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Genetic mosaic in a marine species flock

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Abstract

We used molecular approaches to study the status of speciation in coral reef fishes known as hamlets (Serranidae: Hypoplectrus). Several hamlet morphospecies coexist on Caribbean reefs, and mate assortatively with respect to their strikingly distinct colour patterns. We provide evidence that, genetically, the hamlets display characteristics common in species flocks on land and in freshwaters. Substitutions within two mitochondrial DNA (mtDNA) protein-coding genes place hamlets within a monophyletic group relative to members of two related genera (Serranus and Diplectrum), and establish that the hamlet radiation must have been very recent. mtDNA distances separating hamlet morphospecies were slight (0.6 ± 0.04%), yielding a coalescent estimate for the age of the hamlet flock of approximately 430 000 years. Morphospecies did not sort into distinct mtDNA haplotype phylogroups, and alleles at five hypervariable microsatellite loci were shared broadly across species boundaries. None the less, molecular variation was not distributed at random. Analyses of mtDNA haplotype frequencies and nested clades in haplotype networks revealed significant genetic differences between geographical regions and among colour morphospecies. We also observed significant microsatellite differentiation between geographical regions and in Puerto Rico, among colour morphospecies; the latter providing evidence for reproductive isolation between colour morphospecies at this locale. In our Panama collection, however, colour morphospecies were mostly genetically indistinguishable. This mosaic pattern of DNA differentiation implies a complex interaction between population history, mating behaviour and geography and suggests that porous boundaries separate species in this flock of brilliantly coloured coral reef fishes.

Keywords: coral reef fishes, hamlets, hybridization, microsatellites, mtDNA, speciation

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Introduction

Recent evolutionary radiations such as Darwin's finches and Rift Lake cichlids provide unique snapshots of the ongoing process of species formation. As model systems, these complexes yield many insights into the earlier phases of divergence between closely related, sympatric forms (e.g. Meyer 1993; Seehausen & van Alphen 1998; van Oppen *et al.* 1998), and provide clues as to to how incipient species maintain their integrity in sympatry (e.g. Grant 1993; Grant & Grant 1994). No similar model comes to mind for marine organisms. Although 'ancient' marine species flocks have been described (Wollenberg & Avise 1998; Johns & Avise 1998), it is difficult in such cases to distinguish the factors important in the earliest stages of radiation, from the background of evolutionary divergence that accumulates subsequently. One of the most striking examples of an apparently young marine radiation is the flock of distinctively coloured sea basses in the Caribbean reef fish genus *Hypoplectrus*.

Here, we bring molecular methods to bear on *Hypoplectrus* morphospecies, referred to commonly as hamlets, in an effort to better describe the phylogenetic and population

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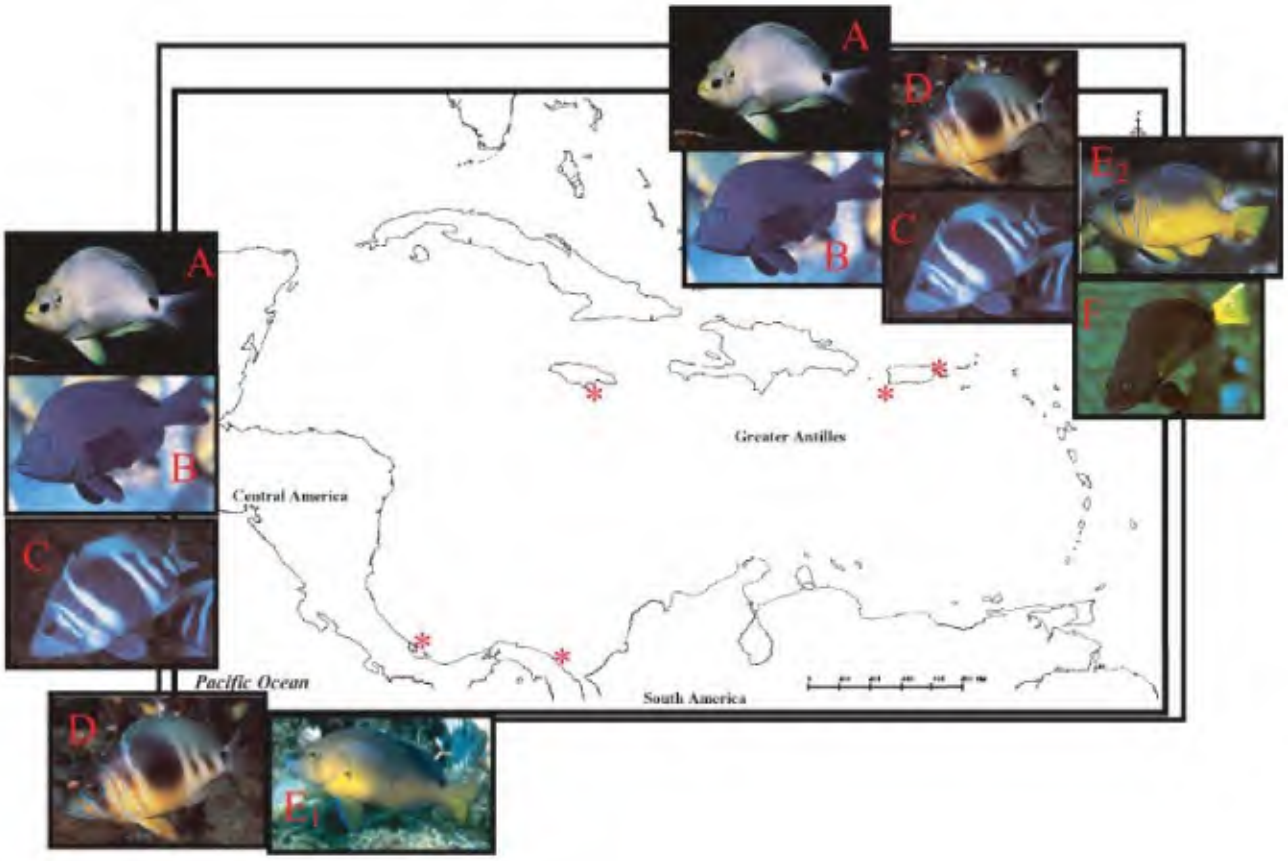

