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Meredith A. Bostrom
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# AN INVESTIGATION OF HYBRIDIZATION BETWEEN TWO SERRANID FISHES THE CONEY (*CEPHALOPHOLIS FULVA*) AND THE CREOLE FISH (*PARANTHIAS FURCIFER*)

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A Thesis

Presented to

The Faculty of the School of Marine Science

The College of William and Mary in Virginia

In Partial Fulfillment

Of the Requirements for the Degree of

Master of Science

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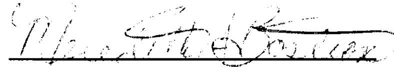
by

Meredith A. Bostrom
2000

APPROVAL SHEET

This thesis is submitted in partial fulfillment of the requirements for the degree of

Master of Science



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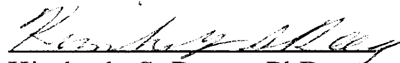

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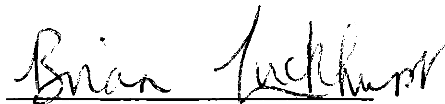

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## Abstract

Hybridization between the epinepheline serranid species *Cephalopholis fulva* and *Paranthias furcifer* was investigated using morphological and molecular analyses. Fifteen putative hybrids, as well as members of each putative parent species were collected in Bermuda. Morphometric and meristic analyses of forty-four characters as well as molecular analyses consisting of allozyme electrophoresis and restriction fragment length polymorphism (RFLP) analysis of mitochondrial and nuclear DNA fragments were used to identify hybridization. Sixteen allozyme loci were screened for fixed differences between *C. fulva* and *P. furcifer*. Seven loci were found to be polymorphic of which four, creatine kinase (*CK-B**), fumarase (*FH**), isocitrate dehydrogenase (*ICDH-S**), and lactate dehydrogenase (*LDH-B**), were fixed for different alleles between *C. fulva* and *P. furcifer*.

Restriction fragments of the mtDNA regions ND4, ATPase 6, and 12S/16S ribosomal RNA were analyzed to identify maternal parentage of hybrids. Restriction fragments of two nuclear DNA intron regions, an actin gene intron and the second intron in the S7 ribosomal protein gene were analyzed to support the detection of hybridization between *C. fulva* and *P. furcifer*. Both morphological data and nuclear genetic data indicated that the putative hybrids were the result of interbreeding between *C. fulva* and *P. furcifer*. Mitochondrial DNA analysis showed the maternal parent of all hybrid individuals was *C. fulva*. Thirteen hybrid individuals were heterozygous at all diagnostic nuclear loci, consistent with pure $F_1$ hybrid individuals. Allozyme evidence indicated that two putative hybrid individuals were post-$F_1$ hybrids based on homozygosity at one nuclear locus. A survey of nuclear and mtDNA loci of 57 *C. fulva* and 37 *P. furcifer* presented no evidence of introgression between the parent species. Nei's (1978) genetic distance calculated between *C. fulva* and *P. furcifer* using the allozyme data was found to be 0.356. The estimated sequence divergence between *C. fulva* and *P. furcifer* for mtDNA was found to be 0.036. Both distance values indicated potential generic misplacement of the putative parent species. Cumulative data support hybridization between *C. fulva* and *P. furcifer*. $F_1$ hybrid individuals are capable of reproducing successfully, and $F_1$ hybridization was mediated by female *C. fulva* and male *P. furcifer*.

# HYBRIDIZATION BETWEEN *C. FULVA* AND *P. FURCIFER*

**Introduction**

In 1860 ichthyologist Felipe Poey described two specimens of a grouper-like fish found off Havana, Cuba that appeared to be a cross between the creole fish, *Paranthias furcifer* and the coney, *Cephalopholis fulva*. A few more specimens of the putative hybrid were collected over the next 100 years and ichthyologists such as David Starr Jordan, Joseph Swain and Carl H. Eigenmann also concluded that these fish were closely related to *C. fulva* and may represent hybrids (Smith 1966). In 1966, C. L. Smith published a review of Poey's specimens, including an analysis of forty-five meristic and morphometric characters. The two putative hybrids were intermediate to *P. furcifer* and *C. fulva* at forty of these characters (Smith 1966). From the morphological data, Smith concluded that the fish (*Menephorus dubius*) were hybrids between *C. fulva* and *P. furcifer*. This apparently successful hybridization across genera was unusual and Smith concluded his study commenting that *P. furcifer* may be more closely related to the epinepheline serranids (such as the *C. fulva*) than previously thought. Interest in the interbreeding of these species was revived in recent years when a number of putative *C. fulva* X *P. furcifer* hybrids were observed by commercial fishermen in the waters off the island of Bermuda (Brian Luckhurst, personal communication).

The goal of the following study was to use molecular genetic and morphological characters to further Smith's analysis of hybridization between *Cephalopholis fulva* and *Paranthias furcifer*. I present an investigation of fifteen recently captured putative hybrid

specimens from Bermuda, along with individuals of both putative parent species and five specimens of a related species, the graysby grouper, *Cephalopholis cruentata*.

***Hybridization***

Hybridization is the interbreeding between two "genetically differentiated forms," including not only species, but also subspecies and populations (Avise 1994). Hybridization occurs in all major animal and plant groups. In some cases, hybridization is successful, but $F_1$ hybrids can be poorly developed or sterile as the result of mixing two different genomes and are generally believed to have reduced fitness relative to pure individuals. In other cases, $F_1$ hybrids are able to effectively compete with members of the pure parent species and reproduce successfully (Avise 1994). Rarely, a hybrid will have vigor and be able to outcompete the pure parent species for resources such as food, space, and mating partners. When an $F_1$ hybrid reproduces with a pure parent, it is called backcrossing and the young are known as backcross hybrid individuals. The product of two $F_1$ hybrids reproducing is known as an $F_2$ hybrid individual, although occurrence of $F_2$s is rare because an $F_1$ hybrid usually has a greater chance of producing successful offspring with a pure individual. This is because half of the genes in a first generation backcrossed individual will have both alleles adapted for one of the parent species' environment, and therefore may have a better chance for survival than progeny of two $F_1$ hybrids (Stebbins 1971).

Extensive hybridization can lead to introgression (Campton 1987), the "movement of genes between species mediated by backcrossing" (Avise 1994). As $F_1$ hybrids backcross to one of the pure parent species and successive offspring repeat the cross with

the same species, genes from one hybridizing species can be transferred into another. Introgression of genes across species is not readily accomplished. There must be a number of fertile $F_1$ and backcross hybrids which reproduce with one parent species over a period of time. It is believed that introgression is not as common as hybridization due to hybrid reproductive inferiority (Avise 1994).

The study of introgression is important from an evolutionary standpoint because it allows for the close up investigation of the merging of two genomes that have been separated for millions of years (Avise 1994) and examination of how a population responds to foreign gene introduction. Even low levels of introgression can be accompanied by changes in behavior, ecology, or morphology of a species and can therefore alter a species' diversity and evolutionary trajectory (Avise and Saunders 1984; Verspoor and Hammar 1991). Investigating introgression also allows a first hand look at speciation and changes in traits involving reproductive isolation (Dowling et al. 1996).

As a result of its strong evolutionary consequences, it is important to understand the extent of interbreeding between species (Campton 1987). Hybridization occurs in varying degrees, from the infrequent production of an $F_1$ hybrid to complete random mating between two former species in a localized environment, resulting in a single, genetically homogeneous population (Avise 1994). Extreme hybridization and introgression, as in the latter case, can result in the local extirpation of species or subspecies, or the production of new species (Campton 1987). For example, the bluegill sunfishes *Lepomis macrochirus macrochirus* and *L. m. purpurescens* of the southeastern United States have introgressed to such a degree in some locations that they can be considered a single randomly mating population (Avise and Saunders 1984). In another

example, past hybridization between *Gila elegans* (bonytail chub) and *Gila robusta* (roundtail chub) is believed to have resulted in the production of the modern species *Gila seminuda* (DeMarais et al. 1992).

*Promoters of Hybridization*

Hybridization is known to be more common in fishes than in other vertebrate taxa (Avise 1994; Campton 1987). Schwartz (1972; 1981) published reviews of 3759 references of natural and artificial fish hybridization. This high incidence of hybridization is believed to be due primarily to the lack of reproductive barriers among closely related fish species. Most fishes have external fertilization, many share spawning habitat, and several species have been introduced through aquaculture; factors that can lead to extensive natural and artificial hybridization (Campton 1987). Several other factors that promote hybridization in vertebrates have been discussed in the literature, including localized low abundance of a species, exotic species introductions, and changes in the local environment.

