ASSORTATIVE MATING AS A BARRIER TO GENE FLOW IN A CORAL REEF FISH SPECIES FLOCK

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ABSTRACT

While the only two recognized marine species flocks, the Pacific rockfishes and Antarctic icefishes, show marked morphological distinctions coupled with obscure genetic relationships due to their rapid radiation, the diversification of these flocks is believed to be very ancient (several millions of years before present). In contrast, the hamlets, Caribbean reef fishes of the genus *Hypoplectrus* (Serranidae), though highly differentiated with respect to their color patterns, do not show monophyletic relationships in mtDNA sequences and are monomorphic in several allozyme loci. Field observations show that mating is strongly assortative with regard to color pattern in sympatric *Hypoplectrus* species in reefs off Panama and Jamaica. In order to determine the strength of assortative mating and genetic differentiation in natural populations of the previously unstudied Florida Keys *Hypoplectrus* species, field surveys were conducted at several reefs and Amplified Fragment Length Polymorphisms were assayed in DNA of specimens collected at the study sites. Hamlet populations in the Upper and Middle Keys were composed mainly of blue (*H. gemma*, 23%) and butter (*H. unicolor*, 63%) hamlets, with black (*H. nigricans*) and barred (*H. puella*) hamlets present at low frequencies. Observation of 68 mating pairs suggested very strong assortative mating, with only mixed pair witnessed. Genetic distances between blue and butter hamlets, estimated from band sharing indeces based on 1108 DNA fragments, resulted in random clustering of individuals, with no monophyly according to color patterns. One AFLP fragment, however, showed strong frequency differences between the two morphospecies. This marker, coupled with the strong assortative mating observed, was regarded as evidence of partial reproductive isolation between *H. gemma* and *H. unicolor*. The lack of overall genomic differentiation thus suggested that the radiation of the 11 morphospecies of *Hypoplectrus* was recent and did not yet reflect species boundaries. The hamlet radiation is a

unique example of marine incipient speciation.

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CHAPTER 1: BACKGROUND AND OBJECTIVES

Background

Studying speciation in wild populations is arguably the most challenging area of evolutionary biology. Characterizing the process of speciation becomes increasingly more difficult as we study marine organisms. The shortage of obvious physical barriers allows the planktonic larvae of many marine organisms to disperse across wide geographic areas. Thus, extensive gene flow among distant regions of a species' range is possible, and little genetic structuring across populations is a commonly observed pattern (Palumbi 1992; 1994). Under this scenario, allopatric speciation is thought to occur at very slow rates (Mayr 1954). Consistent with this scenario, low levels of genetic structuring have been found in coral reef fishes (Shulman and Bermingham 1995), yet these form the most diverse group of vertebrates. Thus, characterizing barriers and mechanisms that initiate and maintain the formation of marine species, even at low levels of genetic differentiation, is of special concern to evolutionary biology.

While more marine physical barriers may yet be found, some alternative mechanisms are starting to receive empirical support. Instances of differentiation due to natural selection along a marine ecological gradient have been observed in mussels (Koehn et al. 1980) and killifish (Brown and Chapman 1991), where strong selection at allozyme loci resulted in clinal gene frequencies. Both of these cases illustrate how strong natural selection may drive differentiation in the face of high dispersal.

Historical events, such as rise and fall of sea levels, have also commonly been invoked to explain current-day patterns of differentiation (Reeb & Avise 1990; Domeier 1994; McMillan et al. 1999). Past low sea levels could have caused temporary geographic isolation in populations

that are now continuous, accounting for current differences among closely related species. On the other hand, populations that are now allopatric were likely once a single population in times of high sea levels, and only little differentiation has yet occurred.

The rapid evolution of genes directly involved in reproductive compatibility has more recently been suggested as a mechanism for creating barriers to free gene flow in the marine environment. Lysin protein found in sperm of the abalone genus *Haliotis* is responsible for allowing the sperm to penetrate the egg. The lysin of certain species is less efficient at promoting egg entry in heterospecific eggs, which reduces fertilization rates between species (Vacquier et al. 1990). Similarly, heterospecific crosses among species of *Echinometra* sea urchins (Palumbi and Metz 1991) resulted, in general, in significantly less fertilizations than conspecific crosses. In the case of the urchins, the reproductive protein involved in this fertilization barrier was bindin, which is found in the sperm and allows the sperm to successfully attach to the egg. As a result of the interaction between sexual conflict between gametes (to reduce polyspermy) and sperm competition, coevolution of the sperm proteins and their egg-bound receptors is believed to have driven prezygotic egg-sperm interactions to a species-specific level in these marine systems (Swanson and Vacquier 2002).

As more marine taxa are studied with the use of modern, more sensitive genetic approaches, we may be able to find novel evidence for genetic structuring. With this evidence in hand, we would then gain valuable insight about mechanisms for differentiation by comparing behavioral, physiological and ecological differences among closely related species.

Hamlets

Fishes of the genus *Hypoplectrus* are commonly known as hamlets. These are small, aggressive, predatory and brightly colored coral reef fishes in the seabass family Serranidae. The

family is characterized by the presence of three opercular spines and a highly mobile maxilla able to slide outside of the suborbital rim (Robins and Douglass 1986). Hamlets are simultaneous hermaphrodites, a common reproductive mode of the subfamily Serraninae (Breder and Rosen 1966; Barlow 1975; Fischer 1981; Robins and Douglass 1986), and their planktonic larvae remain afloat for about 22 days (Domeier 1994). The genus consists of about 11 putative species that are so far indistinguishable based on skeletal and meristic characters (no morphometric study has yet been published), but are easily separated by their remarkable coloration and patterns. The genus is restricted to the western Atlantic Ocean. The taxonomic status of these morphospecies is still argued among ichthyologists, but each "color morph" is now described as a distinct species (Eschmeyer 1998). The group was originally referred to as a monotypic genus with diverse subspecies (Jordan and Evermann 1896), but such classification is problematic under the definition of subspecies. Subspecies are phenotypically distinct subpopulations of a species that do not coexist in the same geographic region (Mayr 1963). Yet, the 11 hamlet morphospecies exhibit largely sympatric distributions, with as many as 7 morphs coexisting on the same reef at certain localities (Barlow 1975; Fischer 1980a; Domeier 1994). Sympatry argues that these color morphs cannot be categorized as subspecies, and they will be referred to in this paper as morphospecies.

Assortative Mating

During the day, hamlets are solitary, and the few interactions with other hamlets are limited to agonistic approaches (Fischer 1980b). At approximately 120 minutes before sunset, individuals form pairs, court and spawn. A pair will spawn several times in one night, alternating sex roles (Fischer 1979).

From 182 observations of hamlet mating pairs in Jamaica and Panama, Fischer (1980a)

found that 96% were between like-colored morphospecies, even when up to 6 morphospecies inhabited the same reef. Such strong assortative mating was also observed in laboratory mate choice experiments, where individuals were given a choice between a con- and a heterospecific fish (color-based assortative pairs formed in 100% of the trials; Domeier 1994).

Genetic Relationships

Although the strength of assortative mating so far observed among sympatric hamlet morphospecies may suggest significant differentiation among them, strong genetic evidence of such divergence has not yet been found. Only two investigations of genetic relationships among hamlet morphs have so far been performed. Graves and Rosenblatt (1980) surveyed a total of 32 allozyme loci across 10 *Hypoplectrus* color morphs, and found extremely low levels of polymorphism and heterozygosity, with no fixed allelic differences to characterize any of the morphs. By sequencing two mitochondrial DNA genes in 6 hamlet color morphs from Panama and Puerto Rico, McCartney et al. (2003) found very close relationships among the morphs, suggesting a recent radiation. A molecular clock for the same regions was calibrated using known divergence time of two geminate species of *Rypticus* (Grammistinae: Serranidae) that were separated by the rise of the Isthmus of Panama. The age of the hamlet radiation was estimated to be in the range of 370,000-430,000 years ago, corroborating the hypothesis of recency.

Hamlets into Perspective

Since genetic differences among hamlets are now understood to be very small, and several morphospecies often coexist, the potential for gene flow among them is very high. Mixed matings, however, were rarely observed in the field (7 out of 182 pairs; Fischer 1980a), while lab mixed matings occurred occasionally and only in no-choice trials (in these trials, the

experimental individual was offered only a heterospecific fish; Domeier 1994).