Fig. 1 Collection locations of hamlet species used in this study. Five species were collected from both Panama and Puerto Rico: (A) *Hypoplectrus unicolor* [Panama (n = 9), Puerto Rico (n = 13)], (B) *H. indigo* [Panama (n = 12), Puerto Rico (n = 21)], (C) *H. nigricans* [Panama (n = 52), Puerto Rico (n = 71)], (D) *H. puella* [Panama (n = 78), Puerto Rico (n = 45)], (E_1 and E_2, respectively) H. *aberrans* [Panama (n = 22), Puerto Rico (n = 18)]. (F) We collected another abundant species, *H. chlorurus* (n = 54), from Puerto Rico (it is absent in Panamá). In eastern Panamá, we collected in the San Blas Islands and 400 km west in Bocas del Toro. IN of Puerto Rico, we collected off the southwestern coast at Parguera and the east coast at Culebra (asterisks label collection sites). A smaller number of fish [*H. puella* (n = 5), *H. indigo* (n = 5) and *H. guttavarius* (n = 1)] were collected in Jamaica for mtDNA analysis. All photographs (except E1) by permission from Reef fish Identification, New World Publications, © 2002, Paul Humann.

genetic distinctiveness among members of the genus. Hamlets are restricted to the Caribbean and Western Atlantic, and comprise 10 recognized species. The different species, or colour morphospecies as they are termed, have identical skeletal features with broad overlap in habitat and diet (Fischer 1979, 1980), but show striking differences in colouration and pattern, ranging from golden yellow to iridescent blue with vertical stripes (Fig. 1). The colour pattern differences that define morphospecies are consistent across their range, which in some morphospecies spans the Caribbean, whereas others (such as the Florida endemic blue hamlet) have more restricted distributions (Domeier 1994). It is not uncommon to observe as many as six hamlets morphospecies co-occurring on a single reef.

Despite colour pattern differences, early ichthyologists pointed to morphometric similarity and occasional colour pattern 'intermediates' to support their description of a single species of hamlets (*H. unicolor*) that displays 'endless variations in colour' (Jordan & Evermann 1896). This view is supported by the only published analysis of genetic differences among the hamlet morphospecies, which reported extremely low levels of polymorphism and no fixed allelic differences between colour types at 32 allozyme loci (Graves & Rosenblatt 1980).

Many biologists have countered the polytypic single-species view of hamlets by noting that mating is strongly and positively assortative between the different colour morphospecies, even in the face of syntopic distributions (Fischer 1980; Domeier 1994). In the Caribbean, hamlets mate nightly throughout the year (Fischer 1979), and display elaborate courtship behaviours that commence about 90 min prior to sunset. Hamlets are simultaneous hermaphrodites that engage in obligate pair spawning, with the pair reciprocally swapping sex roles several times

during an evening's bout of spawning (Fischer 1981). Field observations demonstrate that 96% of the pairings are between like-patterned hamlet individuals (Fischer 1980). Mating is similarly assortative in aquarium choice experiments, although spawning will occur between different colour morphospecies in 'no-choice' trials (Fischer 1980; Domeier 1994). Colour pattern differences among hamlets almost certainly have a genetic basis. Offspring of matings between similarly colour-patterned hamlets resemble the parents, while the few successfully reared hybrids possess intermediate colour patterns unlike any known morphospecies (Domeier 1994).

This study centres its focus on genetic relationships among hamlets inferred from mitochondrial and microsatellite DNA. We used an experimental design that allowed us to judge the molecular distinctiveness of six hamlet colour morphospecies, and to gauge geographical differentiation between populations from the northeastern Caribbean (Puerto Rico) and southwestern Caribbean (Panamá). Collecting opportunities during the course of this investigation permitted us to broaden the scope of our study in several useful directions. A small collection of hamlets from Jamaica increased sample sizes for population genetic tests of differences among colour morphospecies. Collections of *Serranus*, a group of predominately Caribbean sea basses in the same subfamily (Serraninae) as *Hypoplectrus*, permitted comparison of these two Caribbean reef fish radiations. Lastly, analysis of two *Rypticus* (Serranidae, Grammistinae) species putatively split by Pliocene closure of the Panamanian isthmus provided a local calibration of mtDNA substitution rate. We used this to estimate, for the first time, the age of the hamlet radiation.

Materials and methods

The majority of the 412 hamlets examined in this study were collected from reefs in Puerto Rico ($n = 203$) and Panamá ($n = 193$). However, several individuals from Jamaica ($n = 11$) were also examined for mtDNA sequence variation. In addition, we sequenced mtDNA of several additional serranids, including *Serranus phoebe* ($n = 3$), *S. baldwini* ($n = 2$), *S. psittacinus* ($n = 3$), *S. tabacarius* ($n = 1$), *S. tigrinus* ($n = 1$), *S. tortugarum* ($n = 4$), *Diplectrum formosum* ($n = 1$), *Rytpicus saponaceus* ($n = 2$) and *R. bicolor* ($n = 2$) (Fig. 1). All fish were obtained by spear fishing and were identified in the field. Gill tissue was removed from fresh specimens and stored in salt-saturated DMSO/EDTA buffer (Seutin *et al.* 1993) at 4 °C. After removal of tissue, fish were preserved in formalin as voucher specimens and stored subsequently in 70–75% ethanol. Specimens used in this study have been accessioned at the Smithsonian Tropical Research Institute (STRI) (Bermingham *et al.* 1997b). Specimen names, locales, catalogue numbers and microsatellite genotype data for the fishes used in this study are also available upon request from the authors.

