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EXTRAORDINARY AFLP FINGERPRINT SIMILARITY DESPITE STRONG ASSORTATIVE MATING BETWEEN REEF FISH COLOR MORPHOSPECIES

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Recent theoretical models and empirical studies of fruit flies, birds, and fish indicate that assortative mating may initiate speciation when physical barriers to gene flow are absent, and before postzygotic barriers evolve. These are important results for marine animals like coral reef fish, where ocean currents can carry planktonic larvae over broad ranges, interconnecting populations and slowing genetic divergence. The Caribbean hamlets (genus *Hypoplectrus)* are a flock of reef fish morphospecies with highly distinct color pattern that mate like with like, but show little mitochondrial or microsatellite DNA differentiation. Here, we broadly screen genomic diversity using amplified fragment length polymorphisms (AFLP) and survey mating pair formation between two morphospecies in the Florida Keys, the butter hamlet (*H. unicolor*) and the blue hamlet (*H. gemma*). No AFLP was species-diagnostic (fixed), and neighbor-joining analyses revealed no clustering of individuals consistent with morphospecies boundaries. Assignment tests, however, placed most individuals within their morphospecies of origin. Field surveys showed that $>$ 98% of mating pairs, including those of rare morphospecies, were of like color pattern. Spawning by a single mixed pair adds to earlier observations suggesting that infrequent hybridization may be a genetically homogenizing force in *Hypoplectrus*. This study provides a clear example of strong assortative mating in a system with limited genetic differentiation.

KEY WORDS: **AFLP, assortative mating, color pattern, coral reef fish, *Hypoplectrus*, speciation, species flock.**

Mounting evidence shows that assortative mating can evolve without genomic divergence or hybrid incompatibilities (Price and Bouvier 2002; Bolnick and Near 2005). Studies of fruit flies (Coyne and Orr 1989, 1997), Galápagos finches (Grant and Grant 1997), and percid fish (Mendelson 2003) provide examples of strong premating isolation despite weak genetic differentiation and little or no evidence of postzygotic barriers. Further, forced laboratory crosses of species pairs, between which strong assortative mating occurs in sympatry or parapatry, may yield progeny with virtually no reduction in viability or fertility (Wu et al. 1995; McMillan et al. 1997; Mallet et al. 1998). Assortative mating, moreover, can in theory initiate speciation, even when geographic barriers are absent and gene flow is high (Turner and Burrows 1995; Kondrashov and Shpak 1998; Dieckmann and Doebelli 1999). This possibility is of particular interest in the marine environment, where absolute physical barriers are few, and dispersal of planktonic larvae and/or adults can genetically homogenize geographically distant populations (Palumbi 1994; but see Taylor and Hellberg 2004).

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The small sea basses of the genus *Hypoplectrus* (Perciformes: Serranidae), commonly known as hamlets, are strong candidates for this scenario. The genus consists of a "flock" of 11 color morphospecies (Domeier 1994). Hamlets are indistinguishable based on skeletal and meristic characters (Fischer 1979) but are easily separated by striking differences in color pattern. As many as seven morphospecies can be found on the same Caribbean reef (Fischer 1980a; Domeier 1994), and strong color pattern-based assortative mating occurs in natural populations (Fischer 1980a) and in the laboratory (Domeier 1994). Mitochondrial DNA (mtDNA) sequences, however, produce gene trees that are polyphyletic with respect to morphospecies (McCartney et al. 2003; Ramon et al. 2003), and only slight differences in mtDNA haplotype and microsatellite allele frequencies separate members of the flock (McCartney et al. 2003).

Amplified fragment length polymorphism (AFLP) is a rapid PCR-based DNA fingerprinting technique that has resolved the phylogeny of recently evolved species complexes in freshwater fish (Albertson et al. 1999; Seehausen et al. 2003; Sullivan et al. 2004), birds (Wang et al. 2003), and Hawaiian crickets (Parsons and Shaw 2001), examples in which DNA sequences were far less successful. AFLP produces hundreds of dominant Mendelian markers that are well dispersed in animal and plant genomes (reviewed in Mueller and Wolfenbarger 1999), and therefore estimates genome-wide divergence. Here we study two co-occurring *Hypoplectrus* color morphospecies using this method and interpret our results along with new data on assortative mating.