Several authors have noted that the extent of hybridization and introgression is greatest when one parent species is exceptionally rare relative to the other parent species (Avise 1994; Dowling et al. 1989). Avise and Saunders (1984) investigated hybridization between five species of *Lepomis* and found that all occurrences were between an abundant species and a rare species. In six of seven *Lepomis* hybrid broods the maternal parent was shown to be the rare species. Avise and Saunders (1984) concluded that the female of the rare species had a more difficult time finding a conspecific mate and more often encountered males of another species, resulting in hybridization. They also found that when backcrossing was present, it was usually with the more rare parent, because that

species would have less chance of encountering a conspecific mate and may be less discriminating against $F_1$ hybrids. This results in introgression of alleles from the abundant species into the genome of the rare species, often mediated by females (Avise 1994), and has been documented several times in fishes (Dowling et al. 1989; Hubbs 1955).

Hybridization has also been shown to result from the introduction of an exotic species. This is most important in freshwater environments where there is considerable geographic isolation and areas are often stocked with non-native species for recreational fishing purposes. A well known example of hybridization as a result of exotic species introduction is the case of pupfish in the Pecos River, Texas. *Cyprinodon variegatus* was introduced into the river and has since extensively hybridized with the native species, *C. pecosensis* (Echelle and Connor 1989). Five years following the introduction, the two species were shown to comprise a single, randomly mating population over about 400 kilometers of the river. This example also demonstrates that separation over time or space does not necessarily result in complete reproductive isolation between species.

Changes in environmental parameters such as temperature, salinity, and habitat size have also been identified as promoters of hybridization. Reduction of habitat results in crowding and sharing of spawning areas with other species (Campton 1987). Loss of habitat can also result in a severe reduction in the abundance of a species, or the movement of a species into a foreign environment (a natural introduction); both of which can promote hybridization as discussed above. Hubbs (1955) theorized that slight year to year changes in temperature regimes might cause an overlap of spawning times of two species and result in hybridization. For example, the extent of hybridization between the

minnows *Notropis cornutus* and *N. chrysocephalus* varies with geographic location as well as the degree of overlap in spawning seasons. Hubbs (1955) also suggested that there is a clinal change in the occurrence of hybridization, from northern temperate to southern tropical zones. Waters in the temperate zone, which have larger temperature fluctuation, would have a greater frequency of fish hybridization than more stable tropical waters.

A review of literature reveals a significant difference in the frequency of hybridization between freshwater and marine fishes (Hubbs 1955). Several authors have noted a lower frequency of natural hybridizations in marine fishes compared to freshwater fishes (Campton 1987; Hubbs 1955). A portion of this difference can be attributed to increased geographic isolation, a greater loss of habitat and more introductions of exotic species in freshwater ecosystems. In general, oceans are stable for longer periods of time and species may be less affected by the factors that promote hybridization.

### ***Intergeneric Hybridization***

Natural hybridization between distantly related species is rare in most vertebrates. Intergeneric hybridization, however, has been shown to be relatively common in fishes, especially when spawning occurs under similar conditions (Dowling et al. 1989). Verspoor and Hammar (1991) reported eight instances of natural hybridization across genera in fishes within the families Cyprinidae, Salmonidae, Catostomidae, and Pleuronectidae. Loftus (1992) and Aspinwall et al. (1993a and b) reported intergeneric hybridization within the Lutjanidae and Cyprinidae as well. In salmonids, there are fourteen cases of natural and artificial intergeneric hybridization, eight with viable $F_1$ individuals (Verspoor and Hammar 1991). It is generally believed that the more divergent

the hybridizing taxa, the greater the effect introgression will have on the evolution of the hybridizing species (Anderson 1949).

In some cases, however, it is possible species involved in intergeneric hybridization may simply be misplaced taxonomically, and are not as distant as previously thought. This connection between taxonomic misplacement and intergeneric hybridization was discussed in Charles Sibley's (1957) review of the high incidence of intergeneric hybridization in birds. Avian genera have often been assigned based on male physical traits, many of which are secondary sexual characteristics and not necessarily related to species divergence. Sibley's examination of females of these species showed that they were not as distant as previously thought, and that many species of different genera could be considered to be congeneric. Sexual dimorphism to this extent is not common in fishes. This example, however, shows that frequent intergeneric hybridization may signal the need for taxonomic revision.

### *The Putative Hybrid*

Another example of intergeneric hybridization is found in Poey's (1860) description of interbreeding between *Cephalopholis fulva* and *Paranthias furcifer,* although it was largely speculative until Smith's (1966) morphological analysis. Smith was able to demonstrate that the hybrid (*Menephorus dubius*) had traits that were directly intermediate between *C. fulva* and *P. furcifer*. He excluded other species as possible parents by noting that some of the morphological characters of the hybrids were unlike all other serranids in the tropical Atlantic. The geographic extent of the putative hybrid is

largely unknown, although its presence is certain in Bermuda, Cuba (historically), and Jamaica (Thompson and Munroe 1978).

The presumed parents of the putative hybrid are members of different serranid genera that have strikingly different morphologies, occupy different ecological niches, and have different behaviors (Smith 1966). The major distinguishing features of *Paranthias furcifer* are its forked caudal fin and the size and shape of its mouth (Figure 1). *Paranthias furcifer* is dark red in color; darker dorsally, lightening ventrally (Heemstra and Randall 1993). There is an orange spot at the upper end of the base of the pectoral fin and three white spots dorsal of the lateral line. It has a small terminal mouth that facilitates feeding on zooplankton in the water column where it forms large aggregations (Heemstra and Randall 1993) in depths greater than fifty feet. Bermuda is the northern limit of the distribution of *P. furcifer* in the western Atlantic and the species occurs south throughout the Bahamas and Antilles and along the American coast from the Gulf of Mexico to Brazil (Smith 1971).

In contrast, *Cephalopholis fulva* is usually scarlet in color and covered with light blue-green spots, each surrounded by a black ring (Heemstra and Randall 1993). There are two black spots on the edge of the lower jaw as well as on top of the caudal peduncle. *Cephalopholis fulva* has rounded caudal, anal and dorsal fins, similar to other epinepheline serranids (Heemstra and Randall 1993) (Figure 2). The mouth of *C. fulva* is lower on the head than that of *Paranthias furcifer. Cephalopholis fulva* is reported to eat larger prey such as small fishes (Smith 1971). Unlike *P. furcifer, C. fulva* is generally solitary and makes use of crevices in shallow, clear waters. In the northwestern Atlantic

**Figure 1:** Photograph of the creole fish, *Paranthias furcifer*, SL 259mm, Bermuda.

**Figure 2:** Photograph of the coney, *Cephalopholis fulva*, SL 222mm, Bermuda.

*C. fulva* has a distribution similar to that of *P. furcifer*, it occurs as far north as Bermuda and south in the Bahamas, Antilles, and along the American east coast from South Carolina to Panama, including the Gulf of Mexico, Colombia, and Brazil (Heemstra and Randall 1993; Smith 1971).

Morphologically, the putative hybrids which Poey collected are almost exactly intermediate between the parent species (Figure 3). In his 1966 review, Smith noted that the hybrids have characters unique to *Cephalopholis fulva* and *Paranthias furcifer*. For example, the hybrid individuals had both a moderately forked tail and blue spots surrounded by a black ring. *Paranthias furcifer* is the only Atlantic grouper with a forked tail indicating that it is one of the putative parents. Similarly, *C. fulva* is the only Atlantic grouper that has blue spots with a black ring, also indicating parentage of hybrids. The presence of both traits together in a single individual strongly suggests some interbreeding between the two species (Smith 1966). Ecological information about the hybrid would provide an interesting insight, as the putative parent species lead different lifestyles. The ecological niche of the putative hybrids is unknown, although all individuals in this study were caught at depths that overlap those of *C. fulva* and *P. furcifer* (Table 1).