Genomic DNA was extracted from fin clips and gill tissue by standard methods (Sambrook *et al.* 1989). Two mitochondrial gene regions, one coding for the ATP synthase subunits VI and VIII (*ATPase 6/8*) and one for a portion of cytochrome *b* (*cytB*) were amplified using the polymerase chain reaction (PCR: Saiki *et al.* 1988). *ATPase* amplifications used primers ATPase8.2 (L)8331: 5′-AGCRTYRGCCTTTTAAGC and COIII.2 (H)9236: 5′-GTTAGTGGTCAKGGGCTTGGRTC and *cytB* amplifications used GLUDGL: 5′-TGACTTGAARAACCAYCGTTG paired with CytB16460(H): 5′-GGCAAATAGGAARTATCATTC or CB3(H): 5′-TGACTTGAARAACCAYCGTTG. Successful PCR-amplified products were cleaned by agarose gel purification, and 1–2 μL of the cleaned template was cycle-sequenced using ABI Prism™ BigDye Terminator kits. Seqeuenced products were electrophoresed and analysed on an ABI Prism™ 377 DNA Sequencer. All sequences have been deposited in GenBank (cytochrome *b* Accession nos AY321713–AY321802; ATP synthase VIII and VI Accession nos AY321803–Y321888).

Sequence data were aligned, edited and checked for reading-frame errors using SEQUENCHER (version 4.0) and MACCLADE (version 4, Maddison & Maddison 2000). Congruence among tree topologies generated with the three mitochondrial genes was tested using the partition homogeneity test (Farris *et al.* 1995) implemented in PAUP* version 4.0b10 (Swofford 2002). To assess the genetic relationships among hamlets and the other Serrandids, we generated a maximum likelihood (ML) phylogenetic tree using PAUP*, based on the best fitting model of nucleotide substitution identified by MODELTEST version 3.06 (Posada & Crandall 1998) (see Fig. 2A). This tree was generated by successive rounds of tree-bisection–reconnection (TBR) followed by nearset-neighbour interchange (NNI) branch-swapping, updating parameter estimates at each round (Swofford 2002). Confidence in each node was assessed by bootstrapping (1000 replicates, heuristic search with TBR branch swapping). Maximum parsimony (MP) and neighbour-joining trees were also constructed, and were qualitatively similar. The resulting ML tree was compared to the a priori prediction that colour pattern morphospecies fell into monophyletic clusters. Differences between the two topologies was assessed using the methods of Shimodaira & Hasegawa (1999) implemented in PAUP*.

We examined further the relationship between mtDNA clade structure and hamlet colour pattern using the nested-clade approach of Templeton *et al.* (1992, 1993). The haplotype network was was generated using the estimation procedure and nesting rules described by Templeton *et al.* (1992, 1993), extended for sequence data by Crandall & Templeton (1996), and implemented in TCS 1.13 (Clement *et al.* 2001). We determined whether non-random associations