Materials and Methods

CENSUS OF RELATIVE ABUNDANCE AND MATING PAIRS

Belt transect surveys were conducted by two divers during daylight hours in reefs off Key Largo (24°56′–25°00′N, 080°22′–27′W) and Long Key (24°44′–48′N, 080°40′–48′W), Florida, USA, at depths of 6–22 m. A 50-m transect line was laid along the reef, parallel to the seaward edge. Each diver swam along opposite sides of the line, recording the number of *Hypoplectrus* individuals out to 5 m from the transect line. Thus, each transect covered approximately 500 m^2 of reef area. We surveyed eight transects off Key Largo in 2002, and 32 transects off Long Key in 2003. A *G*-test of independence (Sokal and Rohlf 1995) was used to determine whether the relative frequency of the two most common morphospecies, *H. gemma* and *H. unicolor*, was random with respect to transect, in which case assortative mating could not be explained by spatial isolation at the scale surveyed.

Hamlets are simultaneous hermaphrodites that mate nightly with no seasonality. In a typical mating bout, pairs court and spawn several times, switching sex roles successively (Fischer 1980b). At approximately 60 min before sunset (Fischer 1980b), the divers repeated the same transects surveyed earlier in the day. Hamlets that remained less than 1 m from each other, usually displaying courtship behavior, were recorded as a mating pair. As hamlets are solitary during the day (Fischer 1980b; pers. obs.), such interactions were conspicuous. Twenty-eight evening dives were conducted in Long Key only. A *G*-test for goodness-of-fit was used to assess whether morphospecies paired assortatively. Observed numbers of each type of mating pair were compared to expected frequencies calculated from the relative abundances of morphospecies from daytime counts on all reefs off Long Key combined.

AFLP ANALYSIS

Hypoplectrus gemma and *H. unicolor*, which together accounted for 85% of all hamlets, were collected off Long Key after daytime and evening surveys had been completed. Caudal fin clips ($\sim$3 mm^2) were removed underwater, transferred to a salt-saturated dimethyl sulfoxide solution, and stored at 4°C. Extraction of genomic DNA was by standard methods (Maniatis et al. 2000), followed by purification through QIAGEN QIAquick spin columns (Valencia, CA), which yielded more consistent AFLP bands.

AFLPs were generated using the Applied Biosystems (Foster City, CA) AFLP Plant Mapping Protocol designed for large plant genomes. Selective amplification products were electrophoresed on the ABI 3100 Genetic Analyzer using the ROX-500 size standard, and fragments between 75 bp and 500 bp were scored manually with ABI GeneScan 3.1 software, based on default size calling and peak detection thresholds.

Initial screening of two individuals of each morphospecies across 52 selective primer pairs allowed us to select 10 most-polymorphic primer pairs (available from the authors) that were screened in six *H. gemma* and six *H. unicolor*. A neighbor-joining (NJ) dendrogram was estimated in PAUP (Swofford 2002) using Nei and Li's (1979) genetic distance. Although AFLP reproducibility is well documented (Mueller and Wolfenbarger 1999), we are aware of only one assessment in fish (Seehausen et al. 2003). To test for reproducibility in hamlets, we replicated the entire protocol for one individual per morphospecies, screening each fish for all 10 primer pairs. Reproducibility was calculated as the proportion of total peaks that were shared across the two replicates.

No fragments showed fixed differences (i.e., present in all members of one morphospecies and absent in all members of the other) in this initial survey, but several fragments from two primer pairs suggested possible differences in frequency. To increase our power to detect significant frequency differences, we expanded our sample for these two primer pairs to 17 *H. gemma* and 18 *H. unicolor*. An exact test of fragment frequency differences was used with this larger sample, using the Markov Chain Monte

Carlo approach in the Tools For Population Genetics Analysis software (TFPGA: Miller 1997). For each locus, we used 5000 permutations, 1000 dememorization steps, and 50 batches to calculate standard errors for each *P*-value. A sequential Dunn–Sidák's adjustment was used to address type I error from multiple testing (Sokal and Rohlf 1995). Finally, a NJ dendrogram was also constructed, based on fingerprints from these two primer pairs.

