and Avise 1993). However, assuming a moderate per-generation dispersal distance of 200–500 km for butterflyfishes, then per-locus selective coefficients controlling color pattern would fall between 0.05 and 0.34. These values are similar to the values that maintain the distinctive geographic races of *H. erato* (Mallet and Barton 1989b).

Predation is a potent force in maintaining a narrow hybrid zone among races of *Heliconius* and may be playing a similar role in these brightly colored butterflyfishes. Butterflyfishes are heavily spined and it has been suggested that bright coloration advertises their unpalatability (Neudecker 1989). However, butterflyfishes are not obviously involved in mimicry complexes to the extent that occurs in warningly colored butterflies. It seems more probable that sexual selection, not directly related to mate recognition or mate choice, is inhibiting the breakdown of color pattern differences in *C. pelewensis* and *C. punctatofasciatus*. Strong intrasexual selection for territorial space, similar to that proposed for flickers, is a possible mechanism (West-Eberhard 1983). Strong intrasexual aggression was vividly manifested in our pairing experiments, where the outcome was determined by male-male encounters. Likewise, strong female-female aggression was observed ($n = 3$) in behavioral experiments with two females and a male. In our tank experiments, one-on-one encounters determined pairing; but on a coral reef, pairing is probably the result of complex interactions among many individuals. Under these conditions, it may be difficult for an oddly colored recruit to establish or defend a territory, perhaps because its coloration does not convey the appropriate signals to prevent repeated aggressive attacks. An analogous mechanism operates in redwing blackbirds and in swallowtail butterflies (Smith 1972; Lederhouse and Scriber 1996). In blackbirds, males lose their ability to defend breeding territories when their red epaulettes are blackened (Smith 1972). Reproduction in butterflyfishes is linked to territory acquisition, and competition for open territorial space is strong and mainly directed toward individuals of the same sex (Hourigan 1987, 1989). In butterflyfishes, both males and females compete for open space and intrasexual social pressures on color pattern would extend to both sexes.

Speciation in marine taxa with high dispersal potential, like butterflyfishes, is poorly understood (Palumbi 1994). Among species with complex behaviors and conspicuous color differences, it is tempting to ascribe rapid change in color pattern to the effects of selection for mate recognition similar to that envisioned for insects (Lambert and Spencer 1995). For this group of three incipient color forms, we demonstrated that color pattern evolution can occur prior to the evolution of assortative mating. Thus, we caution against the casual acceptance of a role of color pattern in species or mate recognition. When the color pattern change and species recognition are coupled, as they are in *C. multinctus*, genetic differences can quickly build up and persist. However, our

data also show that selective pressures, besides color pattern-based mate choice, can preserve “species-level” differences in color pattern in the presence of high levels of hybridization and introgressive gene flow.

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