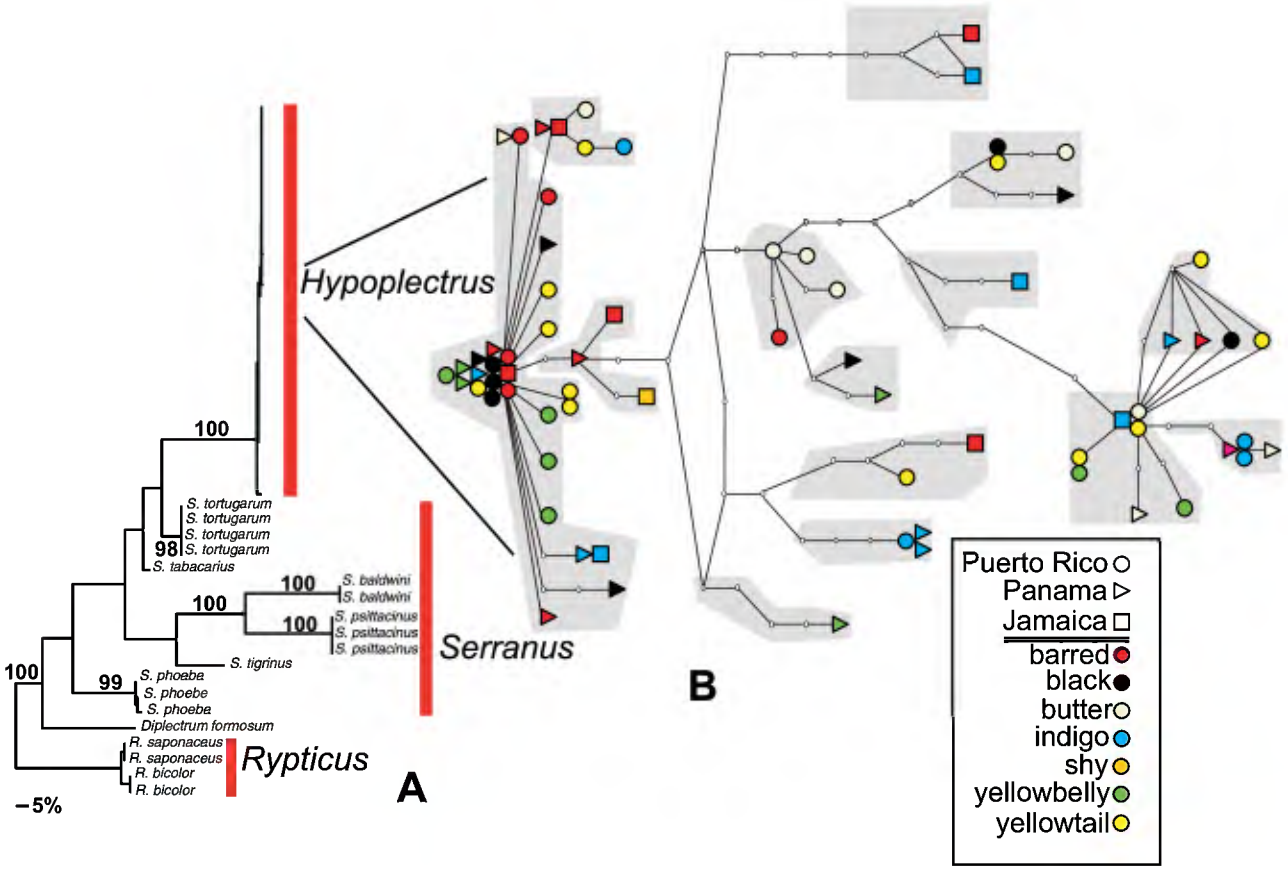

Fig. 2 (A) Maximum-likelihood phylogeny of *Hypoplectrus* and other serranine fishes based on the combined mitochondrial *ATPase 6/8* and *cytB* regions. Bootstrap confidence values > 65 (1000 replicates) label nodes. Maximum parsimony and neighbour-joining trees were qualitatively similar. (B) Statistical parsimony network. Coloured filled circles represent the haplotypes of individuals; clusters of these are haplotypes shared by more than one fish. Thin lines interconnecting haplotypes represent parsimony-derived mutational steps, and unlabelled open circles are 'missing haplotypes' used to connect the network (95%-probability connection limit of 17 steps). Grey shading encloses two-step nested clades.

between membership in mtDNA clades existed, both with regard to colour pattern and geographical location, by permuting the tallies randomly for colour pattern and for source population across clades. Permutations were performed at each nesting level following the procedure of Roff & Bentzen (1989) as recommended in Templeton *et al.* (1992) and later papers.

Microsatellite variation was examined at 5 dinucleotide repeat loci. Three loci: E2/1 (GenBank Accession no. AY316532), G2/1 (Accession no. AY316532), and H24 (Accession no. AY316530), were isolated directly by CR and MAM from a *Hypoplectrus* genomic library. Primers used to amplify these loci were (5′ to 3′): ACATTTGTGGCCAAAGAGTGGC and TCTGCTACGGTTACAGTATGCGG for E2/1, CTGACTAGCCATGTCCTTCCTAC and AGTCAGGCTGTCTTCCTTCAGGC for G2/1, and CACAACCTTCACACCTGCATGC and TCCTGGTTATGAATCCTGGGTC for H24. The two remaining dinucleotide repeats were previously isolated from the commercially important species *Polyprion americanus* (Pam013) and *Mycteroperca microlepis* (Gag10) (Ball *et al.* unpublished; Chapman *et al.* 1999). Gel electrophoresis and data capture were accomplished on an ABI Prism™ 377 DNA Sequencer and analysed using GENESCAN 2.5 and GENOTYPER 2.5 software. Allele sizes were rounded to the nearest integer and individuals were scored independently by JA and CR. Per locus and sample statistics including allele richness, gene diversity and heterozygosities were calculated using FSTAT version 2.9.3 (Goudet 1995). We tested deviations from Hardy–Weinberg equilibrium by comparing the observed F_{IS} value to those generated when alleles were permuted randomly among individuals. Linkage disequilibrium among loci was examined using the procedure outlined in ARLEQUIN version 2.000 (Schneider *et al.* 2000). The degree of genetic subdivision among colour morphospecies and between collection sites were estimated based on both an infinite allele model and a stepwise mutational model, using Weir & Cockerham (1984) θ estimator of F_{ST} & Slatkin (1995) R_{ST}, respectively.

Table 1 Molecular variation in *Hypoplectrus*

Locus	Core repeat	n	No. of alleles	Allele size range	Panamá H_E	Panamá H_O	Puerto Rico H_E	Puerto Rico H_O
(A) Microsatellite loci								
E2	$(CA)_{12}TA(CA)_2$	394	10	136–154	0.633	0.552**	0.608	0.435***
G2	$(CA)_{26}$	396	30	180–240	0.879	0.886	0.873	0.866
H24	$(CA)_{15}$	394	29	180–242	0.941	0.901	0.948	0.925
Gag10	ND	396	30	84–144	0.913	0.912	0.935	0.931
Pam013	ND	391	33	86–152	0.939	0.942	0.954	0.954

Region	Size (bp)	k	No. of variable sites	n	No. of haplotypes	Haplotype diversity
(B) mtDNA						
Total	1641	10.0	93	71	47	0.972
Cyt *b*	789	5.53	48	71	33	0.900
ATP synthase VIII	168	0.30	4	71	5	0.283
ATP synthase VI	684	4.18	41	71	32	0.893

ND = not determined; n = number of hamlets genotyped or sequenced; H_E and H_O = expected and observed heterozygosities, respectively; k = average number of pairwise nucleotide differences. **$P < 0.01$; ***$P < 0.001$.

Results

MtDNA variation in hamlets and other serranine fishes

The pattern of nucleotide substitution within the mitochondrial *ATPase 6/8* and *Cyt b* was similar to that reported in other vertebrates (Kocher & Stepien 1997; Mindell 1997). Among the 1641 nucleotide positions examined the majority of substitutions occurred at 3rd codon positions, with a pronounced bias in the transition/transversion ratio that declined with increasing genetic distance (Table 1). A partition homogeneity test of the three data partitions (*ATPase 8*, *ATPase 6* and *cyt b*) failed to reject the null hypothesis ($P = 0.11$) and hence the data were combined for all subsequent analyses. We found that the evolution of our mtDNA sequences best fit an HKY (Hasegawa *et al.* 1985) model, with transition/transversion substitutional bias = 4.262 and moderate rate heterogeneity across nucleotide sites (proportion of invariant sites = 0.451; rates γ-distributed with $\alpha = 0.7937$). We calculated a crude 'local' molecular clock for the combined mtDNA data of 1.4% sequence divergence per million years (Myr). Our calculation assumed that sequence divergence between the two *Rypticus* species (4.34%) has accumulated in the 3.1 Myr since the final closure of Isthmus of Panamá (Coates & Obando 1996). Our rate estimate is very similar to the value of 1.2% per Myr calculated previously by Bermingham *et al.* (1997a) for *Rypticus* and additional geminate fish species purported to have been isolated by the closure of the Panamanian isthmus.