To assess whether individuals could be correctly assigned to morphospecies based on AFLPs, we used the likelihood approach in *AFLPOP version 1.0* software (Duchesne and Bernatchez 2002). *AFLPOP* estimates the likelihood of an individual genotype belonging to each population, and assigns it to the population with the highest likelihood. We used a threshold log-likelihood difference ($\Delta \log L$) of 1, which represents a 10-fold greater likelihood of assignment. We assessed the reliability of the assignment calls by using the *AFLPOP* simulator to generate 1000 artificial fingerprints, each consisting of AFLP fragments randomly drawn from our sample at their empirical frequencies and after excluding the fingerprint being tested. The calculated *P*-values represent the proportion of artificial fingerprints whose likelihoods of assignment to each morphospecies were lower than that of a sampled individual. Thus, an individual with $P < 0.05$ within morphospecies X, for instance, had a likelihood score of belonging to morphospecies X that was lower than that of 95% of the 1000 simulated fingerprints, suggesting that it could be significantly excluded as a member of that population (Duchesne and Bernatchez 2002).

Results

DAYTIME CENSUS

A total count of 319 fish on Long Key was dominated by *H. unicolor* (63.9% of total) and *H. gemma* (22.3%), with *H. puella* (6.9%) and *H. nigricans* (5.3%) being less common, and tan hamlets (0.9%) and an undescribed olive green color morph (0.6%) being rare. In our smaller survey off Key Largo ($n = 90$ fish), *H. puella* was more common (17%) and *H. nigricans* was absent, whereas *H. unicolor* and *H. gemma* dominated both populations at roughly the same frequencies.

Hypoplectrus gemma and *H. unicolor* were found together on 36 of 39 transects. The relative frequencies of *H. gemma* and *H. unicolor* were random across the 39 transects ($G = 44.075$, $df = 38$, $P > 0.1$), indicating that morphospecies were not spatially isolated on different reefs in the daytime.

MATING SURVEYS

In 14 of 15 like pairs observed for the entire duration of their association, at least one spawn followed, and 13 of those pairs engaged in multiple spawns. The single unlike pair was likewise observed to spawn five times before separating. Hence pairing served as a good surrogate for spawning, as earlier more detailed behavioral observations also confirmed (Fischer 1980b). A total of 66 mating pairs were observed, and only one of these was a mixed pair (Table 1). The number of like pairs was far higher and the number of unlike pairs was far lower than expected under random pairing ($G = 140.1$, $df = 6$, $P < 0.001$, Table 1). Highly positive assortative mating was also evident in matings of uncommon morphospecies. Only three tan hamlets were seen on daytime transects, yet two were observed as a pair that spawned six times. Most notably, we saw two olive-colored fish not fitting any previously described morphospecies several hundred meters apart during the daytime; in the evening, a pair of these fish was seen spawning.

Table 1. Assortative pairing between *Hypoplectrus* morphospecies off Long Key, Florida. Expected numbers of pairs are calculated from binomial encounter frequencies based on relative abundances on Long Key reefs (see Results). A *G*-test for goodness-of-fit, with all categories of mixed pairs pooled for analysis, confirmed that mating was highly nonrandom (G=140.1, df=6, P<0.001).

Mating pair	Observed	Expected
Like		
H. gemma×*H. gemma*	17	3.260
H. unicolor×*H. unicolor*	39	26.90
H. puella×*H. puella*	2	0.314
H. nigricans×*H. nigricans*	5	0.187
Tan×tan	1	0.006
Unknown×unknown	1	0.003
Mixed		
H. gemma×*H. unicolor*	0	18.80
H. gemma×*H. puella*	1	2.030
H. gemma×*H. nigricans*	0	1.570
H. gemma×tan	0	0.276
H. gemma×unknown	0	0.184
H. unicolor×*H. puella*	0	5.820
H. unicolor×*H. nigricans*	0	4.500
H. unicolor×tan	0	0.794
H. unicolor×unknown	0	0.529
H. puella×*H. nigricans*	0	0.485
H. puella×tan	0	0.086
H. puella×unknown	0	0.057
H. nigricans×tan	0	0.066
H. nigricans×unknown	0	0.044
Tan×unknown	0	0.008
Total	66	66

AFLP DIFFERENTIATION

In the two individuals for which AFLP assays were replicated, 99.1% of 445 bands were reproducible. From 10 AFLP primer pairs across 12 individuals, a total of 949 distinct fragments were generated, of which 692 (72.9%) were polymorphic (i.e.,

Table 2. AFLP fragments with significant frequency differences between *Hypoplectrus gemma* and *H. unicolor*. Values in the third and fourth columns are the number of individuals in which the fragment was present divided by the number of individuals assayed.