***Detecting Hybridization***

*Morphology*

When Smith (1966) investigated the parentage of Poey's putative hybrids, examination of morphological characters was the only method available to analyze natural hybridization short of artificial crossing. Hybridization is inferred when the

**Figure 3:** Photograph of the putative hybrid between *Cephalopholis fulva* and *Paranthias furcifer*, SL 210mm, Bermuda

**Table 1:** Collection information on specimens used for genetic study, including species, location, date and depth (where available).

| Species | Total (N) | N/Location | Location | Date |
|---|---|---|---|---|
| *Cephalopholis fulva* (coney) | 57 | 7 | Bermuda | Prior to Aug 97 |
| | | 3 | 3 mi NE St. George's Bda | 8 Aug 98 |
| | | 41 | SW platform, Bermuda | 22 Sept 98 |
| | | 6 | Navassa Island | May 1999 |
| *Paranthias furcifer* (creole fish) | 37 | 5 | Bermuda | Prior to Aug 97 |
| | | 4 | 3 mi NE St. George's, Bda | 8 Aug 98 |
| | | 6 | NW of Bermuda | 22 Sept 98 |
| | | 1 | South of Bermuda | 22 Sept 98 |
| | | 10 | NW of Bermuda | 27 Sept 98 |
| | | 11 | Bermuda | |
| *Cephalopholis cruentata* (graysby) | 5 | 1 | Bermuda | Prior to Aug 97 |
| | | 1 | 3 mi NE St. George's Bda | 8 Aug 98 |
| | | 1 | South of Bermuda | 22 Sept 98 |
| | | 2 | Navassa Island | May 1999 |
| Putative Hybrids | 15 | 1 | Bermuda | Prior to Aug 97 |
| | | 1 | Challenger Bank, Bermuda | Jun 93 |
| | | 1 | off Castle Roads, Bermuda; 28 fathoms | 12 Dec 96 |
| | | 1 | off Castle Roads Bermuda; 28 fathoms | 31 Dec 96 |
| | | 1 | NE Argus Bank, Bermuda; 30 fathoms | 21 Apr 97 |
| | | 1 | SE Challenger Bank, Bermuda; 33 fathoms | 30 Jun 97 |
| | | 1 | off Castle Roads, Bermuda; 10 fathoms | 19 Oct 97 |
| | | 2 | West Blue Cut, Bermuda; 20 fathoms | 15 Oct 98 |
| | | 1 | NW of Sally Tucker's Pt, Bda; 26 fathoms | 28 Oct 98 |
| | | 1 | NE Point, Bermuda; 13 fathoms | 17 Nov 98 |
| | | 1 | NE Point and Kitchen, Bermuda; 12-15 fathoms | 24 Nov 98 |
| | | 1 | inside NE Point | 1 Jul 99 |
| | | 1 | inside NE Point | 6 Jul 99 |
| | | 1 | Brian Luckhurst: SW1 sampling station | 14 Jul 99 |

morphological characters of a putative hybrid are intermediate to those of the putative parents (Hubbs 1955). For some hybrids, characters have been observed to be exactly intermediate, such as counts of gill rakers, while others may be closer to one parent than the other due to allelic interaction (Hubbs 1955). For example, Hubbs found that hybrids of the mosquitofish, *Gambusi affinis* and *G. holbrooki* have the same number of dorsal spines as *G. holbrooki*. Consequently, based on this trait, hybrid individuals would be indistinguishable from pure *G. holbrooki* parents.

There are additional limitations to using morphological characters to detect hybridization. In some cases morphological characters may be phenotypically plastic, influenced not only by the gene products that contribute to the phenotype, but also by the environment (Campton 1987). It may be unknown whether a similarity between two individuals is the product of common ancestry or a common environment. For example, *Paranthias furcifer* individuals have from 41 to 49 caudal peduncle scales, while *Cephalopholis fulva* have from 38 to 43 caudal peduncle scales (Smith 1966). An $F_1$, $F_2$ or a backcross hybrid could potentially have caudal peduncle scales anywhere within this range and it would be impossible to distinguish the three forms based on this character alone. For this reason it can be difficult to assess introgression using morphological characters (Avise and Saunders 1984; Campton 1987).

To quantify morphological data of putative hybrids, Hubbs and Kuronuma (1942) developed a hybrid index. Characters are measured or counted in individuals and compared to mean values calculated for each parent species. All traits are compared separately, equally weighted, and results are averaged to derive a hybrid index for each individual. A value between 30% and 70% is considered indicative of hybridization.

There are some drawbacks to using the hybrid index, notably the assumption that the traits used in the index are independent of one another. Additionally, some traits may have greater resolution of interspecific variation than others and weighting them equally may be inappropriate. These drawbacks can be corrected by using multivariate statistics such as discriminate function analysis and principal component analysis. One can assign each trait a weighting based on its power of resolution and more accurately assess hybridization (Campton 1987).

*Molecular Genetic Data*

Molecular characters provide a different perspective than morphological characters for detecting of hybridization because one is looking at inherited characters that are not readily affected by the environment like morphological traits. For example, in a study of the pocket gopher, *Geomys bursarius* (Baker and Davis 1989) almost half of the hybrids would have been incorrectly classified as pure parents in the absence of genetic data. Genetic characters are inherited from parents and, in the presence of differences between hybridizing species, can be used to identify $F_1$ hybrid individuals. Therefore, identification of hybrid class, ($F_1$, post-$F_1$, backcross etc) and parentage is less ambiguous. Types of genetic characters surveyed in hybridization studies include allozymes, mitochondrial DNA (mtDNA), and nuclear DNA. Allozymes and nuclear DNA markers are biparentally inherited allowing the identification of hybrids, as well as products of backcrossing. MtDNA is maternally inherited in fishes and is a powerful marker for identifying the female parent in hybridizations. Both types of markers are useful in the description of introgression in hybridizing species.

*Allozyme Electrophoresis*

Allozyme electrophoresis is one of the most common genetic methods used to assess hybridization. Enzymes, made up of amino acids, migrate through starch gels mediated by an electric current. The amino acid sequence of the enzyme dictates how rapidly it migrates, and genetic changes that result in a change in the amino acid sequence may result in different mobilities through the gel. The enzymes can be visualized using histochemical stains (Utter et al. 1987). Allozyme electrophoresis is a rapid and cost-effective means of screening the nuclear genome. As in morphological studies, it is necessary to analyze multiple loci in order to distinguish $F_1$ and post-$F_1$ hybrids and detect introgression (Verspoor and Hammar 1991).

There are drawbacks to using allozyme electrophoresis for genetic analysis. Samples must be fresh or frozen soon after collection because enzyme activity drops significantly over time (Utter et al. 1987). Sometimes the genetic diversity in a population can be masked and the estimate of evolutionary distance would be low. For example, two individuals may be different genetically, but their enzymes may still migrate to the same point on the gel due to degeneracy of the genetic code. Additionally, there may be a change in the amino acid sequence that does not result in a change in the enzyme's electrophoretic mobility. Allozymes are also tissue specific and different loci are expressed in different types of tissue. It can be time consuming to screen multiple loci over multiple tissues in several individuals. Once diagnostic loci are indentified, however, the efficiency of enzyme electrophoresis makes analysis of hybridization practical, as shown by Avise and Saunders (1984) and Dowling et al. (1989).

*DNA*

Recent advances in molecular biology have resulted in genetic tools with higher resolution than enzyme electrophoresis for studies in population biology and systematics, including analysis of hybridization. The advantage of directly analyzing DNA for hybridization studies is that the DNA is inherited from the parents, where allozymes are the products of the translation of DNA to proteins. The effects of amino acid changes and degeneracy of the genetic code on electrophoretic mobility are not factors of concern (Dowling et al. 1996). One also has the option of selecting among different loci. Markers with the most appropriate evolutionary rate and mode of inheritance can be chosen to most effectively accomplish the goals of the study (Avise 1994).

*Mitochondrial DNA*

Mitochondrial DNA (mtDNA) is regularly used in hybridization studies of fishes because it is maternally inherited, has a rapid rate of evolution, and has been well characterized. MtDNA is a circular, double-stranded molecule typically comprising 15,000 to 18,000 nucleotide base pairs and 37 genes in vertebrates (Avise 1994). Many of the gene sequences are well conserved, and each animal cell contains thousands of copies of the molecule (Palumbi 1996) allowing ready amplification of the mitochondrial DNA regions with the polymerase chain reaction (PCR).

The rate of nucleotide base substitution is generally higher in animal mtDNA than in nuclear DNA, which allows genetic differences between hybridizing species to be observed with less difficulty. MtDNA also has an effective population size (Ne) that is one fourth that of the nuclear genome because individuals have only one copy and only

females transmit the molecule (Avise 1994). Therefore, mtDNA has a rate of evolution due to genetic drift that is four times that of nuclear DNA (Dowling et al. 1996) allowing higher resolution of variation between closely related taxa. This higher rate of evolution, as well as the nonrecombining nature of mtDNA, makes it useful for identifying introgression.

The maternal inheritance of mtDNA is very important in studies of hybridization. When the parent species of hybrid individuals are known, the maternal parent of each $F_1$ hybrid individual can be unambiguously identified. In the case of backcross species, the sexual preferences of $F_1$ hybrids and their offspring can be studied.

The use of mtDNA to infer hybridization also has limitations. The uniparental transmission of mitochondrial DNA requires that it be used in conjunction with a biparentally inherited (nuclear) marker to accurately detect hybridization or to distinguish $F_1$ and post-$F_1$ hybrids (Campton 1987; Dowling et al. 1996; Verspoor and Hammar 1991).