These studies suggest the potential role of assortative mating as the main force maintaining reproductive isolation of each morphospecies even under low levels of genetic differentiation. The large yet distinct differences in color pattern among hamlet morphospecies is remarkable, considering the recent estimated age for the radiation (McCartney et al. 2003). Variability in color pattern is often believed to be a great source of heritable phenotypic variation upon which evolutionary forces (especially natural and sexual selection) can act, and color-based mate choice is often an effective prezygotic barrier. The cichlid fishes of East African Lake Malawi and Victoria provide a clear illustration of this scenario. Very little genetic differentiation has been found among the hundreds of cichlid species in these lakes (Meyer 1993), but each species can be easily distinguished by characteristic bright colors and patterns. The extreme diversity in color patterns is thought to largely be the result of sexual selection (Seehausen et al. 1999). Assortative mating based on color patterns is strong (Seehausen and van Alphen 1998), and it is believed to maintain species boundaries despite their recent radiation (estimatedto be as recent as 14000 years ago in Lake Victoria; Meyer 1993). Another example involves the strawberry dart-poison frog (*Dendrobates pumilio*), in the Bocas del Toro Archipelago of Panama's Atlantic Coast. Six populations of *D. pumilio* show little genetic divergence but extremely different aposematic colorations (Summers et al. 1997). Lab mate choice trials showed that females preferred males of their own population in 80-90% of the trials. When placed under monochromatic light, which masked color pattern differences between races, females mated at random, serving as evidence that mate choice was based on color pattern (Summers et al. 1999). In contrast to the *Hypoplectrus* group, the different color morphs of *D. pumilio* are allopatric (each morph inhabits a different island). Intraspecific geographic variation

in female preference with regard to male coloration was also found in allopatric populations of Trinidad guppies, in which female preferences were in tune with the amount of orange pigmentation on males of their respective populations (Endler and Houde 1995).

Such strong mate choice divergence and assortative mating are common requirements in recent theoretical models that support the possibility for speciation in the absence of geographic isolation or physical barriers (Turner and Burrows 1995; Kondrashov and Shpak 1998; Dieckmann and Doebelli 1999). These models also predict that assortative mating will evolve, provided that it is genetically linked to a quantitative trait that is evolving under natural selection. In one model, the interplay of sexual and natural selection maintains phenotypic variation on the trait before mate choice diverges (Turner and Burrows 1995), while the others assume the trait is under disruptive selection (Kondrashov and Shpak 1998) or linked to an ecological character involved in resource competition (Dieckmann and Doebelli 1999). These models have been linked to theradiation of cichlid fishes in African (Schliewen et al. 1994) and Central American (Wilson et al. 2000) lakes, in which attempts have been made to identify the conditions under which those species radiated. Exemplified by the cichlids, the distinct divergence in a morphological trait amongst closely related species or races, which still remain only partially reproductively isolated, is referred to as incipient speciation (Wilson et al. 2000).

In the marine realm, incipient speciation has been suggested for morphs of Caribbean *Favia fragum* coral (Carlon and Budd 2002) and Mediterranean *Littorina saxatilis* snails (Johannesson et al. 1995). In the corals, two different coral morphologies were observed to be segregated along a depth gradient. Allozyme analysis showed reduced gene flow between the two forms, while morphological differences were thought to be maintained by selection on the differing habitats (Carlon and Budd 2002). In the snails, two sympatric morphs differ in shell

characters. The smooth morph is mainly confined to the lower rocky shore, while the ridged-banded morph is confined to the upper shore. The two morphs overlap in the middle shore, where some intermediate forms are found. Although genetic structuring is low, assortative mating is strong even in the 'hybrid zone.' The polymorphism in shell character was explained by the action of strong disruptive selection along the microhabitat gradient, and is believed to be maintained by assortative mating (Johannesson et al. 1995).

With evidence for a strong mating barrier, such as assortative mating, even under low genomic differentiation and high potential for gene flow, I would argue that the *Hypoplectrus* system is an example of incipient speciation. In contrast to the examples above, there is no strong evidence for ecological segregations or differences in selectivepressures among hamlet morphospecies. A model of speciation through aggressive mimicry was suggested by Thresher (1978), but was heavily criticized (Fischer 1980a; Domeier 1994). John and Helen Randall (1960) were the first to find morphological and color similarities between hamlets and species of other reef fish families. For instance, they suggested that the yellowtail hamlet can be easily mistaken for the adult form of the yellowtail damselfish *Microspathodon chrysurus* (Pomacentridae), and that the same can be said about the resemblance of the black hamlet to a surgeon fish (Acanthuridae). The most striking comparison was between the blue hamlet *H. gemma* and the blue chromis *Chromis cyanea* (Pomacentridae). Both have vivid blue color over the entire body, black upper and lower caudal fin margins and a deeply incised caudal fin (Randall and Randall 1960). Moreover, the authors observed blue hamlets swimming among small groups of blue chromis and frequently making predatory strikes at the damselfish. This led the Randalls (1960) to suggest the use of coloration for aggressive mimicry, allowing hamlets to get close to their prey (mostly small crustaceans) by disguising as a harmless species.

Thresher (1978) further developed this idea. For aggressive mimicry to occur, he suggested three criteria should be observed: (1) the geographic range of the mimic should be restricted to that of model, (2) mimics should be less common then their model, and (3) the mimic should very closely resemble the model. After comparing color patterns, distribution, abundance and habitat use, Thresher (1978) observed that hamlets fit all three criteria, and concluded that the opportunity for aggressive mimicry has led to the evolution of several hamlet color morphs.

Thresher's theory of aggressive mimicry was not well accepted mainly because hefailed to notice that hamlet behavior was significantly different from that of the proposed models (Fischer 1980a), which is an essential aspect for the success of an aggressive mimic. While the action of natural selection in causing divergence among hamlets cannot be ruled out at this point, significant ecological differences upon which selection could act have yet to be documented. This aspect, in addition to the coexistence of several, genetically similar but morphologically distinct morphospecies makes the *Hypoplectrus* system a unique opportunity for understanding the role of assortative mating in maintaining species barriers in the face of apparent gene flow.

Research Objectives

This study was conducted with the intent to investigate the level of assortative mating and genetic differentiation among hamlet color morphs that coexist on Florida's coral reefs, devoting special attention to the blue hamlet (*Hypoplectrus gemma*). This species is strictly endemic to the Florida Keys and Southeast Florida, while the other sympatric hamlets (*H. nigricans, H. puella* and *H. unicolor;* figure 1) occur at several other Caribbean locations. Florida hamlets have previously not been studied with regard to genetic structuring and field mating behavior.

To determine the level of genomic differentiation among Florida species, AFLP

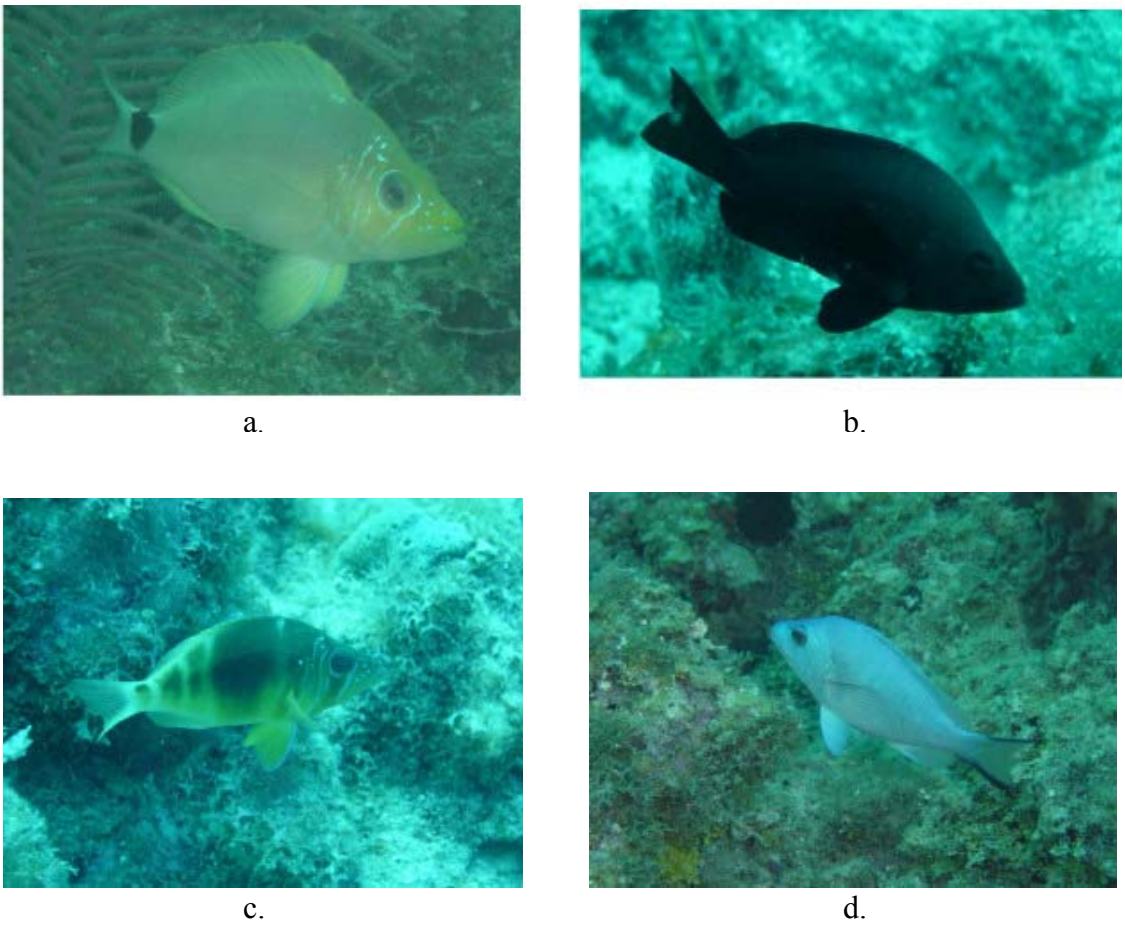


Figure. 1. The four morphospecies of *Hypoplectrus* found in reefs off the Upper and Middle Florida Keys. (Photographs by Dave Wells).