Nucleotide substitutions supported the monophyly of the subfamily Serraninae but did not resolve the branching order of the three genera we examined (*Diplectrum*, *Hypoplectrus* and *Serranus*: Fig. 2A). Interestingly, there was no evidence for the monophyly of *Serranus*. Indeed, the five *Serranus* species examined were highly divergent and even the most closely related pairs, *S. tabacarius* and *S. torturgarum* and *S. psittacinus* and *S. baldwini*, showed considerable differentiation (12.4% and 29%, respectively) consistent with a late Miocene divergence. Branch support for internal nodes was weak, and while trees enforcing the monophyly of *Serranus* were not significantly worse than our ML phylogeny ($\Delta \ln L = 17.640$, $P = 0.138$, Shimodaira–Hasegawa (SH) test), all tree-building methods produced a topology that showed *Serranus* to be paraphyletic with respect to *Hypoplectrus*. Given this trend of lack of support for the monophyly of *Serranus*, previous work on sex allocation theory in this group (Petersen 1987; Petersen 1991) should be revisited.

In contrast to the deep splits within *Serranus*, the hamlet radiation has occurred far more recently. Average genetic differences among the 71 *Hypoplectrus* individuals was slight [0.60 ± 0.04% (SE)] and similar to differences among conspecific individuals of *S. torturgarum* collected from the same reef (0.42 ± 0.12%). There were 47 distinct *Hypoplectrus* mtDNA haplotypes, only one of which was common (Fig. 2B). No clear phylogeographical or colour pattern clustering was apparent on the mtDNA tree — similar mtDNA haplotypes were found on reefs in Panamá and in Puerto Rico and in individuals with very different colour patterns (Fig. 2B). For example, the 13 hamlets carrying the most frequent mtDNA haplotype represented five colour pattern types and came from reefs in Puerto Rico, Panamá and Jamaica. Trees where morphospecies were forced

into monophyletic groups were significantly worse than unconstrained trees ($2\Delta \ln L = 2445.64$; $P < 0.001$, SH test).

Despite the apparent lack of concordance between mtDNA haplotype phylogroups and colour morphospecies, there were haplotype frequency differences among morphospecies ($\chi^2 = 326.97$, $P = 0.001$). We also found differences in the distribution of colour morphospecies across both one-step clades ($\chi^2 = 229.86$, $P = 0.003$) and two-step clades ($\chi^2 = 105.95$, $P = 0.050$) in our haplotype network (see Fig. 1); at higher nesting levels, morphospecies were scattered randomly across clades. Mitochondrial DNA variation was also distributed nonrandomly with respect to collection location. Again this effect was restricted to differences at the lower nesting levels of our haplotype network (one-step: $\chi^2 = 70.58$, $P = 0.012$; two-step: $\chi^2 = 39.08$, $P = 0.022$; and three-step clades $\chi^2 = 19.03$, $P = 0.035$).

Microsatellite differentiation among hamlets. The five microsatellite loci exhibited ample genetic variation. In the nearly 400 hamlets examined from reefs in Puerto Rico and Panamá, the number of alleles per locus varied from nine at locus E2–30 or more at the remaining four loci (data available upon request). All loci were in linkage equilibrium and, with the possible exception of E2, conformed to Hardy–Weinberg expectations within populations. At locus E2, observed heterozygosity was significantly lower than expected in three (*H. puella* — Panama, *H. aberrans* — Puerto Rico, and *H. indigo* — Puerto Rico) of the 11 populations examined ($P < 0.05$, after sequential Bonferoni corrections). Heterozygote deficit may reflect the presence of null alleles at this locus. Null alleles are a recurring problem with genetic data analysis using microsatellites (Paetkau & Strobeck 1995; Jones *et al.* 1998; McGoldrick *et al.* 2000). However, given that heterozygote deficiency was confined to a minority of populations at a single locus in our sample, null alleles are unlikely to affect our conclusions.

As was the case with mtDNA, there was a striking degree of genetic similarity among different colour morphospecies and among populations of the same colour morphospecies collected 1500 km apart. Alleles were shared broadly, and there were no conspicuous differences in within-population measures of variation, such as allele richness and variances in allele size. Nearly 99% of the variation at microsatellite loci occurred within morphospecies at any given geographical location (AMOVA, not shown). None the less, microsatellite variation was not distributed randomly. Pairwise comparisons of the morphospecies for which we had sample sizes of 12 or more individuals collected from Puerto Rico and Panamá revealed significant differences in microsatellite variation between locations. This was true whether the analysis was based on an infinite allele (θ_{ST}) model, a stepwise mutational (R_{ST}) model, or upon comparisons of allele frequency distributions (Table 2).

Table 2 Microsatellite differentiation between Panamá and Puerto Rico populations of hamlet colour morphospecies. Genetic distance measures based on variance in allele frequencies under an infinite allele model ($\theta = F_{ST}$: Weir & Cockerham 1984) and sum of the squared allele size differences (R_{ST}) under a stepwise mutational model (Slatkin 1995) were calculated using ARLEQUIN version 2.001. Significance of these comparisons was determined by permuting genotypes among populations as described in Goudet *et al.* (1996). In addition, we estimated the probability (P) that allele frequency distributions are identical between the two populations based on 10 000 randomizations [see Goudet *et al.* (1996)]. Values in bold denote significant differences between regions at the 0.05 level

	θ	R_{ST}	P
H. aberrans ($n = 40$)	**0.018**	−0.004	**0.0125**
H. indigo ($n = 33$)	0.010	**0.051**	**0.0364**
H. nigricans ($n = 123$)	**0.016**	**0.030**	**0.0001**
H. puella ($n = 123$)	**0.009**	**0.036**	**0.0001**
H. unicolor ($n = 22$)	0.012	−0.004	0.3544