*Nuclear DNA*

An investigation of nuclear gene sequences is the most conclusive way to identify parentage in hybridization studies because one is looking directly at inherited genes from both parents. The number of nuclear genes is vast in animal cells and there are potentially an unlimited number of loci available for study (Dowling et al. 1996). Nuclear regions that are often used in genetic analysis include coding DNA, internal transcribed spacers, intron regions, satellite regions, and randomly amplified polymorphic DNA (RAPDs). Coding DNA is usually well conserved and there may not be enough variation to detect hybridization between closely related species. DNA intron and internal transcribed spacer

regions are noncoding, nontranslated regions of DNA that are subject to high rates of mutation, usually considered effectively neutral to selection, and can be used to discriminate between closely related species. Introns and internal transcribed spacers are usually sufficiently variable to allow interspecific identification, although some may prove to be too variable within a species to be useful for hybridization studies (Palumbi 1996). Satellite DNA regions (mini-, microsatellites) are highly variable repeat regions that are generally used to study within population variation and parentage. RAPD analysis is the amplification of random portions of DNA, with no information about the targeted gene loci (Palumbi 1996). Differences in size or presence of amplification products are used to identify differences in DNA sequence between samples. In general, any of the DNA regions discussed above that shows fixed differences between hybridizing groups are appropriate for the identification of $F_1$ and post-$F_1$ hybrid individuals.

Verspoor and Hammar (1991) cited the need for analysis of genomic nuclear loci in studies of hybridization because it is the only method available to fully assess levels of biparental introgression between animal species without the disadvantages of enzyme electrophoresis. However, the nuclear genome is large and analytical techniques for it are much more expensive and time consuming than enzyme electrophoresis. Often nuclear gene families comprise more than one locus complicating the assignment of homology. Because only one locus per marker is desired, the presence of multiple loci makes PCR amplification and interpretation difficult. Once a single locus has been identified and amplified, however, nuclear DNA analysis can be an efficient way to study natural hybridization.

*RFLP Analysis*

Mitochondrial and nuclear DNA loci can be screened for sequence variation using restriction fragment length polymorphism (RFLP) analysis. PCR amplified segments of DNA, such as introns, are digested with restriction enzymes that cut the DNA strand at specific four, five, or six nucleotide base pair sequences. The fragments are separated on an agarose gel to determine differences in size and number of fragments among individuals, indicating nucleotide sequence polymorphism (Dowling et al. 1996). The result is a series of DNA fragments which summed, equal the length of the amplified DNA segment in homozygous individuals or up to two times the length in heterozygous individuals. RFLP analysis is a quick and relatively inexpensive method to estimate genetic variation within and between species.

*Genetic Data Analysis*

The key to using molecular markers to identify hybridization is to find multiple independent nuclear and mitochondrial loci that are fixed for different alleles in each putative parent species (Dowling et al. 1996). An $F_1$ hybrid would be consistently heterozygous at all nuclear loci and have a mitochondrial haplotype identical to one parent species (Campton 1987; Dowling 1989). A backcrossed individual, however would be heterozygous at some nuclear loci and homozygous at others. The power of demonstrating an $F_1$ hybrid as opposed to a backcross or pure parent individual increases with the number of nuclear loci examined, because one may mistake an individual as a hybrid, when it is heterozygous at only the one or two loci examined. The chance of misclassifying an $F_1$ individual is equal to $0.5^n$ where n is the number of loci surveyed that are fixed differently between the hybridizing species (Avise 1994). Therefore, the

most reliable studies use the greatest number of diagnostic loci. If only one or two loci are studied, the chances of identifying a backcrossing event correctly are greatly reduced.

The presence of shared alleles between parent species does not necessarily exclude the possibility of detecting hybridization, especially if there are major allele frequency differences between the species. One can calculate the allele frequencies of the parents and the probability of obtaining the observed hybrid genotype from random mating (Campton 1987). This calculation is another type of hybrid index developed by Campton and Utter (1985). However, without fixed differences between the loci, the extent of post-$F_1$ hybridization can only be an estimate.

Introgression is detected by the presence of an allele fixed in one species that is present as a rare allele in the other hybridizing species. However, there are other reasons that a rare allele may occur, such as symplesiomorphy and convergent evolution. Multiple diagnostic loci with rare alleles in the hybrid zone individuals is generally considered an indicator of introgression (Avise and Saunders 1984).

Using nuclear and mitochondrial loci together, along with morphological characters, hybridization can be ascertained and the extent of introgression, if any, can be quantified. However, there are instances when inferences based on mtDNA and nuclear DNA do not agree. Dowling et al. (1989) reported conflicting results between mtDNA, allozyme data, and morphology in reference to the direction and extent of introgression in *Notropis* sp. For example, in all localities, the mtDNA data estimated a much higher level of introgression than that estimated from the allozyme data. At some locations, the direction of introgression was also variable between the mitochondrial and nuclear data.

Although all possible methods were employed, the process of interbreeding between these species was so complicated that results were ambiguous.

*Genetic Distance*

In studies of intergeneric hybridization as described here, a means of measuring the evolutionary divergence between the putative parent species can be informative. For molecular data, a genetic distance can be calculated to estimate the evolutionary distance between two species (Avise 1994). Genetic distance lends uniformity to data, so even taxonomically distinct groups can be compared. Essentially, the calculation of genetic distance is a proportion of shared genetic characters (alleles, for example) to the number of unshared characters. There are a number of different ways to calculate genetic distance, depending on the type of data (Avise 1994). However, the general calculation is consistent between authors. In cases of intergeneric hybridization, the calculation of genetic distance provides insight as to whether the taxonomic placement of the hybridizing species is appropriate.

**Objectives**

The objective of my thesis research was to test the hypothesis that the putative hybrid is a product of *Cephalopholis fulva* and *Paranthias furcifer*, and to assess whether introgression has occurred between the parent species in Bermuda via hybrid backcrossing. The study used genetic information from mitochondrial and nuclear loci to test Smith's (1966) conclusion that the putative hybrids are the result of interbreeding between *C. fulva* and *P. furcifer*. It was hypothesized that:

(1) hybridization occurs between *C. fulva* and *P. furcifer*;

(2) hybridization is not gender biased;

(3) post-$F_1$ hybridization has not occurred; and

(4) no significant introgression has occurred between *C. fulva* and *P. furcifer* in Bermuda as a result of hybridization.

Hybridization was tested by analyzing allozymes, mitochondrial DNA, and single copy nuclear DNA. Gender bias of hybridization was tested using mtDNA haplotypes to identify the species of the female parent of each hybrid individual. $F_1$, and post-$F_1$ hybrids were identified by analysis of allozymes and single copy nuclear DNA.

**Methods**

*Cephalopholis fulva, C. cruentata,* and *Paranthias furcifer* were collected in Bermuda by Dr. Brian Luckhurst, Bermuda Department of Agriculture and Fisheries, and Dr. Bruce Collette, National Systematics Laboratory, National Marine Fisheries Service, using baited handlines or rotenone (Table 1). In addition, six *C. fulva* and two *C. cruentata* were sampled from Navassa Island by Bruce Collette. All putative hybrids except one were captured by Bermudian fishermen using handlines or lobster traps. Hybrid specimens and collection data were compiled by Brian Luckhurst. *Cephalopholis cruentata* was included in the study as a possible parent species of the putative hybrid and three *Epinephelus guttatus* specimens from the Bahamas were used in the preliminary mitochondrial DNA study as a putative parent species.

Specimens were frozen upon capture and transported to the laboratory at the Bermuda Department of Agriculture and Fisheries or the Virginia Institute of Marine Science for dissection. Tissues were sampled using different methods for allozyme and DNA analysis. For mitochondrial and nuclear DNA analysis muscle tissue was removed from frozen fish and placed in storage buffer (0.25M EDTA, 20% DMSO and saturated with NaCl). For allozyme analysis eye, heart, liver, brain and muscle tissues were removed from each specimen. A $1.5cm^3$ piece of tissue was ground in 250μl of chilled (4°C) grinding buffer (0.1M Tris, 0.9mM EDTA, and 0.05mM $NADP^+$, pH 7.2; Waples 1986) in a microfuge tube using a small pestle until tissue was homogenized. Samples

were centrifuged in a tabletop microcentrifuge for three minutes at 16,000 X g. Extractions were stored at -80°C or used immediately for allozyme analysis at the Virginia Institute of Marine Science. Following tissue extraction, whole fish were stored at –20°C or –80°C.

Genomic DNA was isolated from a 1.5cm$^3$ piece of muscle tissue using the phenol chloroform protocol of Winnepenninckx and Wachter (1993) with the following modifications. CTAB was not added to the extraction and phenol was added immediately following incubation of the tissue at 37°C. DNA was precipitated by the addition of 0.04 X the volume of 5M NaCl and 1X the volume isopropanol. DNA was resuspended in 150μl of sterile 1X TE (Tris-EDTA) and stored at –20ºC. A small amount (4μl) of each DNA isolation was separated on a 0.8% agarose gel, stained with ethidium bromide and visualized with ultraviolet light to assess extraction quality.