a. *Hypoplectrus unicolor*, butter hamlet

b. *Hypoplectrus nigricans,* black hamlet

c. *Hypoplectrus puella*, barred hamlet

d. *Hypoplectrus gemma,* blue hamlet

(Amplified Fragment Length Polymorphism) markers were used. AFLP is a PCR-based fingerprinting technique that has been used for several important applications, such as linkage mapping, positional cloning of genes, identification of bacterial and fungal strains, as well as estimating genetic diversity and relationships (Hill et al. 1996; Blears et al. 1998; Mueller and Wolfenbarger 1999; Riek et al. 2001). After digestion of genomic DNA by two restriction endonucleases, adaptor pairs are ligated to the ends of the fragments. These adaptors have known sequences that serve as priming sites, and subsequent PCR is performed to amplify fragments non-selectively. Finally, selective amplification is performed by using primers that have a known 3-bp extension into the unknown part of the fragment sequences. Therefore, a selective primer pair amplifies only a fraction of the preselectively amplified fragments. Each selective primer pair thus produces a different banding pattern. Each fragment can be considered a distinct independent character, and only 6-8 selective amplifications may produce a total of 700 to over 1000 fragments (Blears et al. 1998). Such a multilocus approach provides a good estimate of intra and/or interspecific genome-wide differences. Other advantages of AFLP include time and cost efficiency, reproducibility, and no need for *a priori* knowledge of the DNA under study. With AFLP markers, however, homologous alleles cannot be readily identified, and dominant inheritance precludes the ability to distinguish homozygous from heterozygous individuals. This reduces the applicability of the technique in population genetics (Mueller and Wolfenbarger 1999).

The objectives of the study were to:

1) Ascertain the relative abundances of Florida hamlet morphospecies, as well as determine whether the morphospecies co-occur or are segregated with conspecifics along a reef.

2) Determine whether Florida morphospecies mate at random or assortatively with respect to

color pattern.

3) Determine whether a molecular signature of marked non-random mating exists, as well as estimate an overall level of genomic differentiation by using AFLP.

CHAPTER 2: ABUNDANCE, DISTRIBUTION, AND MATING PREFERENCES

Introduction

The 11 recognized morphospecies of *Hypoplectrus* are distributed throughout the Caribbean, ranging from South Florida to the islands off Venezuela. In certain areas, up to 7 different morphospecies have been observed to coexist (e.g. Discovery Bay, and Holandes Cay; Domeier 1994). The relative abundance of each morphospecies varies considerably at different localities. The barred hamlet *H. puella* is the most abundant morphospecies in most places where it occurs. In the Florida Keys, however, the most abundant hamlet, according to Domeier (1994) is the butter hamlet, *H. unicolor*. This is the only locality in which this morphospecies is the most abundant. Only 3 other morphospecies are consistently found in the Florida Keys: *H. puella*, *H. nigricans* and *H. gemma.* The blue hamlet *H. gemma* is the only recognized *Hypoplectrus* morphospecies that is strictly endemic; all others occur in more than 2 localities throughout the range. The three other Florida hamlets contrast especially with the endemic blue hamlet in that they are the three most widely distributed morphospecies. *Hypoplectrus puella* is found throughout most Caribbean reefs, and is the only morphospecies found in the Gulf of Mexico. Although absent from the Gulf of Mexico, *H. unicolor* shows a similar distribution to *H. puella*, being found throughout the remainder of the range. *Hypoplectrus nigricans* is found throughout Central America, from Panama to Belize, and is present in all the islands in the Greater Antilles. It is absent from the Lesser Antilles, except from Dominica, and from the Yucatan Peninsula and Venezuelan islands (Domeier 1994). The presence of an endemic morphospecies, as well as the unusual relative abundances of the other Florida hamlets, makes the *Hypoplectrus* population inthe Florida Keys unique in composition.

Domeier's (1994) field surveys, though the most complete to date, were concerned solely

with broad-scale patterns in the distributions and abundances of all *Hypoplectrus* morphospecies. Moreover, much of his data came from answers to questionnaires. The only previous mating observations involving Florida hamlets were all performed in the lab (N=15; Domeier 1994). The most extensive field mating observations were conducted in Panama and Jamaica (N=182, Table 1; Fischer 1980a). Since the relative abundances of the different morphospecies differ with location, the strength of assortative mating may vary with morph and geography. Thus, this study also intended to document the strength of assortative mating in Florida Keys hamlet populations in the wild, given the likely unique composition of morphospecies found there.

Materials and Methods

Relative Abundances and Distributions

In order to determine which morphospecies inhabit Florida Keys reefs, as well as their relative abundances and distributions, daytime censuses were conducted using SCUBA. While at the National Undersea Research Center in Key Largo (November 18-22, 2002), and at the Keys Marine Laboratory (July 14-August 16, 2003), the following method was used for surveying hamlet populations in several reefs between 8:30am and 3:00pm:

A 50-meter yellow polypropylene line was used as a transect line. The line was marked at every 5-meter interval with a small strip of bright flagging tape, and each strip was numbered according to the length from it to one end of the line (i.e. 5m, 10m, 15m, etc…). These markings served to divide the line into 20 quadrats (10 on each side), with the perpendicular lines being imaginary projections across each marking. The color of the line and the strips allowed divers to maintain visual contact with the line, as well as keep track of their position along the transect.

The transect line was laid along the reef, generally parallel to the seaward edge of the

Table 1. Number of mating pairs of *Hypoplectrus* morphospecies of populations from Panama and Jamaica (from Fischer 1980a).

	Like Pairs						
Morphospecies	*H. unicolor*	*H. puella*	*H. indigo*	*H. aberrans*	*H. nigricans*	*H. guttavarius*	Total
Number of pairs	16	75	25	29	28	2	175

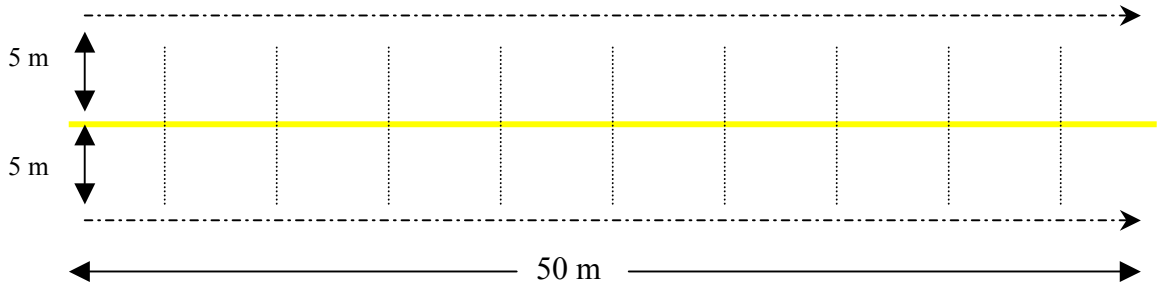
	Mixed Pairs			
Morphospecies	*H. unicolor* x *H. puella*	*H. aberrans* x *H. puella*	*H. aberrans* x *H. nigricans*	
Number of pairs	3	1	3	7
			TOTAL	182

reef, and secured at the ends with 12-inch spikes hammered to the sediment. Two divers swam along either side of the line, maintaining a distance of 5 meters from it (Figure 2a). On slates, the position of each individual was recorded by noting in which quadrat the fish was located. Even though most hamlets were solitary during the day census, any interaction observed between different individuals was recorded.

A total of 8 transects in two reef areas (Conch and Molasses reefs) were censused off Key Largo, and 32 transects in three reefs (East Tennessee, West Tennessee and X-Muta) off Long Key (see appendix A for coordinates). Initially, reefs at a wide range of depths (6m – 22m) were surveyed. However, very few individuals were found in reefs shallower than 13m. Thus, most of the surveys were performed in reefs in depths between 15m and 22m.

By swimming along the transect and 5m away from it, a total of 500m^2 were covered per transect, allowing the density of hamlets to be calculated.

Statistical analysis of daytime distributions was performed with EcoSim software (Gotelli and Entsminger 2001). This software performs null model tests on ecological parameters by using the observed data and creating expected values through random iterations according to commonly used statistical models. In order to format the censusdata for appropriate analysis in EcoSim, sets 4 adjacent quadrats were considered a spatial unit, or site (Figure 2b). Since 40 transects were surveyed, there were a total of 200 sites (40 transects x 5 units per transect). Only sites with more than one individual, regardless of species, were used in the analysis. The presence or absence of each morphospecies in each site was then entered in the EcoSim data matrix following the software's procedure (Figure 2c). A co-occurrence analysis was performed by testing for the index of number of species combinations (Pielou and Pielou 1968). This index is commonly used to detect patterns in certain species co-occurrences, usually due to



a.

Site 1		Site 2		Site 3		Site 4		Site 5	
blue	butter	butter			butter			black	blue
blue	black		butter		butter			barred	blue

b.

	Site 1	Site 2	Site 3	Site 4
Blue	1	0	0	1
Butter	1	1	1	0
Black	1	0	0	1
Barred	0	0	0	1

c.