Analysis of morphospecies collected from the same region of the Caribbean revealed a intriguing pattern of genetic differentiation. On reefs in Puerto Rico, pairwise θ_{ST}s among colour morphospecies were generally significant, providing genetic evidence of assortative mating (Table 3a). Interestingly, only *H. indigo* was differentiated from the other morphospecies with respect to both allele sizes and allele frequencies. Between other colour morphospecies genetic differentiation was less extreme, but all comparisons except two (*H. nigricans* × *H. chlorurus* and *H. puella* × *H. abberans*) differed significantly from random expectations. Conclusions about genetic differences between *H. unicolor* and the other Puerto Rican colour morphospecies must be made with caution — the small sample size of this and of the other less abundant morphospecies (including *H. indigo*) is likely to limit our ability to estimate their true levels of genetic differentiation. Nevertheless, despite small sample sizes *H. unicolor* could be distinguished from *H. nigricans*, *H. chlorurus* and *H. indigo* in Puerto Rico, and *H. indigo* was distinguishable from all other morphospecies at this location.

In contrast, microsatellite evidence for colour-pattern based assortative mating among sympatric hamlet colour morphospecies in our Panamanian collection was much weaker. Pairwise θ_{ST} values between the same colour morphospecies were generally 4–5 times lower in Panamá than in Puerto Rico, and none of these comparisons were significantly different from zero in the Panamá population. As an example, *H. indigo*, which was clearly differentiated from *H. aberrans*, *H. nigricans*, *H. puella* and *H. unicolor* in Puerto Rico, was indistinguishable from these same colour morphospecies in Panamá (Table 3B).

Table 3 Pairwise $\theta = F_{ST}$ (below diagonal) and R_{ST} (above diagonal) at microsatellite loci between six hamlet species collected in Puerto Rico (A) and five species collected in Panamá (B). Values in bold are significantly different from zero at the 0.05 level; those in bold and italics are significantly different from zero at the 0.10 level after sequential Bonferroni adjustments (Rice 1989)

(A)	*H. chlorurus*	*H. aberrans*	*H. indigo*	*H. nigricans*	*H. puella*	*H. unicolor*
H. chlorurus ($n = 54$)		−0.01489	**0.09260**	0.0077	−0.00475	−0.01428
H. aberrans ($n = 18$)	**0.01837**		**0.09856**	−0.00958	−0.00816	−0.02852
H. indigo ($n = 21$)	**0.02782**	**0.03311**		**0.12996**	**0.06177**	**0.10457**
H. nigricans ($n = 52$)	0.00069	**0.02826**	**0.04414**		0.00768	−0.00053
H. puella ($n = 45$)	*0.00752*	0.00417	**0.02034**	**0.017261**		0.0074
H. unicolor ($n = 11$)	*0.01356*	−0.00370	*0.02660*	0.03066	−0.00339	

(B)	*H. aberrans*	*H. indigo*	*H. nigricans*	*H. puella*	*H. unicolor*
H. aberrans ($n = 22$)		0.06526	0.00199	−0.01308	−0.001430
H. indigo ($n = 12$)	0.00613		0.0864	**0.06679**	0.11304
H. nigricans ($n = 71$)	0.00633	0.00793		0.00303	0.02954
H. puella ($n = 78$)	0.00054	0.00618	0.00456		−0.00187
H. unicolor ($n = 13$)	−0.00530	0.00776	−0.00128	−0.00311	

Discussion

The sympatric coexistence of the closely related hamlets brings to mind Darwin's finches and species flocks of haplochromine cichlids (Freeland & Boag 1999; Meyer *et al.* 1990; Meyer 1993; Grant & Grant 1994, 1997; Parker & Kornfield 1997; Petren *et al.* 1999; Sato *et al.* 1999). As in these classic radiations, our addition of high-resolution genetic information helps to refine questions about speciation and the nature of species boundaries. Perhaps the most striking result from our work is the conspicuous genetic similarity among the six hamlet morphospecies we examined, at both mtDNA and at hypervariable nuclear loci. This is true despite the dramatic differences in colour pattern that distinguish the morphospecies and in spite of published evidence for strong assortative mating (Domeier 1994; Fischer 1980).

We found no obvious clustering of molecular variation by morphospecies; both mtDNA haplotypes and microsatellite alleles were shared broadly across colour pattern boundaries. None the less, overlaid on this homogeneity were subtle differences between colour morphospecies and between regions. The complex genetic mosaic that we observe among sympatric colour pattern types has three possible explanations.

First, *Hypoplectrus* colour morphs may simply represent variants of a single biological species. If this were the case, no significant genetic differentiation would be predicted among morphs, and this was precisely the outcome of the only previous molecular study of hamlets (Graves & Rosenblatt 1980). However, Graves and Rosenblatt's allozyme survey suffered from extremely low levels of detectable variation. Of the 32 loci they examined, only five were variable and in only one of these five loci was the rare allele found in more than a single specimen of 132 assayed. In contrast, our study uncovered ample genetic variation within hamlets. MtDNA haplotype diversity was high and microsatellite heterozygosity was 100 times greater than that observed at allozyme loci. All five microsatellite loci were polymorphic and the most common allele occurred at a frequency > 0.5 at only the one locus (E2) with low allelic diversity; at the four other loci the majority allele showed a frequency < 0.25.

Other aspects of our genetic findings are inconsistent with a single polytypic species view. Colour morphospecies were not distributed randomly within the mtDNA network. That this pattern was present among only the more closely related (i.e. most recently arisen) mtDNA haplotypes suggests that some degree of reproductive isolation has evolved between the colour morphospecies, and that it has appeared recently (see below). In addition, at least on reefs in Puerto Rico, pairwise comparisons of hypervariable microsatellite loci provide genetic evidence for nonrandom mating between most of the colour pattern morphospecies (Table 3A). Curiously, this conclusion appears to break down in Panamá.