### *Morphology*

Nine putative hybrids, ten *Cephalopholis fulva* and ten *Paranthias furcifer* were fixed in 10% buffered formalin and preserved in 75% ethanol for morphological analysis. Specimens of *C. fulva* and *P. furcifer* used in the morphological analysis were not the same specimens as those in the genetic study and were obtained from the National Museum of Natural History. Museum numbers for those specimens are listed in Appendix B. Four *P. furcifer* and nine hybrid individuals have not yet been catalogued. Morphological traits of ten *P. furcifer*, ten *C. fulva* and nine putative hybrids were examined as described in Smith 1971 (Appendix B) using dial calipers and a meter stick.

A principal component analysis of the morphological data was performed using the computer program Minitab (Minitab Inc. 1998) to spatially define *Cephalopholis fulva* and *Paranthias furcifer* and compare the morphological characters of the hybrid individuals to those of the parent species.

### *Allozyme Analysis*

Allozyme electrophoresis followed the protocols described in Murphy et al. (1996) for horizontal starch gels. Gels were 12% (w/v) hydrolysed potato starch (Starch Art Corporation) and polymerized in Tris-citrate II buffer (TC II), lithium hydroxide buffer (LIOH), or Tris borate-EDTA buffer (EBT). TCII and EBT gels were run with a current of 30 mAmps for 14 hours and LiOH gels were run with a current of 25 mAmps for 14 hours. Histochemical stains and corresponding buffer systems are listed in Table 2. Stain recipes are listed in Appendix A of this text and were taken from Waples (1986).

A preliminary survey of 16 loci in 16 individuals of each species was done to identify those loci fixed for different alleles among the putative parent species, *Cephalopholis fulva, C. cruentata* and *Paranthias furcifer* (Table 2). All putative parent individuals were surveyed for loci with fixed differences between the species. Alleles were assigned in the following manner; the most common allele at a locus was 100 and less common alleles were assigned values based on their migration distance relative to the 100 allele. Genotypes at the loci demonstrating fixed differences were recorded for all putative parent and ten putative hybrid individuals.

**Table 2:** Allozyme analysis: information including loci, buffer systems and tissues.

| Locus | Buffer | Tissue |
|---|---|---|
| Alcohol dehydrogenase (*ADH-1** 1.1.1.1) | EBT | Liver |
| Creatine kinase (*CK-B** 2.7.3.2) | LIOH | Liver |
| Creatine kinase (*CK-C**2.7.3.2) | LIOH | Liver |
| Esterase, (*EST-1** 3.1.1.1) | EBT | Liver |
| Esterase, (*EST-2** 3.1.1.1) | EBT | Liver |
| Fumarase, (*FH** 4.2.1.2) | TCII | Liver |
| Glucosephosphate isomerase (*GPI-A** 1.1.1.49) | EBT | Liver |
| Isocitrate dehydrogenase (*ICDH-S** 1.1.1.42) | TCII | Liver |
| Lactate dehydrogenase (*LDH-A** 1.1.1.27) | TCII | Muscle |
| Lactate dehydrogenase (*LDH-B** 1.1.1.27) | TCII | Liver |
| Malate dehydrogenase (*MDH-A** 1.1.1.37) | EBT | Muscle |
| Malate dehydrogenase (*MDH-B** 1.1.1.37) | EBT | Muscle |
| Peptidase-B (*PEP-B** 3.4.11) | LIOH | Liver |
| Peptidase-S (*PEP-S** 3.4.11) | LIOH | Liver |
| Peptidase-C (*PEP-C** 3.4.11) | LIOH | Liver |
| Xanthine Dehydrogenase (*XDH** 1.1.1.204) | EBT | Liver |

***Nuclear DNA Analysis***

Two nuclear DNA loci were investigated: an actin gene intron and the second intron in the S7 ribosomal protein gene. PCR primers and reaction conditions are listed in Table 3. The primers for the actin intron were designed by Jan Cordes (unpublished data) for weakfish (*Cynosion regalis*). The $2^{nd}$ S7 ribosomal protein intron was amplified with universal fish primers of Chow and Hazama (1998). The regions were amplified using the PCR Reagent System (GIBCO/BRL Life Technologies®). The 25μl PCR cocktail contained a 1X concentration of PCR buffer with $MgCl_2$, 0.2mM dNTP, 0.5μM of each primer, 2.5 units of *Taq* DNA polymerase, and 0.25μl (approximately 25-50ng) of genomic DNA as template. Some PCR reactions were performed with Platinum® *Taq* High Fidelity (Gibco/BRL Life Technologies®) with the following cocktail: 1X High Fidelity PCR buffer, 2mM $MgSO_4$, 0.2mM dNTP, 0.2μM of each primer, and 2.5 units of Platinum® *Taq* DNA polymerase High Fidelity. In other cases, 1μl dimethyl sulfoxide (DMSO, Fisher Scientific BP231-1) was added to the reaction to increase sensitivity. Following amplification PCR products were separated electrophoretically on 1% agarose (Gibco/BRL Life Technologies®) gels, stained with ethidium bromide and visualized using ultraviolet light.

The PCR products from two individuals of each species for both loci were digested with a panel of restriction enzymes to identify those that potentially exhibited fixed differences between the putative parent species. The actin intron PCR fragments were screened with fourteen enzymes and the S7 intron fragments were screened with thirty-five enzymes (Table 4). All samples were digested with the enzymes that demonstrated fixed differences between the putative parent species in the preliminary

analysis. The reaction cocktail contained; 1.5µl 10X reaction buffer, 3 units restriction enzyme, and 4µl PCR amplification reaction product. All restriction digestion reactions were incubated at 37°C for 2 to 18 hours. Digestion products were separated on a 1.25% agarose/1.25% Nuseive agarose (Gibco/BRL Technologies®) gel and visualized with ultraviolet light after staining with ethidium bromide. Restriction fragment patterns were used to assign genotypes to putative parent and hybrid individuals.

***MtDNA Analysis***

The following regions of the mitochondrial genome were surveyed in the three putative parent species and hybrids; adenosine 5'-triphosphatase subunit 6 (ATPase 6), 12S/16S ribosomal RNA, and NADH dehydrogenase 4 (ND4). Primer sequences and PCR conditions used to amplify the loci are provided in Table 3.

Two individuals of each putative parent species were screened with restriction enzymes to identify fixed differences between them. The 12S/16S region was screened with seven enzymes, the ND4 region was screened with nine enzymes, and the ATPase 6 region was screened with six enzymes (Table 4). All individuals were screened at the three regions with those enzymes that revealed fixed differences in the preliminary study. Restriction digestion reactions were performed as described for mtDNA and incubated at 37°C for 2 to 18 hours. Digested fragments were separated on a 1.25% agarose/1.25% Nuseive agarose (Gibco/BRL Life Technologies®) gel, stained with ethidium bromide and visualized under ultraviolet light. Each restriction fragment pattern was given a haplotype designation, letters, in this case. For each individual, the haplotype designations of each region were combined in sequence creating a composite haplotype.

**Table 3:** PCR primers and conditions used in the RFLP analysis of the mtDNA and nuclear intron regions. The forward primer is on top, reverse is on the bottom.

| Region | Primer Sequence | PCR Conditions | Citation |
|---|---|---|---|
| 12S/16S | 12SA-L: 5' AAA CTG GGA TTA GAT ACC CCA CTA T 3'<br>16SA-H: 5' ATG TTT TTG ATA AAC AGG CG 3' | 94°c for 1 min., 45°C for 1 min., 65° for 3 min. | Palumbi et al. 1991 |
| ATPase 6 | H8969: 5' GGG GNC GRA TRA ANA GRC T 3'<br>L8331: 5' TAA GCR NYA GCC TTT TAA G 3' | 95°C for 1 min., 45°C for 1 min., 65°C for 3 min. | Joe Quattro, unpublished data |
| ND4 | ARG-BL: 5'CAA GAC CCT TGA TTT CGG CTC A 3'<br>LEU: 5' CCA GAG TTT CAG GCT CCT AAG ACC A 3' | 95°C for 1 min., 45°C for 1 min., 65°C for 3 min. | Bielawski and Gold, 1996 |
| S7 Ribosomal | S7RPEX2F: 5' AGC GCC AAA ATA GTG AAG CC 3'<br>S7RPEX2R: 5' GCC TTC AGG TCA GAG TTC AT 3' | 95°C for 30 secs., 60°C for 1 min., 72°C for 2 min. | Chow and Hazama, 1998 |
| Short Actin Intron | F3: 5' ATG CCT CTG GTC GTA CCA CTG G 3'<br>R1: 5' CAG GTC CTTACG GAT GTC G 3' | 94°C for 1 min., 48°C for 1 min., 65°C for 3 min | Jan Cordes, unpublished data |