Figure. 2. Procedure for data collection and analysis of hamlet abundances and co-occurrence.

a. Diagram of the transect line laid underwater to census hamlets. The position of each individual was plotted on slate according to the quadrants on the line.
b. Each four adjacent quadrants were considered a site for the purpose of scoring the occurrence of each morphospecies. Examples of occurrence data are given.
c. Scoring of the presence (1) or absence (0) of each morphospecies in each site.

competition. Even though this study did not attempt to measure competition, this index was useful in determining whether the different hamlet morphospecies co-occur randomly or certain pairs of species co-occur more often. Thus, if hamlet species pairs co-occur randomly, the observed number of species combinations should not differ significantly from that expected by chance. Conversely, an observed index significantly smaller than expected by chance would indicate that certain morphospecies rarely co-occur, such as is the case in competitively structured communities.

Mating Census

In transects where more than one morphospecies was observed, evening dives were conducted with the intent of censusing mating pairs. At approximately 90 minutes before sunset, the divers revisited one of the transects surveyed during the day and swam the same pattern. This choice of time corresponds to the beginning of hamlet reproductive behavior (Fischer 1980b). Individuals that remained less than 1m away from each other were assumed to be courting, and were counted as a mating pair. Divers recorded the position on the grid in which each mating pair was observed, as well as that of any non-paired hamlet. A small number of pairs were observed for the entire duration of their interaction to determine the frequency with which spawning could be assumed to occur following the observed courtship events. Moreover, all mating pairs between unlike morphospecies were observed until their interaction was finished, since the failure of such pairs to successfully spawn could suggest the presence of interspecific incompatibilities in mating behavior.

A Chi-square test for goodness-of-fit was used to determine whether the observed numbers of each type of mating pair were significantly different from those expected by chance. According to a binomial distribution, the relative abundances data collected during the day were

used to generate expected frequencies of mating encounters among all morphospecies on a reef. Since the number of mating pairs observed per census was low, the numbers for all transects were combined in a global measure.

Tissue Collection

For the genetic component of the study, tissue samples from a total of 68 fish were collected during the field seasons. One *H. gemma* was collected from East Tennessee, 56 (22 *H. gemma*, 28 *H. unicolor*, and 6 *H. puella*) from West Tennessee, and 11 (7 *H. unicolor* and 4 *H. gemma*) from X-Muta. The fish were collected by divers using drop nets and hand nets. In the first 55 captures, the fish were placed in "bongo" holds and brought to the surface slowly, where a small piece of caudal fin was clipped and placed in DMSO preservative. They were soon released and only 5 fish perished as a result of handling, even though most fish suffered from stress and barotrauma. To further reduce stress on the fish, the last 13 individuals were clipped and released underwater, without ever being placed in a hold. The latter technique was greatly successful inreducing mortality, since no fish died during handling and some clipped fish were even seen alive and seemingly in excellent condition weeks after capture.

Results

Abundance and Distributions

In both locations, the number of hamlets observed ranged from 0 to 22 individuals per transect. In the three dives conducted at depths shallower than 8m, only a total of 4 hamlets (1 *H. gemma,* 1 *H. nigricans*, and 2 *H. unicolor*) were observed. The highest densities were found between 15-22m depths, in reef tracts usually located near the reef edge. These reefs also had relatively low coral relief and wide hard coral cover. Reefs dominated by soft corals and

sponges, even at depths of 15-20m, had relatively low hamlet densities. Including only transects with 5 or more individuals, the average densities of hamlets were 0.024 fish/m^2 in Key Largo and 0.022 fish/m^2 in Long Key. *Hypoplectrus gemma* and *H. unicolor* together comprised over 86% of Long Key populations, and ~83% of Key Largo (Table 2). While *H. nigricans* were not observed in Key Largo, they were nearly as abundant as *H. puella* in Long Key. Besides the four morphospecies that were expected to be found, a few rare morphs were observed. A shy hamlet (*H. guttavarius*) was seen alone at one location, while at another location, 3 tan (*Hypoplectrus* sp.) hamlets were found in the evening on the same quadrat. Finally, 2 individuals with an unidentifiable color pattern (but identical to each other) were observed in courtship.

For the co-occurrence analysis, a total of 139 sites were identified and analyzed, and only the four main morphospecies were considered. The index of number of species combinations observed was not significantly different from that expected by chance (observed = 9.000, expected = 9.972, p=0.307, table 3). Thus, among *H. gemma*, *H. unicolor*, *H. puella* and *H. nigricans*, there was co-occurrence of each species pair. Also, numerous aggressive chases were observed between individuals of the same and of different morphospecies.

Mating Census

A total of 68 mating pairs were observed during 28 evening dives, and only one of those was between unlike morphospecies. The pair of unlike morphospecies (*H. gemma* x *H. unicolor*) was observed to spawn 5 times during the spawning period before separating, while the only pair of tan hamlets spawned 6 times. Since there were only 2 pairs observed in Key Largo, statistical analysis was performed only in the Long Key data (Table 4). The number of like pairs was significantly larger and that of unlike pairs significantly lower than if mating was random (X^2=93.8, df=5, p<0.0001), indicating the presence of strong assortative mating.

Table 2. Relative abundances of *Hypoplectrus*. Censuses were conducted during daytime off Long Key (July-August 2003) and Key Largo (November 2002).

	Long Key (% total)	Key Largo (% total)	TOTAL (% total)
Butter (*H. unicolor*)	204 (63.9%)	48 (53.3%)	252 (61.6%)
Blue (*H. gemma*)	71 (22.3%)	26 (28.9%)	97 (23.7%)
Barred (*H. puella*)	22 (6.9%)	15 (16.7%)	37 (9.0%)
Black (*H. nigricans*)	17 (5.3%)	0	17 (4.2%)
Tan	3 (0.9%)	0	3 (0.7%)
Unknowns	2 (0.6%)	0	2 (0.5%)
Shy (*H. guttavarius*)	0	1(1.1%)	1 (0.2%)
TOTAL	319	90	409

Table 3. Total pairwise co-occurrences. Number of sites in which each *Hypoplectrus* species combination was found. A site was defined as a unit composed of four adjacent transect quadrants; only sites with more than one individual were considered. Censuses were conducted in reefs off Long Key and Key Largo.

	H. gemma	*H. unicolor*	*H. nigricans*	*H. puella*
H. gemma	4			
H. unicolor	83	33		
H. nigricans	10	11	0	
H. puella	16	26	0	0

Table 4. Number of mating pairs observed and expected during evening censuses off Long Key. Divers entered the water at ~90 minutes before sunset. Expected values were estimated through a binomial distribution using observed relative abundances.

	observed	expected
Blue x Blue	17	3.26
Butter X Butter	39	26.9
Barred x Barred	2	0.314
Black x Black	5	0.187
Tan x Tan	1	0.006
unknown x unknown	1	0.003
Blue x Butter	0	18.8
Blue x Barred	1	2.03
Blue x Black	0	1.57
Blue x Tan	0	0.276
Blue x unknown	0	0.184
Butter x Barred	0	5.82
Butter x Black	0	4.50
Butter x Tan	0	0.794
Butter x unknown	0	0.529
Barred x Black	0	0.485
Barred x Tan	0	0.086
Barred x unknown	0	0.057
Black x Tan	0	0.066
Black x unknown	0	0.044
Tan x unknown	0	0.008
TOTAL	66	66

Discussion

Although parameters in ecology and microhabitat use of hamlets have yet to be studied, the results of the co-occurrence analysis (as well as observations of aggressive interactions among the different hamlet morphospecies) strongly suggest that morphospecies co-occur randomly in a given reef. This result indicates that the high level of assortative mating observed was due to active mate choice, instead of simple likelihood of encounter. In other words, since individuals were not found around only conspecifics, each had to make a choice of with which morphospecies to mate. This is important because it implies that behavioral interactions culminating in assortative mating are essential for maintaining color pattern distinctiveness, and not habitat choice.

Concordant with previous estimates of levels of assortative mating (96% in 182 pairs, Fischer 1980a), strong color-based assortative mating was observed. Besides the statistical evidence obtained from the census data, the importance of certain individual cases should not be overlooked. If mating was random with respect to color, the rarest morphs would more often mate with unlike individuals by simple probability. Further support for this prediction comes from Domeier's (1994) no-choice experiments, in which mixed matings occurred much more often when individuals were not given a choice of a conspecific.

Thus, even under assortative mating, mate choice was predicted to be dependent on the frequency of each morphospecies, with the rarest ones being more 'promiscuous.' This pattern was observed in Darwin's finches in the island of Daphne Major, in the Galapagos Archipelago. From the four species present on the island, *Geospiza fuliginosa* composed, on average, less than 2% of the breeding population (7 out of 396 breeding individuals). While on average only 0.8-1.8% of the individuals of the other three species hybridized, 73% of the *G. fuliginosa* breeding

population hybridized with another species (Grant and Grant 1998). The difficulty in finding mates due to its rarity on the island was the reason attributed to this species' frequent hybridization events.