A second explanation would hold that high genetic similarity simply reflects the very recent origin of the hamlet species flock. If so, polyphyly of mtDNA and similarity of microsatellite allele frequencies among morphospecies may reflect historical associations among mtDNA haplotypes and microsatellite alleles present in an ancestral undifferentiated population. This is expected in the very earliest stages of differentiation, and discordance between molecular data and recognized species boundaries are observed commonly in species flocks in both terrestrial and freshwater habitats. For instance, fish flocks in lakes and complexes of finches on the Galápagos and Cocos

Islands show overlap of intra- and interspecific divergence, and an absence of clustering of mtDNA lineages into species-diagnostic clades (Meyer *et al.* 1990; Meyer 1993; Strecker *et al.* 1996; Parker & Kornfield 1997; Freeland & Boag 1999; Sato *et al.* 1999).

For neutral markers, the length of time before there is a strong correspondence between recognized species boundaries and mtDNA lineages or multilocus genotypic clusters depends on the genetic structure of the ancestral population, the demographic history of speciation and the effective population sizes of the emergent forms (Avise *et al.* 1984; Neigel & Avise 1983; Pamilo & Nei 1988; Hudson 1992; Edwards & Beerli 2000). In marine species such as hamlets, broad geographical ranges and large population sizes will retard the lineage sorting process. Present-day population sizes are large for all morphospecies examined. Five of the six morphospecies are found on reefs throughout the Caribbean, a reef area of approximately 65 000 km^2 (Roberts 1997) in densities typically approaching 1–20 hamlets/100 m^2 (Fischer 1979). This yields a conservative estimate of census number of these morphospecies at ~6.5 million individuals. The sixth morphospecies, *H. chlorurus*, has a more restricted distribution but is still found over large areas. In the absence of severe genetic bottlenecks during speciation, it could take many hundreds of thousands of generations to erase the genetic signature of a large ancestral population. Moreover, expected coalescence times of mtDNA will be protracted further in hamlets relative to other vertebrates with similar population histories. This is because hamlets are simultaneous hermaphrodites and the mitochondrial genome will be transmitted through the eggs of both parents, effectively doubling the expected coalescence time.

Under a no-gene flow scenario, our molecular data imply that the hamlet radiation has occurred very recently. Using an estimated rate of evolution of 1.4% per million years for our mtDNA gene regions, the observed 0.6% within-group mtDNA divergence suggests that members of the hamlet species complex studied here shared a common ancestor within the last 429 000 years. This estimate (with 95% confidence limits of 485 000 and 373 000 years, not including the unknown error in the estimated evolutionary rate), corresponds to about 215 000 generations for hamlets (Fischer 1979). In fact, if our estimates of population size are even roughly accurate, and gene flow between morphospecies has been low enough, cladogenesis may have been far more recent than this. For Pleistocene radiations such as the hamlets appear to be, the bulk of DNA divergence is likely to date to the common ancestral population, and overestimation of the age of splits between species and geographical populations is predicted be particularly severe (Edwards & Beerli 2000). Supporting this view is our finding that mtDNA differentiation among colour pattern forms was restricted to haplotype frequency differences and to the most recently evolved mtDNA lineages. Similarly, there were only slight frequency differences at microsatellite loci. These types of frequency shifts are expected in the very earliest stages of divergence, before drift and mutation clearly separate incipient populations (Nei 1987; Avise 2000; Edwards & Beerli 2000).

The issue of gene flow between recently diverged forms brings us to the third explanation for patterns of DNA differentiation in the hamlets. This explanation suggests that the genetic mosaic we observe reflects a recent history marked by gene flow across incomplete species boundaries. This scenario is consistent with previous field observations, which have documented occasional mixed-morph matings (Barlow 1975; Fischer 1980; Lobel & Neudecker 1985) and adults with 'intermediate' colour pattern (Thresher 1978). While the frequency of mixed matings are low [less than 4% (Fischer 1981)], theoretically this level of hybridization would be enough to homogenize neutral genetic variation among morphospecies (cf. Takahata & Slatkin 1984).

The existence of strong assortative mating with occasional hybridization led Fischer to describe hamlets as a 'multispecies' complex (Fischer 1980). His theory, roughly analogous to a sympatric species ring (Moritz *et al.* 1982; Wake 1997), holds that hamlets form a broad complex of incipient species, each of which can exchange genes with at least one of the other members of the group. Mating probabilities between morphospecies are expected to vary and some colour morphospecies may never directly interbreed. Nevertheless, in a multispecies assemblage there exists a conduit for gene exchange throughout the complex, indirectly linking incompatible forms. Fischer (1980) suggested that similarly patterned fishes would be more likely to interbreed than would fish with more distinct colour patterns.

While Fischer's prediction that colouration serves as a primary cue for mate choice awaits further testing, field and laboratory observations, coupled with our new genetic data, provide some support for this idea. The indigo hamlet *H. indigo*, although superficially similar to *H. puella* (see Fig. 1), has never been observed to interbreed with other morphospecies (Fischer 1980; McCartney, pers. obs.). It is also the only morphospecies so far that has shown consistent ecological and life history differences. Across their geographical range, indigo hamlets prefer more intermediate over shallower depths and mature at a larger body size than other common morphospecies (Fischer 1979; Fischer 1980). Interestingly, *H. indigo* was the most distinctive morphospecies we studied, both at mtDNA and at microsatellite loci (Table 3a). In contrast, assortative mating appears to be weaker for other morphospecies that we found to be weakly or not genetically differentiated from the rest of the flock. For example, Barlow (1975) commonly observed mixed mating between black hamlets and the phenotypically similar yellowtailed hamlet

on reefs in Puerto Rico, and we found these two colour pattern forms sampled from the same region to show identical microsatellite profiles (Table 3a).