**Table 4:** Restriction digest enzymes used to screen mtDNA and nuclear DNA PCR products and those that showed fixed differences between *Cephalopholis fulva* and *Paranthias furcifer*.

| Type | Locus | Enzymes Screened | Fixed Differenes |
|---|---|---|---|
| MtDNA | 12S/16S | *Ban* II, *Cla* I, *Dra* I, *Mbo* II, *Mse* I, *Rsa* I, *Tha* I | *Ban* II, *Rsa* I |
| | ATPase 6 | *Apa* I, *Dde* I, *Nci* I, *Pvu* I, *Sty* I, and *Spe* I | *Dde* I |
| | ND4 | *Apa* I, *Bst*O I, *Hha* I, *Hpa* II, *Mbo* I, *Nci* I, *Pvu* I, *Rsa* I, *Sty* I | *Bst*O I, *Hpa* II, *Mbo* I, *Rsa* I |
| Nuclear DNA | 2nd S7 Intron | *Alu* I, *Bam*H I, *Ban* II, *Bcl* I, *Bgl* I & II, *Bst*E I, *Bst*O I, *Dra* I, *Eco*R I & V, *Hae* II & III, *Hin*C II, *Hin*f I, *Hha* I, *Hpa* I & II, *Mbo* I, *Mse* I, *Nru* I, *Nsi* I, *Pst* I, *Pvu* II, *Rsa* I, *Sca* I, *Spe* I, *Sph* I, *Ssp* I, *Sst* I & II, *Stu* I, *Sty* I, *Taq* I, *Tha* I. | *Alu* I, *Dra* I |
| | Short Actin Intron | *Alu* I, *Ava* II, *Bst*O I, *Dra* I, *Eco*R I, *Hha* I, *Hin*f I, *Mbo* I & II, *Mse* I, *Msp* I, *Nci* I *Rsa* I, *Taq* I, | *Hin*f I |

***Statistical Analysis***

All specimens were characterized as pure parental, $F_1$ hybrid or post-$F_1$ hybrid individuals based on analysis of nuclear genotypes. $F_1$ hybrids were identified based on heterozygosity at all diagnostic nuclear loci. The maternal parent species of the $F_1$ hybrid and post-$F_1$ hybrid individuals was identified from mitochondrial DNA composite haplotypes. Post-$F_1$ hybrid individuals were identified by homozygosity at one or more diagnostic nuclear loci.

Sequence divergence was calculated for the mitochondrial DNA locus using the computer program REAP (Restriction Enzyme Analysis Program) version 4.0 (McElroy et al. 1991) which uses the equation of Nei and Li (1979) for fragment data with weighting based on Nei and Tajima (1983). The divergence for the allozyme data was calculated using the computer program BIOSYS2 (Swofford and Selander 1997) using the unbiased genetic distance of Nei (1978). Genetic distance could not be calculated using the nuclear DNA loci due to an absence of shared fragments between *Cephalopholis fulva* and *Paranthias furcifer*.

## Results

### *Morphology*

The range and mean of the counts and measurements for each species as well as the hybrids are given in Table 5. Measurements for each individual are reported as millimeters, divided by the standard length and multiplied by 1000 (Appendix C). Thirty-seven of forty-four morphological characters demonstrated differences between *Cephalopholis fulva* and *Paranthias furcifer,* as well as the intermediacy of the hybrid individuals. For example, the head length of *C. fulva* ranged from 374 to 427 and for *P. furcifer* it was 259 to 301. As expected, the range of the head length for the hybrids was intermediate to the ranges for the parent species, from 320 to 353. Other head features of the putative hybrids were also intermediate. For example the maxillary length of *C. fulva* ranged from 177 to 194, while that of *P. furcifer* was 96 to 115. Putative hybrids had intermediate maxillary lengths that ranged from 130 to 163. Another intermediate feature of the putative hybrids was the tail, particularly the distances from the caudal base to the top, middle, and bottom tips of the caudal fins. The putative hybrids were intermediate to *C. fulva* and *P. furcifer* at all three of these measurements.

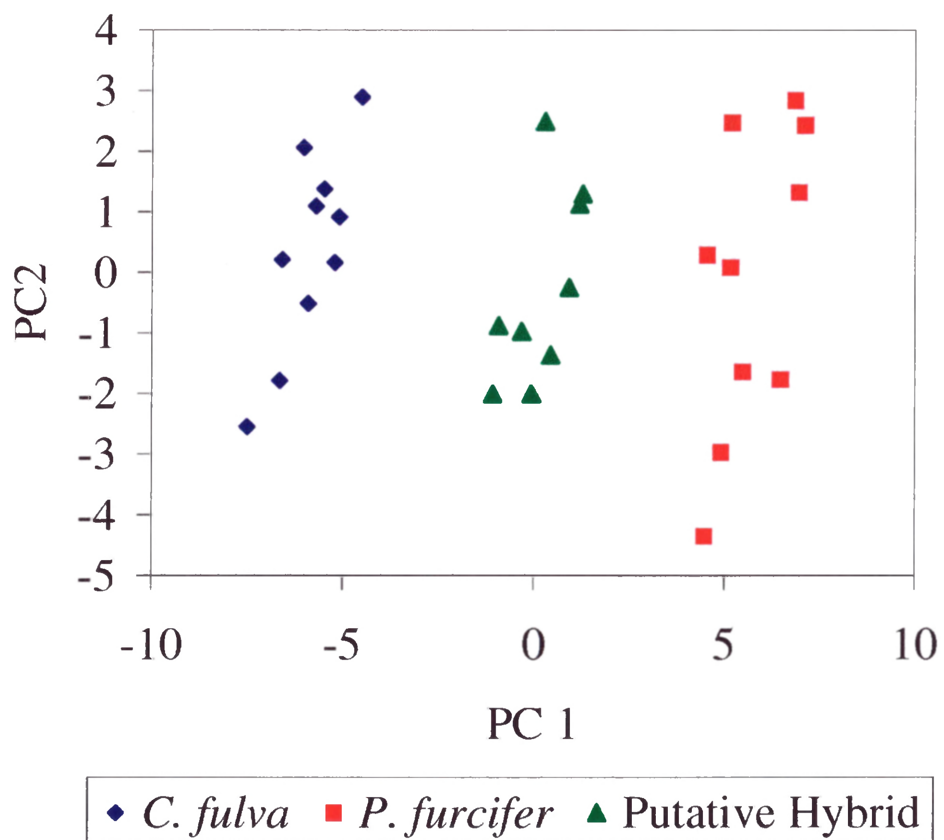
In the principal component analysis of the morphological data. The first three components accounted for 75% of all the variation in the morphological data, the first component was the most important, accounting for 60% of the variation. A plot of the first two principal components, which accounted for most of the variation, shows two well defined groups, made up of the two parent species (Figure 4). All nine putative hybrids fell on or near 0 for the first principal component, halfway between the two putative parent groups.

**Table 5:** Measurement ranges for ten *Paranthias furcifer*, ten *Cephalopholis fulva*, and nine putative hybrids. Measurements are in millimeters. Raw measurements and counts were divided by the standard length and multiplied by 1000. Means are in parentheses. L.L. = lateral line; * The measurement for one hybrid was discarded due to a broken third dorsal spine.

| Measurement | *C. fulva* | putative hybrids | *P. furcifer* |
|---|---|---|---|
| dorsal rays | IX, 15-16 (16) | IX, 17-18 (17) | IX, 18-19 (19) |
| anal soft rays | 8-10 (9) | 9-10 (9) | 9-11 (10) |
| pectoral rays | 32-35 (34) | 35-37 (36) | 36-39 (38) |
| gill rakers | 22-28 (25) | 29-35 (31) | 32-39 (36) |
| scales above the L.L. | 6-8 (8) | 10-11 (10) | 10-14 (12) |
| scales below the L.L. | 22-27 (25) | 26-29 (28) | 26-32 (30) |
| transverse scale rows | 64-84 (71) | 82-91 (85) | 85-96 (91) |
| caudal peduncle scales | 40-49 (46) | 44-51 (47) | 43-48 (46) |
| head length | 374-427 (402) | 320-353 (336) | 259-301 (280) |
| head width | 173-227 (200) | 150-189 (162) | 130-162 (142) |
| head depth | 248-288 (266) | 212-250 (231) | 183-241 (212) |
| snout length | 86-122 (107) | 79-111 (94) | 53-79 (67) |
| suborbital width | 44-52 (47) | 29-35 (33) | 20-24 (22) |
| interorbital width | 64-78 (71) | 73-89 (81) | 76-89 (80) |
| orbit length (diameter) | 62-76 (68) | 56-67 (62) | 52-89 (65) |
| postorbital head length | 217-243 (232) | 185-210 (194) | 146-171 (159) |
| maxillary length | 166-194 (182) | 130-163 (143) | 96-115 (104) |
| lower jaw length | 176-200 (186) | 127-149 (140) | 98-114 (105) |
| snout to angle of preopercle | 257-306 (282) | 213-246 (230) | 176-200 (188) |
| maxillary width | 42-56 (48) | 37-45 (42) | 28-38 (34) |
| tip of lower jaw to gular notch | 119-181 (145) | 90-174 (120) | 69-121 (89) |
| body width | 154-223 (178) | 155-169 (163) | 138-162 (150) |
| body depth | 325-385 (357) | 302-348 (329) | 282-354 (316) |
| caudal peduncle depth | 128-139 (134) | 118-145 (129) | 102-122 (111) |
| tip of snout to dorsal origin | 386-422 (407) | 334-373 (356) | 321-361 (329) |
| tip of snout to pectoral base | 287-415 (371) | 293-340 (320) | 265-293 (277) |
| tip of lower jaw to pelvic base | 398-449 (420) | 359-420 (381) | 321-384 (351) |
| dorsal base length | 523-549 (538) | 532-561 (544) | 543-613 (583) |
| depressed dorsal length | 609-663 (629) | 574-643 (610) | 603-658 (635) |
| anal base length | 166-187 (174) | 171-192 (182) | 165-204 (180) |