This prediction did not hold true for Florida hamlets based on this study's field surveys. Black hamlets (*H. nigricans*) mated with one another 25 times as often as expected from chance, and the single tan hamlet mating observed exceed its likelihood of occurring by chance over 150-fold. With a relative abundance of only 0.9%, theprobability that two tan hamlets encountered each other among all hamlets observed was only 0.000081. The fact they did find each other and spawned successfully provides strong evidence that very strong species recognition is operating. While rarity might be largely the reason why *Geospiza fuliginosa* hybridizes frequently, the similarity in song and phenotype between *G. fuliginosa* and *G. fortis* probably explains the fact that all promiscuous *G. fuliginosa* individuals mated only with *G. fortis*, and never with the other two species in Daphne Major (Grant and Grant 1998). In other words, weak distinctions in the species recognition system (song and phenotype) of these two species likely resulted in "mistaken" mate choice. In *Hypoplectrus*, as suggested by evidence in other brightly colored reef fishes (Thresher and Moyer 1983; Warner et al. 1975), color pattern is likely the main trait used in social communication. Nevertheless, the observation of a mixed mating between blue (*H. gemma*) and barred (*H. puella*) hamlets, two extremely different color patterns, suggests that other cues complement color in mate choice assessments. Laboratory mate choice experiments would be useful in controlling for chemical and acoustic signaling. Moreover, color pattern could also be controlled in those experiments by using monochromatic light, as performed with frogs (Summers et al. 1999) and African cichlids (Seehausen and van Alphen 1998). Although the complex courtship behavior described in *Hypoplectrus* is believed to be involved in

egg-trading and avoidance of gamete wastage (a pair of simultaneous hermaphrodites need to decide on respective sex roles; Fischer 1979, 1981), the presence of such behaviors provides many opportunities for incompatibilities and mate preference differences to evolve as well. Whatever combination of cues is involved in species recognition and mate choicein *Hypoplectrus*, the strength of positive assortative mating in common as well as rare hamlets suggests that a strong behavioral barrier to gene flow exists. Theoretical models predict that genetic linkage between mate preference (assortative mating) and signaling traits (color or behavior) must exist for sympatric forms to diverge (Turner and Burrows 1995; Kondrashov and Shpak 1998; Dieckmann and Doebelli 1999). This suggests the same linkage in hamlets may have facilitated their divergence.

Observations of color morphs that cannot be visually categorized as any of the known morphospecies, such as the ones seen in this study, may represent intermediate forms that resulted from mixed matings (see also Thresher 1978). The frequency and abundance of these forms, however, are very low, and are far from forming a 'hybrid swarm.' In a system where color pattern identity seems to be essential in mate choice, hybrid phenotypes are likely selected against during courtship. Thesher's (1978) aggressive mimicry hypothesis cannot be ruled out as possible explanation for reduced hybrid fitness. Under this scenario, hybrid phenotypes would likely be poor mimics of a planktivorous fish species, and would therefore be less successful in obtaining food. Finally, inviability and infertility of hybrids have also not yet been tested, and they could account for the low frequency of intermediate phenotypes.

Geographic environmental variation throughout the broad range of *Hypoplectrus* distribution is likely to create different natural selective pressures at different localities. Therefore, differential fitness associated with color pattern or pleiotropic traits would result in

largely different relative abundances at different locations, as found in this system. Variation in fitness among hamlet morphospecies could also explain the disjunct (non-continuous) distribution exhibited by some (e.g. *H. nigricans, H. chlorurus, H.aberrans, H. gummigutta* and *H. indigo*; Domeier 1994). Even though *Hypoplectrus* larvae are known to remain afloat for approximately 22 days (Domeier 1994), the possibility of regional larval retention, as recently evidenced in other reef fishes (Swearer et. al 1999; Jones et al. 1999; Taylor and Hellberg 2003), also cannot be ruled out as partially involved in explaining these patterns.

CHAPTER 3: GENETIC ANALYSES

Introduction

Although the original study examining *Hypoplectrus* genetic relationships failed to find polymorphism in allozymes (Graves and Rosenblatt 1980), significant genetic variation was found in mtDNA and nuclear DNA microsatellite loci (McCartney et al. 2003). The latter study found significant levels of frequency differences in microsatellite alleles among most morphospecies of Panama and Puerto Rico, revealing a signature of non-random mating. The variation found in mtDNA sequences, though substantial, was not concordant with species boundaries and did not form monophyletic clusters according to morphospecies.

These previous results are consistent with other studies of mtDNA divergence in young species flocks. As mentioned above, the cichlids of eastern African lakes have been widely studied as models for the scenario of divergence in the face of gene flow. Genetic differentiation in mtDNA genes have not been concordant with the extreme color pattern differences among sympatric cichlid species, and this has been taken as evidence of a very recent radiation (Meyer 1993). The first study to document strong monophyly of African cichlid species used Amplified Fragment Length Polymorphisms (AFLP) instead of DNA sequences. Albertson et al. (1999) were able to provide a strong hypothesis for the phylogeny of 9 species of "mbuna" cichlids from Lake Malawi with the use of 11 AFLP selective primer pairs. High levels of polymorphism were found (over 50%) and, although pairwise genetic distances were low, AFLP frequency differences were concordant with species boundaries. The use of hundreds of genome-wide polymorphisms may provide a more sensitive detection of frequency differencesamong hamlet morphospecies, and the AFLP technique was used in this study to determine whether there is molecular evidence of at least partial reproductive isolation.

Materials and Methods

From initial abundance estimates in the field, *H. gemma* and butter hamlets were estimated to compose over 85% of the hamlet populations in the areas surveyed. Thus, individuals of these two morphospecies were more likely to encounter conspecifics and consequently have more opportunities for assortative mating. Hence, only *H. gemma* and *H. unicolor* were selected for the AFLP analysis.

DNA Extraction

From fin clips collected in the field, DNA from 6 *H. gemma* and 6 *H. unicolor* was extracted following a standard mammalian DNA extraction protocol (Maniatis et al. 2000). Since a large number of polymorphic characters are usually produced by AFLP amplifications, a small number of individuals is typically sufficient for a reliable analysis. A small piece (5 x 8mm) of fin was placed in a microcentrifuge tube containing 600μL extraction buffer (appendix B) and homogenized with a pestel. Three μL proteinase K (20 mg/ml) were added and tubes inverted to mix. Samples were then incubated at a 55°C water bath for 8-16 hours. After cooling to room temperature, 200μL potassium acetate solution was added to each and samples were vortexed vigorously for 10 seconds. Sample were centrifuged at maximum speed at 4°C for 3 minutes. Approximately 650μL of supernatant of each sample were transferred to new tubes and pellets discarded. Six hundred μL 100% isopropyl alcohol were added and mixed to precipitate DNA, and samples were centrifuged at max at room temperature for 5 minutes. Supernatantwas poured out, 600μL 70% ethanol were added, and tubes were inverted to wash the pellets. Samples were then centrifuged at max speed for 1 minute. Supernatant was poured out, and the remaining alcohol was evaporated by roto-suction, leaving a dried pellet. After extraction, DNA pellets were resuspended in 50 μL ddH_2O instead of TE (Tris-EDTA) buffer, since the presence of

EDTA would inhibit ligation of adaptors during the AFLP restriction-ligation reactions. The DNA samples were finally cleaned with QIAGEN Quick columns following the kit's PCR purification protocol, and stored at -20°C.

AFLP Fingerprinting

For DNA restriction, the endonucleases *EcoRI* and *MseI* were used, and the entire restriction-ligation reaction procedure closely followed the Applied Biosystems AFLP Plant Mapping Protocol (P/N 4303186; Appendix C). After overnight incubation, the 11μL restriction-ligation reactions were diluted in 189μL $TE_{0.1}$ (where 0.1 refers to $1/10^{th}$ the EDTA concentration). For pre-selective amplification, 4μL diluted restriction-ligation reactions, 1μL pre-selective primers and 15μL AFLP Core Mix were mixed in PCR tubes. All amplifications were performed in a MJ-Research PTC-100 thermal cycler, and the pre-selective PRC profile was as follows: 72°C/2 minutes, followed by 20 cycles of 94°C/20 seconds, 56°C/30 sec., 72°C/2 min., then held at 60°C/30 min. After amplification, 10μL of the product were run on a 1.5% Agarose gel in 1X TBE at 9 V/cm for 1.5 hours. The gel was then post-stained in 0.5 mg/ml EtBr and visualized under UV. In successful amplifications, a smear ranging from 50bp to 500bp was observed. The remaining 10μL of the pre-selective amplifications were diluted in 190μL $TE_{0.1}$ and stored at 4°C. To generate individual AFLP fingerprints, selective amplification wasperformed. Selective primers have the same sequence as the preselective primers, but with a 3-bp extension, which allows the primers to anneal only to selected fragments. Thus, each selective primer combination amplifies a different set of fragments. The 12 samples were assayed across 52 selective primer combinations (Table 5). For selective amplifications, 3μL diluted pre-amplification products, 1μL *Eco*RI primer, 1μL *Mse*I primer, and 15μL AFLP Core Mix were mixed and amplified with the following profile: 94°C/2 min., followed by 10 cycles of

94°C/20 sec., 66°C/30 sec. with -1°C per cycle, 72°C/2 min., then 20 cycles of 94°C/20 sec., 56°C/30 sec., 72°C/2 min., and finally held at 60°C/30 minutes.