Primer pair	Fragment size (bp)	*H. gemma*	*H. unicolor*
EcoRI-ACT/MseI-CAG	107	0/17	12/18***[1]
EcoRI-ACT/MseI-CAG	111	8/17	2/18*
EcoRI-ACT/MseI-CAG	252	4/17	13/18**
EcoRI-ACT/MseI-CAG	285	8/17	2/18*
EcoRI-ACT/MseI-CTC	174	1/17	7/18*

*$P<0.05$; **$P<0.01$; ***$P<0.001$ by Fisher's exact test, without correction for multiple comparisons.

[1] Significant at table-wide $P = 0.05$ after sequential Dunn–Sidák's correction.

present in at least one but not all individuals). Despite high polymorphism, no fragments were diagnostic, and NJ analysis (not shown) revealed no tendency for individuals to cluster according to morphospecies. Mean (± standard error) pairwise genetic distance was 0.046 (± 0.002) within *H. gemma*, 0.038 (± 0.001) within *H. unicolor*, and 0.044 (± 0.001) between morphospecies.

When 35 individuals were assayed across the two potentially most discriminating primer pairs, 288 fragments were generated, 253 (87.8%) of which were polymorphic. Of these, five fragments showed significant frequency differences between morphospecies (Table 2), but only one remained significant after sequential Dunn–Sidák correction for multiple testing (adjusted $P = 0.000203$ after 253 tests, Sokal and Rohlf 1995). This fragment was present in 12 of 18 *H. unicolor*, but absent in all 17 *H. gemma*. Consistent with results from exact tests, the θ estimator of F_{ST}, calculated using TFPGA and based on Lynch and Milligan's (1994) Taylor expansion estimate of allele frequencies, indicated moderate differentiation between morphospecies ($\theta = 0.0524$, 95% confidence limits = 0.0318, 0.0739). The distribution of θ was "L-shaped," with $\theta \leq 0$ across 139 loci, $0 \leq \theta \leq 0.1$ across 89 loci, $0.1 \leq \theta \leq 0.3$ across 17 loci, and $0.3 \leq \theta \leq 0.5$ across only eight loci.

NJ analysis (Fig. 1) revealed no clusters that were concordant with morphospecies boundaries. Individuals sorted into two clusters. Cluster 1 contained 29 of 35 fish (14 *H. gemma,* 15 *H. unicolor*) and no morphospecies-specific subclusters. Fourteen AFLPs distinguished cluster 1 from cluster 2 (bootstrap support = 99%), which contained three members of each morphospecies.

In contrast to phenetic analysis, assignment tests correctly assigned most individuals to groups of like color pattern. Based on likelihood scores, 13 of 17 *H. gemma* and 13 of 18 *H. unicolor* were correctly assigned, whereas two *H. gemma* and three