| | | | |
|---|---|---|---|
| depressed anal length | 268-320 (294) | 248-273 (262) | 232-278 (253) |
| end of dorsal to caudal base | 129-150 (141) | 148-165 (158) | 145-175 (162) |
| length of caudal peduncle | 166-198 (178) | 194-217 (203) | 174-241 (219) |
| pectoral length | 257-292 (274) | 253-295 (280) | 249-290 (274) |
| pelvic length | 186-211 (197) | 174-192 (184) | 150-187 (173) |
| dorsal spine I length | 54-66 (62) | 48-70 (63) | 40-64 (53) |
| dorsal spine III length | 103-138 (123) | 90*-119 (106) | 86-117 (105) |
| dorsal spine IX length | 97-150 (127) | 104-123 (114) | 76-105 (94) |
| anal spine I length | 52-72 (62) | 43-66 (55) | 36-48 (43) |
| anal spine II length | 95-121 (106) | 93-112 (102) | 78-98 (86) |
| anal spine III length | 78-118 (108) | 92-121 (107) | 74-101 (87) |
| caudal base to tip of upper rays | 197-246 (216) | 228-302 (277) | 321-373 (347) |
| caudal base to tip of middle rays | 204-248 (222) | 166-192 (176) | 122-143 (128) |
| caudal base to tip of lower rays | 197-242 (217) | 278-318 (295) | 308-346 (322) |

**Figure 4:** Graph of principal components 1 and 2 for the morphological data for 10 *Cephalopholis fulva*, 10 *Paranthias furcifer*, and 9 putative hybrids.



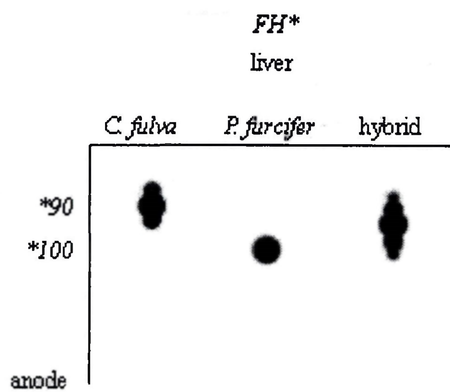
4
3
2
1
0
-1
-2
-3
-4
-5
PC2
-10
-5
0
5
10
PC 1
C. fulva
P. furcifer
Putative Hybrid

***Allozyme Analysis***

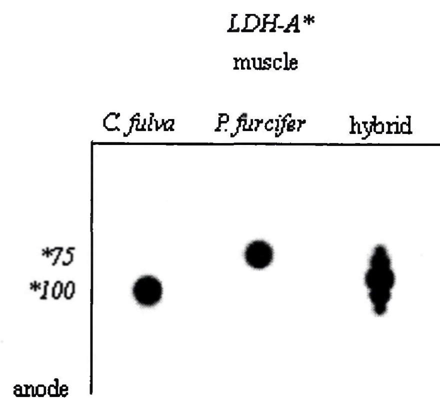
Sixteen allozyme loci were surveyed in 17 *Cephalopholis fulva* and 16 *Paranthias furcifer* (Table 2). The criteria of Waples (1986) were used to identiy loci and nomeclature followed Shaklee et al., (1990). Four loci, *CK-B*, FH*, LDH-B*,* and *ICDH-S**, were fixed for different alleles in *C. fulva* and *P. furcifer* (Figure 5). Seven loci were monomorphic, *ADH-1*, GPI-A*, CK-C*, LDH-A*, MDH-A*, MDH-B*,* and *XDH** and five loci, *EST-1*, EST-2*, PEP-1*, PEP-2**, and *PEP-3**, were variable within one or both species (Table 6). The creatine kinase (CK) loci were visualized with a general protein stain, which has been shown in fishes to reveal the creatine kinase enzyme, (Dayton et al. 1994; Sezaki et al. 1994).

Forty *Cephalopholis fulva*, twenty-eight *Paranthias furcifer*, one *C. cruentata* and ten putative hybrids were screened at the four loci with fixed differences between the putative parent species. At the *CK-B** locus, all the *C. fulva* and *C. cruentata* individuals were homozygous for the **100* allele, while the *P. furcifer* individuals were homozygous for the **50* allele. All ten putative hybrids were heterozygous for the **50* and **100* alleles (Table 7). At the *ICDH-S** locus, all *C. fulva* were homozygous for the **90* allele, all *P. furcifer* were homozygous for the **100* allele, and *C. cruentata* was homozygous for a third allele, **105*. All ten putative hybrids were heterozygous for the **95* and **100* alleles. At the *FH** locus, *C. fulva* and *C. cruentata* were homozygous for the **90* allele and *P. furcifer* individuals were homozygous for the **100* allele. Nine of the putative hybrids were heterozygous for the **90* and **100* alleles, while one putative hybrid was homozygous for the **90* allele. At the *LDH-A** locus, all *C. fulva* individuals were homozygous for the **100* allele and *P. furcifer* and *C. cruentata* were homozygous for

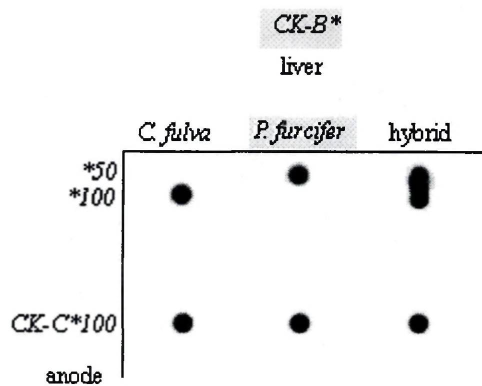
**Figure 5:** Allozyme allele patterns for the four diagnostic loci, *FH*, LDH-B*, CK-B**, and *ICDH-S**, in *Cephalopholis fulva*, *C. cruentata, Paranthias furcifer*, and putative hybrids.



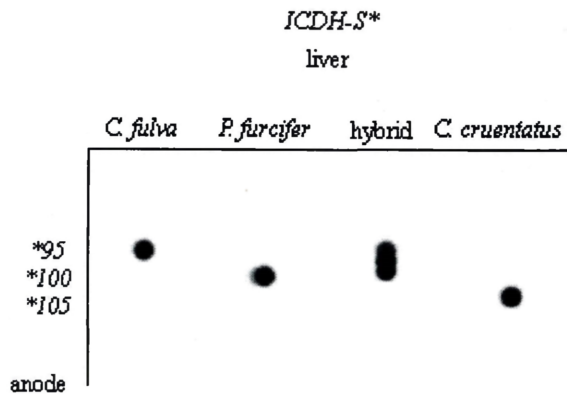
*FH**
liver
*C. fulva*
*P. furcifer*
hybrid
*90
*100
anode

*LDH-A**
muscle
*C. fulva*
*P. furcifer*
hybrid
*75
*100
anode

*CK-B**
liver
*C. fulva*
*P. furcifer*
hybrid
*50
*100
CK-C*100
anode

*ICDH-S**
liver
*C. fulva*
*P. furcifer*
hybrid
*C. cruentatus*
*95
*100
*105
anode

**Table 6:** Allozyme genotypes of *Cephalopholis fulva, Paranthias furcifer*, *C. cruentata* and putative hybrid individuals for all 16 loci screened.