After selective amplification, fragments were electrophoresed and visualized in the automated ABI 3100 Genetic Analyzer with ROX-500 size standard. Such a method was made possible because the selective *EcoRI* primers were factory-labeled with a fluorescent dye. This allowed for up to 3 different PCR products to be electrophoresed together, given that each reaction used a different dye. Thus, time and cost were greatly decreased. Scoring and sizing of fragments were performed by ABI GeneScan 3.1 software, using the Local Southern Method of size calling and lowest peak detection settings (amplitude threshold of 50 rft for all dyes and minimum peak half width of 2 pts.).

For each of the 52 primer combinations assayed, the electropherogram of the 12 individuals (6 *H. gemma* and 6 *H. unicolor*) were aligned by size and each site (peak) was visually checked for state (presence/absence) in each individual. The number of polymorphic sites was counted in each primer combination, paying special attention to those that differed between *H. gemma* and *H. unicolor*. A polymorphic site was defined as a fragment that is present in at least one but not all individuals. This was done in order to find a subset of primer sets that showed the largest amount of polymorphism.

As a result of this initial investigation, ten primer combinations were chosen as most polymorphic and were thus used for the complete analysis. These were P3, P5, P6, P18, P37, P38, P41, P45, P46 and P50. Using ABI Genotyper software, the fragments produced across all 12 individuals for each primer were aligned by size and then exported to an Excel spreadsheet. The presence or absence of each fragment was then scored across all primers, creating a binary matrix. These data were finally used to estimate overall genetic distances among the individuals

Table 5. AFLP selective primers across which 12 hamlets were assayed. The 3-bp extensions are shown in the first row and column. The *Eco*RI primers contained a fluorescent dye, represented by the different colors (FAM, NED, and JOE), and amplification products were electrophoresed and scored in a ABI 3100 Genetic Analyzer.

Mse I primers

*Eco*RI primers	CAA	CAC	CAG	CAT	CTA	CTC	CTG
ACT	P1	P2	P3	P4	P5	P6	P7
ACA	P9	P10	P11	P12	P13	P14	P15
AAC	P17	P18	P19	P20	P21	P22	P23
ACC	P25	P26	P27	P28	P29	P30	P31
AGC	P33	P34	P35	P36	P37	P38	
AAG	P41	P42	P43	P44	P45	P46	
AGG	P49	P50	P51	P52	P53	P54	
ACG	P57	P58	P59	P60	P61	P62	

using two different methods. First, pairwise band-sharing was calculated from the binary matrix by adding the number of differences between each pair of individuals and dividing these numbers by the total number of fragments produced. For the second estimate, the binary matrix was imported into TFPGA software (Tools For Population Genetic Analysis; Miller 1997) and analyzed using Nei's coefficient (1978) of genetic distance. Finally, both distance matrices were imported into PAUP (Swofford 1997) and dendrograms were constructed using neighbor-joining and UPGMA methods.

In addition to allowing ten primer sets to be selected, the initial visual assessment of fragment polymorphisms indicated the presence of two sites, one in P3 and one in P6, that showed strong frequency differences between *H. gemma* and *H. unicolor*. Thus, in order to fully examine these potential genetic differences, the number of individuals assayed on these two primer pairs was increased to 18 of each morphospecies. After the state of these sites was determined for all individuals, an exact test was conducted using TFPGA in order to test for significant frequency differences.

Since the AFLP procedure involves PCR that is dependent on efficient restriction and ligation, it is important to ensure the technique is reproducible, in order to avoid comparing 'false' polymorphisms. To test for reproducibility, DNA from one individual of each morphospecies was taken through the entire procedure in two replicates, and amplified across the ten chosen primer combinations. The replicate fingerprints were compared visually to ensure that nearly identical patterns were observed.

Results

AFLP Reproducibility

By aligning the two replicates of each individual used in this assessment, the number of reproduced fragments was counted visually in ABI GeneScan software. In 7 of the primers used, the fragments smaller than 75 bp and greater the 600 bp, including the ROX size standard, were misaligned, indicating perhaps a mobility inconsistency in such small pieces during electrophoresis. The ROX size standard of size 75 bp was the first to be sized and aligned correctly in both replicates, with all larger size markers behaving consistently. Thus, for all analyses, including the reproducibility assessment and genetic distances estimates, only fragments between 75 and 600 bp were considered. In each of the two individuals used for this assessment, a total of 445 fragments were generated across the ten chosen primer pairs. While in the butter hamlet used (id FL 26) 3 peaks were not reproduced, only one peak failed to reappear in the blue hamlet (FL 38). Thus, between the two individuals, the average reproducibility was 99.6%. Since the level of confidence in this technique was found to be high, all peaks produced in the entire dataset (within the 75-600 bp range) were used for the distance measures.

Genetic Distances

The individuals assayed across the ten chosen polymorphic primer pairs were FL 1, FL 3, FL 4, FL 38, FL 50, FL 60 (*H. gemma*), and FL 20, FL 26, FL 39, FL 42, FL 39 and FL 53 (*H. unicolor*). A total of 1108 distinct fragments were generated across the entire data set, 913 of which were polymorphic. None of the fragments produced was diagnostic to either morphospecies. Even though the two distance estimates used produced slightly different values (Table 6), the positions of the individuals in the dendrograms were identical for each clustering method used. In other words, the neighbor-joining dendrograms of both Nei's coefficient and

Table 6. Genetic distances matrix. Distances were estimated for *Hypoplectrus gemma* (blue font) and *H. unicolor* (red font) based on 1108 AFLP fragments generated across 10 selective primer combinations. All individuals were collected from reefs off Long Key, Florida. Two pairwise distance measures were calculated: simple band-sharing (above diagonal) and Nei's (1978) coefficient (below diagonal).

	FL 1	FL 3	FL 4	FL 38	FL 50	FL 60	FL 42	FL 49	FL 39	FL 53	FL 20	FL 26
FL 1		0.367	0.276	0.272	0.339	0.303	0.265	0.310	0.301	0.293	0.311	0.280
FL 3	0.4572		0.317	0.330	0.257	0.373	0.331	0.323	0.373	0.354	0.377	0.329
FL 4	0.3229	0.3809		0.237	0.321	0.274	0.211	0.268	0.280	0.261	0.282	0.252
FL 38	0.3175	0.401	0.271		0.290	0.212	0.221	0.227	0.216	0.233	0.202	0.166
FL 50	0.4144	0.2974	0.3876	0.3421		0.327	0.329	0.277	0.316	0.310	0.322	0.292
FL 60	0.3605	0.4664	0.3207	0.2384	0.3956		0.267	0.281	0.188	0.281	0.192	0.201
FL 42	0.308	0.4023	0.2372	0.2499	0.3983	0.3108		0.245	0.271	0.232	0.268	0.239
FL 49	0.3705	0.3902	0.312	0.258	0.3245	0.3295	0.2805		0.241	0.217	0.258	0.218
FL 39	0.3577	0.4664	0.3282	0.243	0.3796	0.2079	0.3157	0.2757		0.270	0.189	0.210
FL 53	0.3464	0.4366	0.3022	0.2651	0.3704	0.3295	0.2639	0.2441	0.3145		0.258	0.224
FL 20	0.3719	0.4736	0.332	0.2259	0.3889	0.2135	0.312	0.2986	0.209	0.2986		0.213
FL 26	0.3284	0.3983	0.2901	0.1816	0.3446	0.2247	0.2733	0.2464	0.2361	0.2534	0.2395	

band-sharing methods were identical to each other, and the same was true for the UPGMA trees. In fact, all four trees had nearly identical clustering, with the only difference being the position of FL 1. Regardless of which dendrogram was examined, the same information was conveyed: there were no monophyletic clusterings with respect to color pattern (Figure 3).

Character Frequency Differences

In the primer combination P6, the site at 141 bp showed potential for frequency differences based on the initial investigation. From the 36 individuals (18 of each morphospecies) assayed on this primer, 9 blue hamlets and 4 butter hamlets exhibited this fragment. This small frequency difference was not significant according to an exact test ($p=0.167$).

The same procedure was performed on site 107 bp from P3, on which 35 individuals (17 blue hamlets) were amplified. In this case, the exact test detected a significant frequency difference ($p=0.0107$), with the fragment present in 16 butter hamlets and in 8 blue hamlets. Upon closer examination of all 35 electropherograms, it was noticed that peak 107 was consistently higher in butter hamlets than in blue hamlets (Figure 4). Since the peak height is largely dependent on PCR efficiency, the overall peak heights may differ across different pherograms, which would prohibit direct comparison of this trait among different individuals. Thus, corrections were designed with the intention of appropriately scaling the height of peak 107 in all individuals. In order to further investigate this quantitative difference, two corrections were employed. First, the average peak height of each electropherogram was calculated. By dividing each average by the smallest average obtained, a conversion factor was calculated for each pherogram. The original height of peak 107 was then divided by each respective conversion factor, scaling each height to that of the individual with lowest peaks. Considering