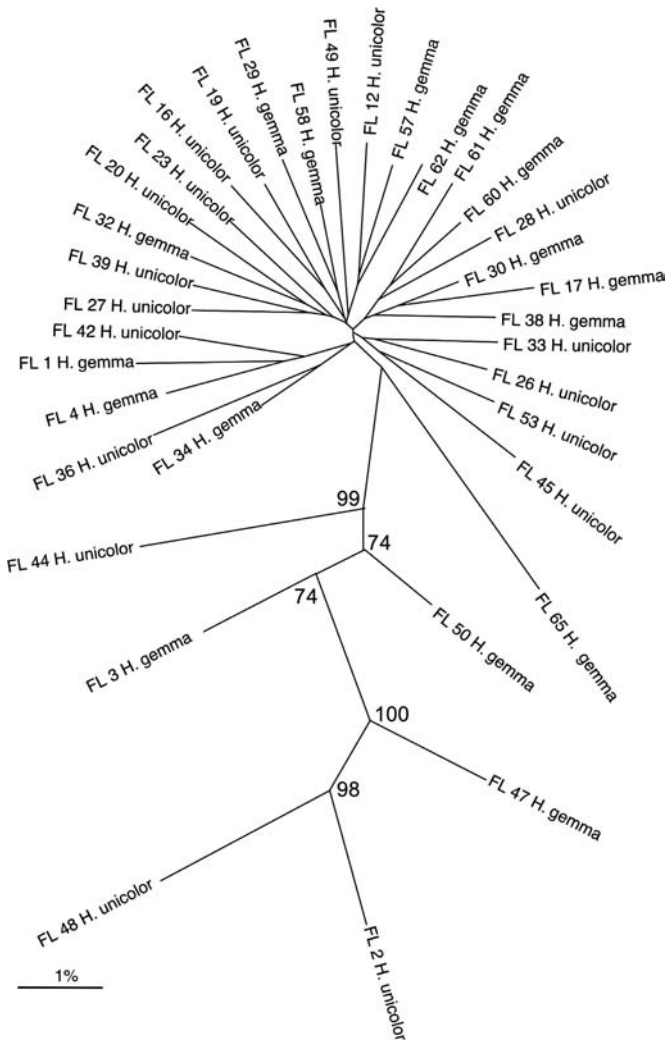

Figure 1. Unrooted NJ phylogram of *Hypoplectrus gemma* and *H. unicolor* AFLPs. Branch lengths are Nei and Li (1979) genetic distances estimated from 288 AFLP fragments generated using two selective primer combinations. Values label nodes with bootstrap support (1000 replicates) > 70%.

H. unicolor were incorrectly assigned, and two *H. gemma* and two *H. unicolor* were below the threshold Δ log *L* of 1 (Fig. 2). *P*-values from the allocation procedure (Fig. 2) sorted individuals into three classes. Individuals in the first class showed significant ($P > 0.05$) allocation only to their morphospecies of origin, whereas individuals in the second class received significant allocation to both morphospecies. This second outcome, suggesting some ambiguity in assignment, occurred with 12 of 17 *H. gemma,* and eight of these fish showed "high" ($P > 0.5$) heterospecific allocation values. In contrast, fewer (8 of 18) *H. unicolor* showed significant heterospecific allocation, and only one fish showed high heterospecific *P*-values. Along with greater Δ log *L* for *H. unicolor,* this pattern seems to reflect greater distinctiveness of *H. unicolor* fingerprints.