Our high-resolution genetic data, moreover, argue that Fisher's multispecies concept is more complex than previous envisioned. In particular, greater genetic differentiation among colour morphospecies in Puerto Rico than in Panamá suggests that mating probabilities among morphospecies might also vary spatially. Despite widespread co-occurrence and lack of clear ecological differences, the composition of hamlet assemblages can change dramatically over small spatial scales (McMillan and McCartney, pers. obs.). For example, although at least six morphospecies are known to occur on reefs in Puerto Rico, patches of reef can be dominated by one or two colour forms with the remaining types rare or absent. The morphospecies that dominate can change from patch reef to patch reef or across contiguous reef habitat. These casual observations, together with tank experiments that demonstrate the breakdown of assortative mating in 'no-choice' experiments (Fischer 1980; Domeier 1994), suggest that mating behaviour is likely to depend on the composition of hamlet types on a reef. Rarer colour pattern morphospecies might mate more promiscuously and raise the likelihood of introgressive gene flow. Clearly, more observations of mating behaviour at more locations are needed to address these hypotheses, as are measures of DNA differentiation across more loci, and from more individuals sampled at much greater spatial resolution than we have in the present data set.

The multispecies view of hamlets raises important questions about the maintenance of colour pattern distinctiveness in the presence of gene flow. Similar genetic patterns were observed between incipient colour pattern forms of Pacific *Chaetodon* butterflyfishes. However, in these species, geography plays an important role in the maintenance of pattern differences (McMillan *et al.* 1999). *Chaetodon* species are largely allopatric. In regions of sympatry, there is no evidence for colour pattern-based mate choice, and the colour pattern differences that define species are eroded. In contrast, in *Hypoplectrus* there is strong colour-pattern based mate choice, morphospecies overlap broadly, and colour pattern differences are maintained despite potentially homogenizing levels of gene flow and little genetic differentiation. It is likely that strong selection against hybrid colour pattern types is preventing homogenization of colour pattern differences. Hybrid phenotypes may have reduced mating success because of sterility or inviability or because they are selected against during courtship. Additionally, hybrid phenotypes may be poorly adapted to aspects of reef life not related directly to mating success. Hamlet colour morphospecies have been described as aggressive mimics (Thresher 1978) that match their colouration to that of other herbivorous and planktivorous fishes in order to fool their mainly crustacean prey. Thus hybrids that lack appropriate mimetic signals may suffer higher mortality because of reduced feeding success.

Our somewhat preliminary analysis of *Serranus* provides an informative framework in which to view the evolution of *Hypoplectrus*. *Hypoplectrus* is confined entirely to the western Atlantic, but *Serranus* contains at least one species in the Indian Ocean, three in the eastern and central Pacific, 12 in the western Atlantic and six in the eastern and central Atlantic (Robins & Starck 1961; Eschmeyer 1998). The one eastern Pacific and five western Atlantic *Serranus* species we studied are each deeply divergent from one other, and our phylogeny suggests that *Hypoplectrus* is nested within *Serranus*. More work will be required to verify this result and to resolve relationships within the genus and subfamily, both groups whose phylogeny based on morphological characters remains problematic (Eschmeyer 1998; Robins & Starck 1961). Nevertheless, our results so far question the status of *Serranus* and *Hypoplectrus* as natural groups. Resolving phylogenetic relationships would inform efforts to understand the unique reproductive biology of the serranines. *Serranus* shares with *Diplectrum* and *Hypoplectrus* a trait that is very unusual in the vertebrates — simultaneous hermaphroditism (Smith 1965). Previous studies of mating systems and sex allocation in these hermaphroditic genera (Fischer 1981; Fischer 1984; Petersen 1987; Petersen 1991) might be revisited profitably with a resolved phylogeny in hand. For instance, all our trees clustered *Hypoplectrus* with *S. tortugarum* and *S. tabacarius*, and all of these show a serially monogamous mating system (Petersen 1991). Moreover, the unusual system of reciprocal courtship and spawning may have played a role in the very recent flocklike multiplication of species in *Hypoplectrus*. Of course, in *Serranus* our results would suggest that if a similar diversification occurred, it happened long ago.

Marine radiations provide an important but poorly exploited contrast to other, more well-studied systems. Relative to terrestrial and freshwater groups, members of marine radiations have larger geographical ranges and greater powers of dispersal through long-lived planktonic stages (Palumbi 1992; Palumbi 1994). Furthermore, contact zones between incipient marine species, at least in fishes (McMillan *et al.* 1999; Roques *et al.* 1999), do not appear to coincide with obvious dispersal barriers. These patterns challenge classic models of allopatric divergence and suggest that the population and evolutionary dynamics underlying marine radiations may differ from their terrestrial counterparts. Ultimately, the continued unraveling of the enigma of the hamlets (*sensu* Fischer 1981) promises a richer understanding of speciation in the sea, with particular relevance to the diversity of vividly coloured fishes on coral reefs, and with results that will compliment those from terrestrial and freshwater species radiations.

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This paper represents the result of a collaborative effort between Drs McCartney, Bermingham and McMillan, who share interests in understanding the evolutionary radiation of the remarkable hamlet fishes. Dr McCartney studies marine speciation and marine molecular ecology and was a postdoctoral at the Smithsonian Tropical Research Institute (STRI) when he began this investigation. Dr Rico was a visiting researcher from Norwich, whose interests include the evolution of cichlid species flocks in Lake Malawi, and he, Brice Quenoville and Dr McCartney cloned the microsatellite loci. Dr Bermingham is a staff scientist at STRI with a long-standing research programme focused on neotropical vertebrate biogeography and evolutionary genetics. Dr McMillan is at the University of Puerto Rico and has interests in diversification of species and colour pattern in butterflies and fishes. Ms Acevedo genotyped fish at microsatellite loci and Ms Heredia sequenced hamlet mtDNAs as part of their master's degree research in Dr McMillan's laboratory.