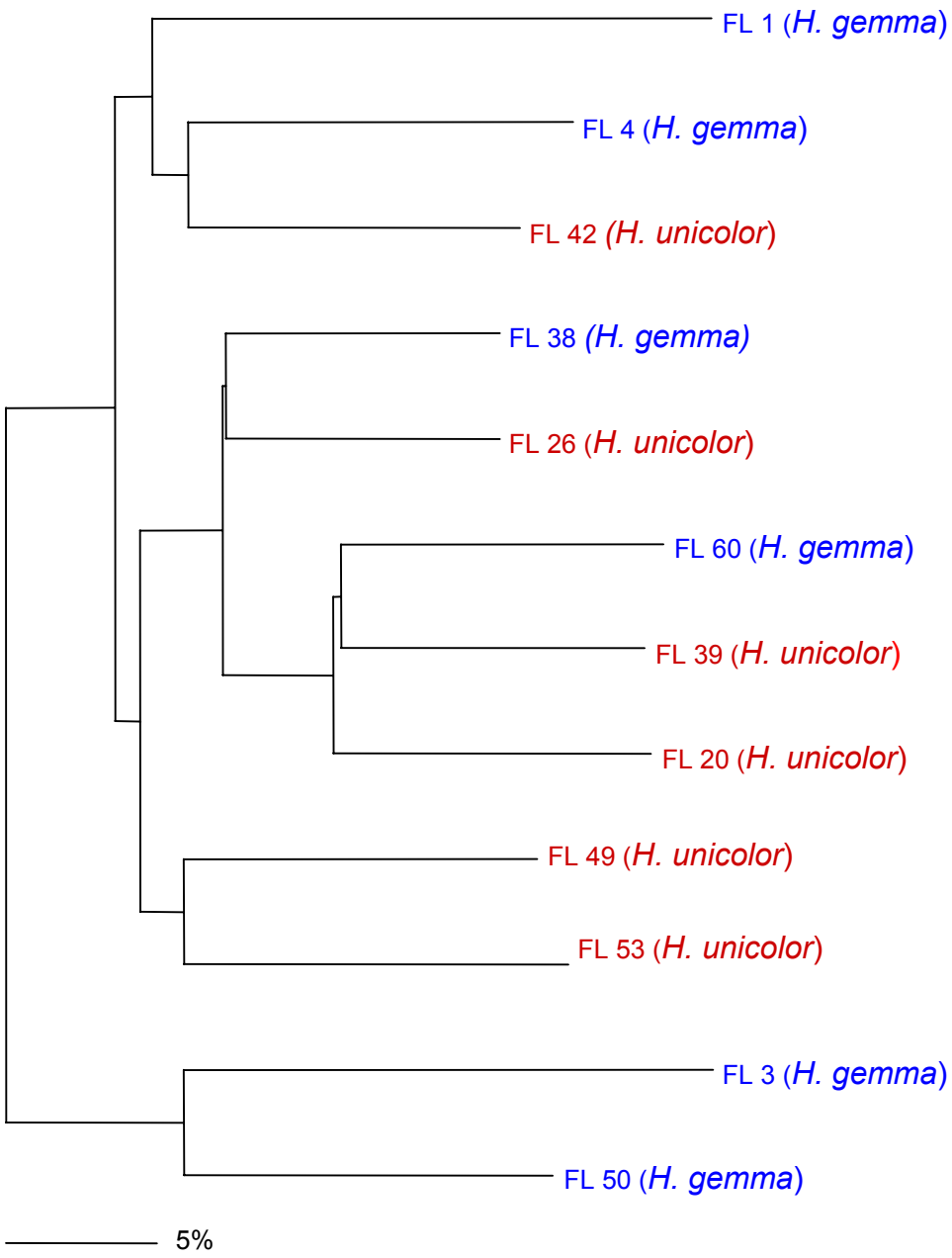


Figure 3. Neighbor-joining dendrogram of *Hypoplectrus gemma* and *H. unicolor*. Relationships based on Nei's coefficient of genetic distances estimated across 1108 AFLP fragments generated from 10 selective primer combinations. All samples were collected from reefs off Long Key, Florida.

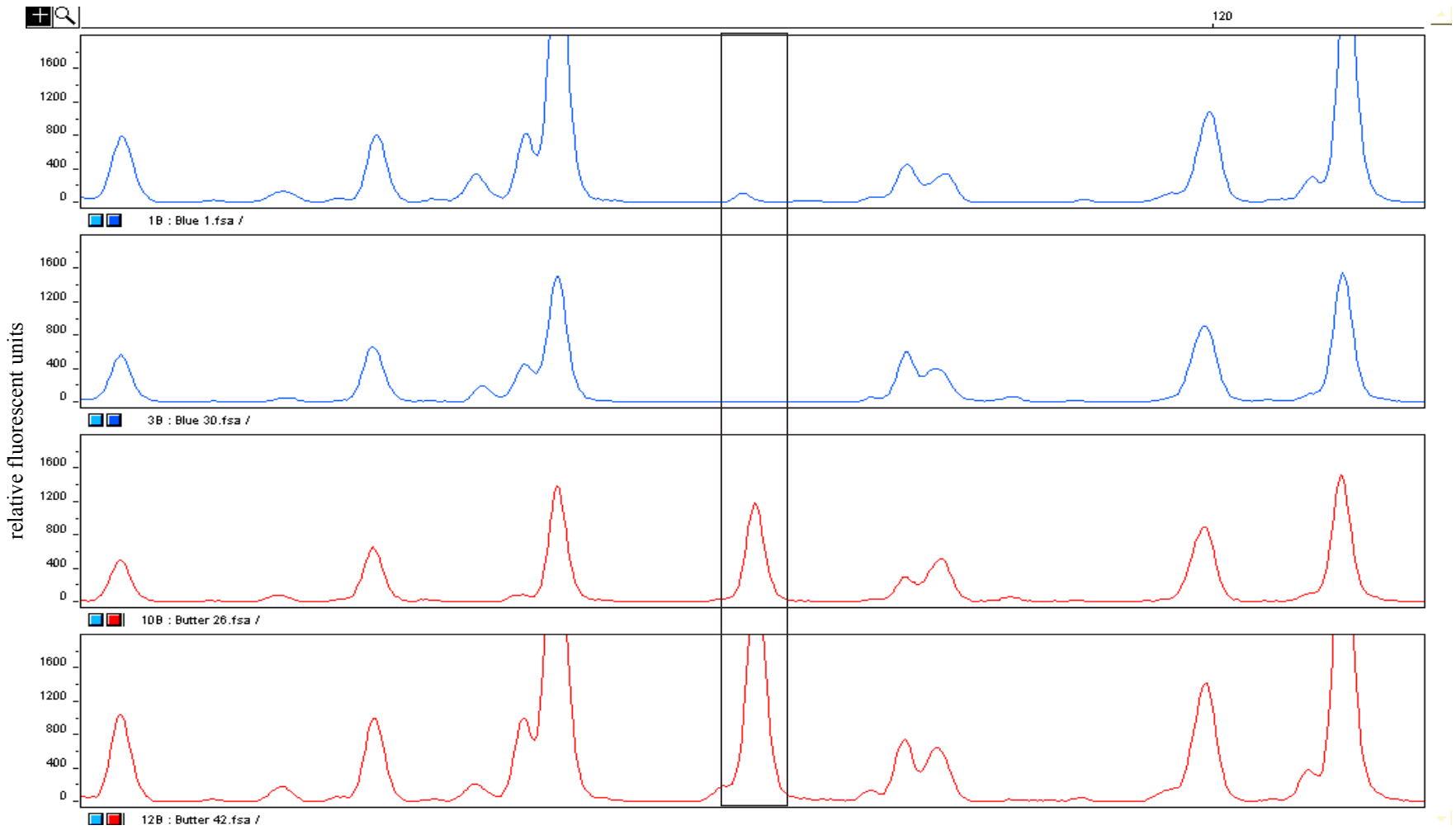


Figure 4. Section of AFLP electropherograms. The region inside the box is peak size 107, showing a difference in height between the two morphospecies *H. gemma* (blue) and two *H. unicolor* (red). Electropherograms were generated in ABI GeneScan software.

the original peak amplitude threshold of 50 rft, a new count was performed on the corrected heights, eliminating all peaks lower than 50 rft. As a result of this correction, 3 extra peaks from blue hamlets were considered "undetected," comprising even stronger significant frequency difference ($p=0.0002$).

The second adjustment on the height of peak 107 was performed by dividing the original height of the peak by the sum of all peak heights in its respective pherogram. This estimated the proportion of that peak's height with respect to the total height generated in the amplification. Proportions were then arcsine transformed and a t-test was used to test for difference in mean proportions. Average proportion of height of peak 107 was significantly higher in *H. unicolor* than in *H. gemma* ($p<0.0001$; table 7).

Discussion

Even though a high level of overall genetic polymorphism was observed (82% of all AFLP sites generated were polymorphic), there were not enough frequency differences between *H. gemma* and *H. unicolor* to separate the two into distinct clusters. A similar result using AFLP was found in the fungus *Melampsora* epitea, in which four geographically separated populations (in Chile, France, Ireland, and Sweden) failed to cluster despite high levels of polymorphism (87% polymorphic AFLP markers; Hurtado and Ramstedt 2002). In contrast, Albertson et al. (1999) found strong phylogenetic resolution among four species of *mbuna* cichlids even though less polymorphic AFLP markers were produced (53%). Since feeding morphology adaptation and sexual selection are thought to have played important roles in the cichlid radiation (Meyer 1993; Albertson et al. 1999), strong and widespread species-specific genetic linkages were likely formed, accounting for the overall frequency differences in AFLP markers.