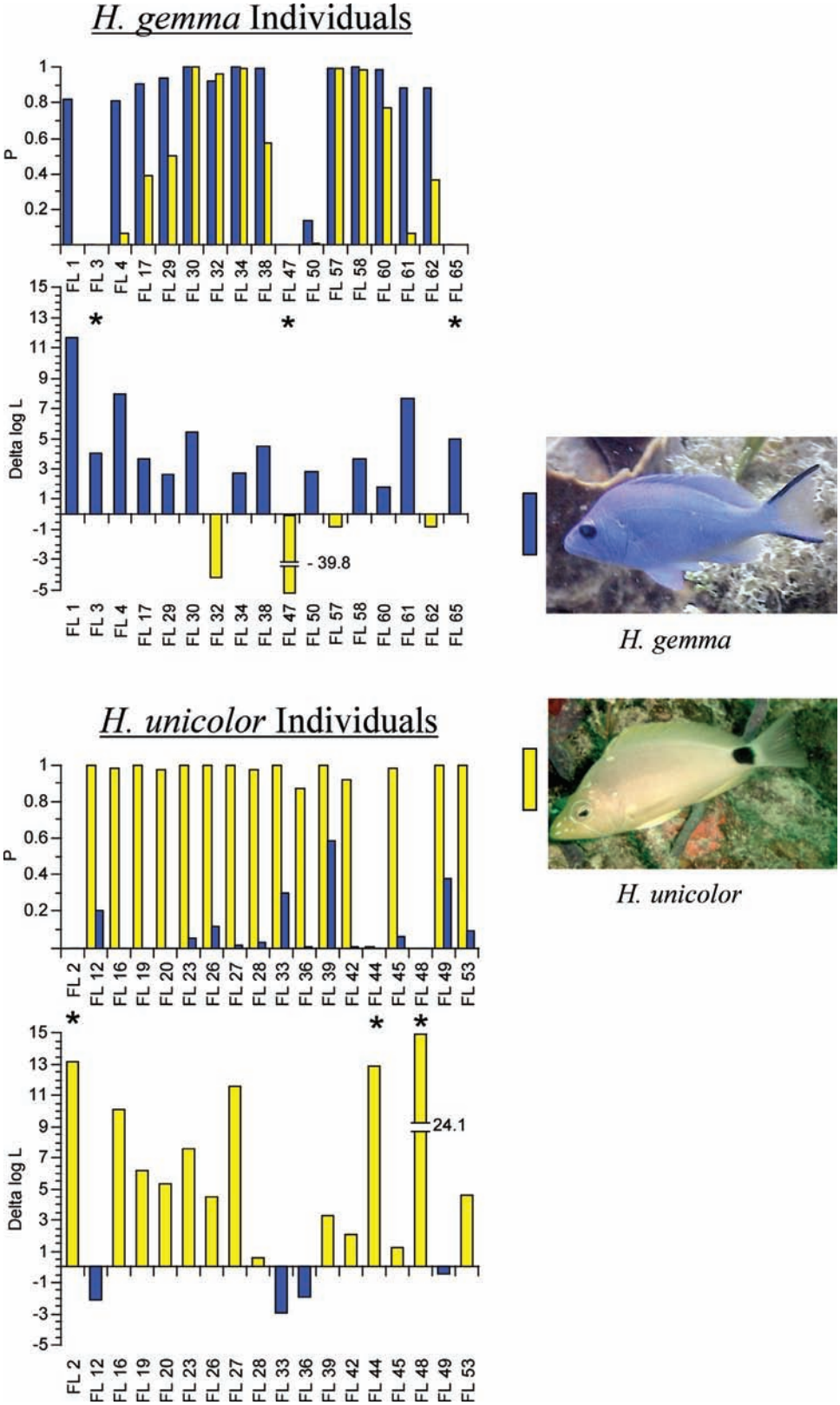

Figure 2. Assignment of individuals to morphospecies based on AFLP fingerprints. The top panels show the probability (*P*) of allocation and the bottom panels show log-likelihood differences favoring assignment to either morphospecies. Asterisks indicate individuals with relatively divergent AFLP fingerprints (see Fig. 1) and for which *P*-values for allocation to both morphospecies were low.

The third class of individuals, each of which belonged to phenetic cluster 2 noted above (Fig. 1), showed low *P*-values (< 0.05) for allocation to both morphospecies. Although all but one of these six individuals were correctly assigned based on Δ log *L*, low *P*-values and low absolute values of log *L* for both morphospecies suggest that the fingerprints of these fish (see asterisks in Fig. 2) did not conform well to either morphospecies. The absence of significant allocation to either group suggests that these may be migrants from other, genetically distinctive subpopulations. To determine whether poorer allocation of *H. gemma*, noted above, was caused by the presence of these atypical fish, we reran the *AFLPOP* analysis with them removed. Five of the 14 remaining *H. gemma* but none of 15 *H. unicolor* received significant allocation to the heterospecific group. Hence, the greater genetic distinctiveness of *H. unicolor* remains, and cannot be attributed solely to data heterogeneity due to the atypical fish. Finally, the AFLP protocol was repeated with new DNA extracts from the six individuals from cluster 2. All fragments were reproduced, so we can exclude PCR artifacts as explanations for the uniqueness of these six fish.

Discussion

ASSORTATIVE MATING

Taken together, our results demonstrate strong assortative mating of *Hypoplectrus* morphospecies, between which genomic divergence is minimal. Conspecific pair formation occurred in $> 98\%$ of cases, and did not result from clumped distributions of conspecifics on daytime reefs, but must involve active mate choice. Like earlier studies (Fischer 1980a), we showed that uncommon morphs are not more likely to form mixed pairs. Individuals of the rarest morph we saw (two in 16,000 m^2 reef area) spawned as a pair. Hence, keen preferences for like color pattern (or associated cues) exist in hamlets.

On the other hand, our one mixed pair adds to others regularly documented throughout the Caribbean. In the Florida Keys, the frequency of heterospecific spawns (1.5%) was similar to the frequency (1.6%) in 251 spawnings in Barbados, Belize, and Panamá (Puebla et al. 2007), but slightly lower than the frequency (3.8%) in 182 spawns in Jamaica and Panamá (Fischer 1980a). Possible color pattern hybrids comprised only 0.5% of > 400 fish in our survey, and 2% of 585 fish across Florida and the Caribbean (Domeier 1994). Although some F_1 hybrids might be cryptic due to dominance of color pattern alleles, color pattern traits are codominant in laboratory crosses between *H. unicolor* and *H. gemma* (Domeier 1994). Hence, although *Hypoplectrus* does not form a hybrid swarm anywhere, it is possible that occasional hybridization and backcrossing, the progeny of which may be cryptic, promote introgression at neutral marker loci.

GENOMIC SIMILARITY

Our AFLP data indicate that polyphyly of mtDNA markers with respect to morphospecies (McCartney et al. 2003; Ramon et al. 2003) also pervades the nuclear genome. Assuming these presumptive loci are unlinked, we estimate (from exact tests) that only 0.4–2% of them (after and before Bonferroni correction, respectively) show significant differentiation between morphospecies. A low but significant F_{ST} across all 253 loci suggests incomplete and/or recent isolation between these two Florida morphospecies. The level of differentiation is only slightly greater than that between several Panamá morphospecies, estimated with five microsatellite loci (McCartney et al. 2003). *AFLPOP* detected this signal and assigned most but not all individuals to the correct morphospecies.

Phenetic analysis of AFLPs revealed a subcluster of six individuals that also received low likelihood scores from *AFLPOP*, suggesting they are not part of the same genetic pool that contains the remaining 29 fish. Because these fish were collected from several nearby reefs off Long Key, and because PCR artifacts were not responsible for their unusual AFLP fragments, we suspect the six hamlets were immigrants from one or multiple distant source populations. Reexamination of this hypothesis is warranted, perhaps using recently developed microsatellite markers (McCartney et al. 2003; Puebla et al. 2007).

Ongoing hybridization provides an alternative hypothesis for the genomic similarity of hamlets. Although mixed matings between *H. gemma* and *H. unicolor* have never been documented, indirect gene exchange between them might still occur, as both are known to mate with *H. puella* (Fischer 1980a, this study, and Puebla et al. 2007). Our genetic findings may be consistent with introgression in large populations, perhaps realized as indirect gene exchange in a "multispecies" complex, as Fisher (1980a) envisioned. Traditional island models suggest that one migrant per generation is sufficient to slow genetic differentiation between subpopulations (Slatkin 1987). If mixed matings, observed at a frequency of 1–3.5% in this study and others (Fischer 1980a, Puebla et al. 2007), produce hybrid progeny that are at least occasionally successful in backcrossing with one or both parental forms, gene flow between morphospecies may easily exceed this threshold. Future multilocus studies, analyzed with explicit models of divergence with gene flow (Hey and Nielsen 2004), are needed to quantitatively evaluate the contribution of hybridization. Based on our results of greater AFLP distinctiveness of *H. unicolor*, it is possible that rates of introgression are asymmetric between pairs of morphospecies, as Fisher (1980a) suggested.

Each of these hypotheses assume neutrality of AFLP markers, when in fact the L-shaped distribution of F_{ST} across hamlet loci is not consistent with neutral allopatric divergence (Beaumont 2005). Some of the markers showing higher levels of differentiation perhaps fall within or are closely linked to loci encoding

color pattern, color preferences, or other traits under disruptive selection. The viability of this hypothesis depends in part on the genomic coverage in our AFLP screen. The family Serranidae has a cellular DNA content of 1.12 picograms (Gregory et al. 2007), as does *Oreochromis niloticus*, with a linkage map of ~1000 cM (Kocher et al. 1998). Assuming a similar *Hypoplectrus* map, our initial screen of 692 AFLPs sampled one marker per 1.4 cM (~1400 kb), or about 23 markers per linkage group (Kocher et al. 1998).

AFLP surveys at comparable scale have resolved phylogenies of other species complexes. For example, Albertson et al. (1999) used 1205 AFLPs to resolve the phylogeny of nine Lake Malawi *mbuna* cichlid species. The AFLP phylogeny was congruent with male color pattern and female mate preference despite polyphyly of mtDNA sequences (Parker and Kornfield 1997). Similarly, Kai et al. (2002) found five fixed and 16 significant frequency differences from 230 AFLPs across three color morphs of *Sebastes inermis*. Our 253 AFLPs produced no marker with fixed differences, and only one with a significant frequency difference. Compared to other species complexes, genomic similarity in *Hypoplectrus* appears to be exceptional.

Conclusions

Hypoplectrus is best viewed as a complex of incipient species. Mating behavior, morphology, and syntopic distributions support this view, whereas AFLP and earlier molecular work might be taken to conflict with it. We suggest that future quantitative analyses of introgression can help account for the remarkable genomic homogeneity of hamlet species. Our study suggests that color pattern-based assortative mating is a primary force maintaining reproductive isolation between sympatric morphospecies. It also provides strong evidence that assortative mating has evolved in this system despite limited differentiation over broad stretches of the genome. Mate choice based on vivid color patterns may be an engine of speciation in hamlets and other coral reef fish, the most speciose of vertebrate groups.

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