Table 7. Normalized peak heights. Theses were calculated as proportion of the height of peak 107 (primer combination P3) with respect to the sum of the heights of all peaks in its respective electropherogram.

H. gemma	Proportion	*H. unicolor*	Proportion
FL 17	0	**FL 12**	0
FL 1	0.002	**FL 16**	0
FL 29	0	**FL 19**	0.032
FL 30	0	**FL 20**	0.03
FL 32	0	**FL 23**	0.029
FL 34	0.004	**FL 26**	0.038
FL 38	0.009	**FL 27**	0.037
FL 3	0.003	**FL 28**	0.01
FL 47	0	**FL 2**	0.042
FL 4	0.002	**FL 33**	0.008
FL 50	0.006	**FL 36**	0.03
FL 57	0	**FL 39**	0.036
FL 58	0	**FL 42**	0.046
FL 60	0.012	**FL 44**	0.084
FL 61	0.006	**FL 45**	0.006
FL 62	0	**FL 48**	0.032
FL 65	0	**FL 49**	0.017
Mean	0.00265	**FL 53**	0.038
		Mean	0.0286

High levels of genetic polymorphism were also found by McCartney et al. (2003) using DNA microsatellites in Panamanian and Puerto Rican hamlets, and both studies contrast with the initial allozyme study (Graves and Rosenblatt 1980). Since the AFLP technique is assumed to survey most of the genome, most of the polymorphisms are likely found in neutral genetic regions. According to theoretical models, a considerable amount of time is required for neutral markers to sort into monophyletic clusters, even in the presence of reproductive barriers (Avise et al. 1984). These models also predict that lineage sorting is even slower in large effective populations. Given the wide geographic ranges of most *Hypoplectrus* morphospecies, populations of these fishes can be assumed to be large, retarding lineage sorting. Thus, the current polyphyletic state of genomic differentiation between *H. gemma* and *H. unicolor* is likely due to recent divergence.

An obvious alternative explanation for the lack of phylogenetic resolution andhigh genetic similarity between the two morphospecies is hybridization. However, the presence of a significant frequency difference in one AFLP site (peak 107 in P3) is molecular evidence of at least partial reproductive isolation between *H. gemma* and *H. unicolor*. This signature, when coupled with the fact that no mixed mating between them has yet been reported from the field, supports the hypothesis of recency for explaining the lack of monophyletic differentiation in the rest of the genome. Thus, significant yet largely incomplete genetic differentiation has occurred. A thorough search for AFLP differences among the other morphs coupled with more mating data among them would be essential in investigating this hypothesis. Finally, gene flow between two morphspecies would require introgression, or backcrossing of hybrids with parental (pure) morphs. Such events have not been observed in the field.

CHAPTER 4: CONCLUSIONS

While overall genetic variation among *Hypoplectrus* morphospecies is high, sorting of these differences according to their species boundaries is largely incomplete. However, molecular evidence of partial reproductive isolation between *H. gemma* and *H. unicolor* was detected in this study and should not be understated. Based on the current genetic and mating evidence (low differentiation despite strong mating barriers), as well as their distinct color pattern differences, the *Hypoplectrus* system can be comfortably classified as an incipient species flock. Species flocks are groups of closely related species that underwent fast morphological radiation. Only two other marine taxa have been recognized as species flocks, the Pacific rockfishes (*Sebastes*, Johns and Avise 1998) and the Antarctic icefishes (Bargelloni et al. 1994). Although the branching order among the different species on phylogenies is unclear, which suggests a rapid radiation of forms, the ancient age of these groups has allowed monophyletic clustering of each species to occur. In contrast, as evidenced by the polyphyletic condition of AFLP markers (this study) and mtDNA sequences (McCartney et al. 2003), as well as based on McCartney et al.'s mtDNA molecular clock, the radiation of *Hypoplectrus* morphs was likely to have been very recent.

In order to accept a scenario of recency, we need to acknowledge that extreme color pattern divergence, as shown among the 11 *Hypoplectrus* morphs, can evolve much faster than the remainder of the genome. In other words, color pattern is a labile trait that can undergo change without correlated genetic differentiation. Evidence that this is possible has been found in other systems. In *Dendrobates pumilio* (see above), six strikingly different aposematic colorations have evolved in approximately 6000 years (Summers et al. 1997). Neotropical butterflies of the genus *Heliconius* (Nymphalidae) have undergone great radiations of mimetic

patterns across Central and South America. Some species of the genus have up to 29 parapatric subspecies (races), and each of these subspecies is a Mullerian mimic of a sympatric subspecies from another species of *Heliconius* (Mallet et al. 1998). Although some hybridization occurs, hybrid phenotypes have high mortality because they are poor mimics of the parental residents (Mallet and Barton 1989). The *Heliconius* radiation has been estimated to be as young as 200,000 years (Brower 1994). Like in *Hypoplectrus*, strong color-based assortative mating is present in *Dendrobates* (Summers et al. 1999) and *Heliconius* (Mallet et al. 1998), and is considered a key mechanism maintaining race color pattern distinctions.

Although these systems may serve as evidence that coloration can evolve in a short time period, the scenario for the hamlet radiation is more complex. In *D. pumilio*, each of the color morphs inhabits a different island, and this complete allopatry could allow genetic drift to diverge mating preferences and correlated color pattern (West-Ebenhard 1983). In *Heliconius*, mimetic races are distributed parapatrically, and this separation is maintained by frequency-dependent selection due to predation on uncommon phenotypes (Mallet and Barton 1989). In contrast, hamlet morphospecies exhibit largely sympatric distributions, and no extrinsic selective forces have yet been detected. Thus, the opportunity for gene flow among hamlets is considerably higher, highlighting the effectiveness of assortative mating in maintaining color pattern identities.

Even though initial divergence of hamlet color patterns in sympatry cannot be ruled out, the disjunct distribution of several morphospecies suggests that demographicand biogeographical history of the flock has undergone variations. Domeier (1994) envisioned a scenario of transient allopatry due to low sea levels. During low sea levels, ancestral undifferentiated *Hypoplectrus* populations may have become allopatric in isolated pockets of suitable reef. Since color patterns

are believed to be labile (see above), variation among the isolated populations in preference for certain color characters could have initiated divergence of colorations. If coloration was a socially or sexually selected trait, and the direction of preference was toward the fittest form of the trait, divergence would be rapid (West-Ebenhard 1983). Under this scenario, different color patterns could have evolved during the short periods of geographic isolation. In periods of high sea levels, the populations would meet, but the presence of strong color-based mate preference would have created an effective pre-mating barrier. It is believed that sea levels in the Caribbean oscillated 5-6 times in the last 135,000 years (reviewed in Domeier 1994), which would indicate that several instances of allopatry occurred.

Although genetic distinction of species boundaries among *Hypoplectrus* morphs is largely incomplete, phenotypic integrities remain strong even in locations where several morphospecies coexist. The strength of assortative mating and the low frequency of intermediate phenotypes (whether due to selection or inviability) suggest strong reproductive isolation. These are the defining features of an incipient species flock.

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APPENDICES

Appendix A: Coordinates for the sites where transect surveys were conducted. These were the approximate locations where divers entered the water; in some of these locations more than one transect survey was conducted.

Long Key, Florida

West Tennessee Reef

N 24°44.136’ / W 080°47.989’
N 24°44.129’ / W 080°48.018’
N 24°44.094’ / W 080°48.048’
N 24°44.039’ / W 080°48.063’
N 24°43.991’ / W 080°48.078’

East Tennessee Reef

N 24°45.054’ / W 0.80°45.502

X-Muta Reef

N 24°48.114’ / W 080°40.911’
N 24°48.060’ / W 080°40.919’
N 24°48.004’ / W 080°41.013’
N 24°47.961’ / W 080°41.093’
N 24°47.856’ / W 080°41.225’
N 24°47.761’ / W 080°41.351’

Key Largo, Florida

Molasses Reef

N 25°00.441’ / W 080°22.396’

Conch Reef

N 24°56.821’ / W 080°27.339’
N 24°56.869’ / W 080°27.255’
N 24°57.060’ / W 080°27.064’
N 24°57.090’ / W 080°27.098’

Appendix B: DNA extraction buffer.

- 1ml 1M Tris-HCl pH 8.0
- 20ml 0.5M EDTA pH8.0
- 0.5g SDS
- 100μL RNase (20mg/ml)

Bring volume to 100ml with ddH_2O

Appendix C: Recipe for restriction-ligation reaction of AFLP fragments.
This procedure was designed according to the reactant concentrations indicated in ABI AFLP Plant Mapping Protocol.

Enzyme Master Mix

- 10X T4 DNA Ligase buffer.....1.0µL
- 0.5M NaCl.....1.0µL
- 1mg/ml BSA.....0.5µL
- *Mse* I.....1.5µL
- *Eco*R I.....3.0µL
- T4 DNA Ligase.....0.5µL
- deionized H_2O.....2.5µL

Restriction-ligation Reaction

- DNA extract (0.5-0.8µg).....5.5µL
- 10X T4 DNA Ligase buffer.....1.0µL
- 0.5M NaCl.....1.0µL
- 1mg/ml BSA.....0.5µL
- *Mse* I adaptors..... 1.0µL
- *Eco*R I adaptors.....1.0µL
- Enzyme Master Mix.....1.0µL