| Locus | *ADH-1** 1.1.1.1 | *CK-B** 2.7.3.2 | *CK-C** 2.7.3.2 | *EST-1** 3.1.1.1 | *EST-2** 3.1.1.1 | *FH** 4.2.1.2 | *GPI-A** 1.1.1.49 |
|---|---|---|---|---|---|---|---|
| Genotypes | *100/100 | *100/100<br>*50/50<br>*50/100 | *100/100 | *100/100<br>*100/105 | *90/90<br>*90/85<br>*90/80<br>*80/80 | *90/90<br>*100/100<br>*90/100 | *100/100 |

| *ICDH-S** 1.1.1.42 | *LDH-A** 1.1.1.27 | *LDH-B** 1.1.1.27 | *MDH-A** 1.1.1.27 | *MDH-B** 1.1.1.37 | *PEP-1** 3.4.11 | *PEP-3** 3.4.11 | *PEP-2** 3.4.11 | *XDH** 1.1.1.204 |
|---|---|---|---|---|---|---|---|---|
| *95/95<br>*100/100<br>*95/100 | *25/25 | *100/100<br>*75/100<br>*75/75 | *40/40 | *100/100 | *130/130<br>*120/120<br>*110/130<br>*100/130<br>*100/120<br>*100/100 | *100/100<br>*100/105 | *120/130<br>*125/125<br>*120/120<br>*110/130<br>*110/120<br>*110/110<br>*100/130<br>*100/100 | *100/100 |

**Table 7:** Allozyme genotypes of *Cephalopholis fulva, Paranthias furcifer, C. cruentata* and putative hybrid individuals at the four diagnostic loci, *CK-B**, *FH**, *ICDH-S**, and *LDH-B**.

| | N | *CK-B** | *FH** | *ICDH-S** | *LDH-B** |
|---|---|---|---|---|---|
| *C. fulva* | 40 | **100/100* | **90/90* | **95/95* | **100/100* |
| *P. furcifer* | 28 | **50/50* | **100/100* | **100/100* | **75/75* |
| *C. cruentata* | 1 | **100/100* | **90/90* | **105/105* | **75/75* |
| Putative hybrids | 8 | **50/100* | **90/100* | **100/95* | **75/100* |
| | 1 | **50/100* | **90/100* | **100/95* | **75/75* |
| | 1 | **50/100* | **90/90* | **100/95* | **75/100* |

the **75* allele. Nine of the putative hybrids were heterozygous for the **75* and **100* alleles while one putative hybrid was homozygous at the **75* allele. It should be noted that the homozygosity in the *FH** and *LDH-A** loci among the putative hybrids occurred in two separate individuals, one homozygous at the *FH** locus and one homozygous at the *LDH-A** locus.

### ***Nuclear Intron Regions***

An actin intron approximately 450 base pairs in length amplified in these species. The PCR amplified region was surveyed with fourteen restriction enzymes, of which one, *Hin*f I, showed a fixed difference between species (Figure 6, Table 8). Following digestion, all *Cephalopholis fulva* had two fragments, 400 and 50 base pairs (allele A), while the regions in *P. furcifer* and *C. cruentata* were not cleaved by the enzyme (allele B). Because the fragment sizes summed to equal the size of the PCR fragment, it was concluded that the individuals surveyed were homozygous at this locus. *Cephalopholis fulva* individuals were homozygous for allele A, while *Paranthias furcifer* were homozygous for allele B (Table 9). All fifteen putative hybrids were heterozygous for both alleles.

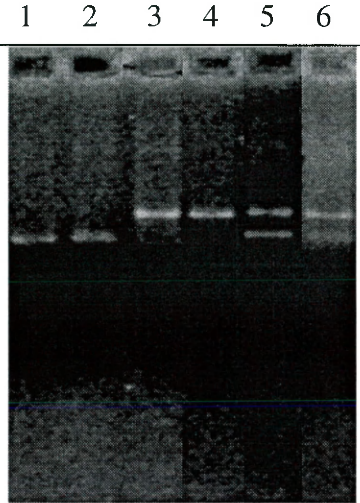
The second intron region of the S7 ribosomal protein, which was approximately 1200 base pairs in length, was screened with thirty-five enzymes. Two enzymes demonstrated fixed differences between *Paranthias furcifer* and *Cephalopholis fulva*, *Dra* I and *Alu* I. *Dra* I digestions produced four different fragment patterns in these species. Fifty *C. fulva* individuals had three fragments, 575, 550 and 75 base pairs (allele A), three *C. fulva* had four fragments 575, 525, 75, and 25 base pairs (allele E),

**Figure 6:** RFLP fragment patterns of *Cephalopholis fulva*, *Paranthias furcifer*, *C. cruentata* and putative hybrids for the actin intron (digested with *Hin*f I) and the 2nd intron in the S7 ribosomal protein region (digested with *Alu* I and *Dra* I).

Actin Intron

1. *C. fulva*
2. *C. fulva*
3. *P. furcifer*
4. *C. cruentata*
5. hybrid
6. hybrid

1 2 3 4 5 6

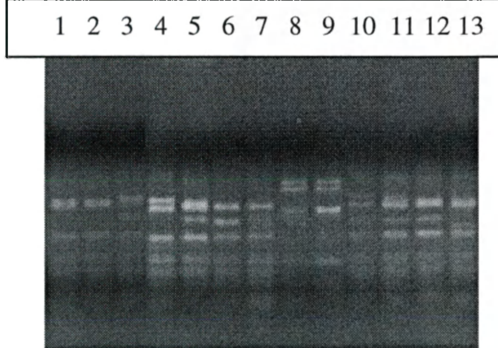


RP2 Intron

1. *C. fulva*
2. *C. fulva*
3. *C. fulva*
4. *C. fulva*
5. *P. furcifer*
6. *P. furcifer*
7. *P. furcifer*
8. *C. cruentata*
9. *C. cruentata*
10. hybrid
11. hybrid
12. hybrid
13. hybrid

*Alu* I

1 2 3 4 5 6 7 8 9 10 11 12 13



1. *C. fulva*
2. *C. fulva*
3. *C. fulva*
4. *P. furcifer*
5. hybrid

*Dra* I

1 2 3 4 5

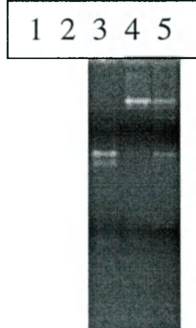


**Table 8:** Restriction fragment sizes of the actin intron digested with *Hin*f I. The asterisk* refers to inferred bands so fragments would sum to the total (uncut) fragment size.

**Actin Intron**
*Hin*fI

| A | B |
|---|---|
| - | 450 |
| 400 | - |
| 50* | - |

**Table 9:** Genotypes of *Cephalopholis fulva, Paranthias furcifer, C. cruentata,* and putative hybrids for the short actin intron and the second intron in the S7 ribosomal protein region.

| **Species** | **Genotypes** | | | |
|---|---|---|---|---|
| | Actin Intron | | S7 Intron | |
| | N | *Hinf* I | N | *Dra* I |
| ***Cephalopholis fulva*** | 57 | A/A | 50<br>3<br>4 | A/A<br>E/E<br>A/E |
| ***Paranthias furcifer*** | 37 | B/B | 37 | D/D |
| ***C. cruentata*** | 5 | B/B | 5 | D/D |
| **Putative hybrid** | 15 | A/B | 14<br>1 | A/D<br>E/D |

and four individuals had a combination of these patterns (i.e. were heterozygous) (Figure 6; Table 10). All *P. furcifer* and *C. cruentata* were uncut by the enzyme (allele D). As shown in Table 9, *C. fulva* was variable at this locus, with fifty individuals being homozygous for allele A, three were homozygous for allele E, and four were heterozygous for alleles A and E. All thirty-seven *P. furcifer* and five *C. cruentata* were homozygous for the D allele. The fifteen putative hybrids were heterozygous at this locus with one of the *C. fulva* alleles (A or E), and the *P. furcifer* allele (D). *Epinephelus guttatus* was not able to be amplified at this locus, indicating variation at the primer binding sites. It was concluded that it was not a putative parent species and therefore, it was not included further analyses.

Digestion of the second intron in the S7 region by *Alu* I produced a large number of small fragments that were not easily interpreted. Therefore, the data were not used to identify hybridization between *Cephalopholis fulva* and *Paranthias furcifer*. However, *C. cruentata* was distinguished from *P. furcifer* using this enzyme, eliminating it as a putative parent for the hybrid individuals (Figure 6).

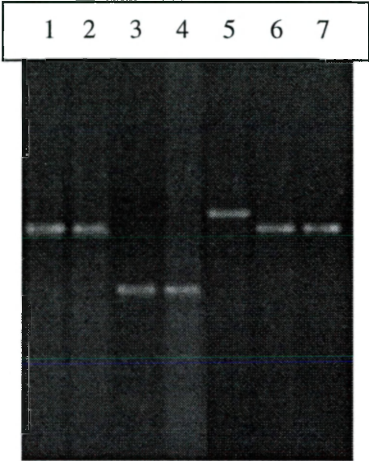
***Mitochondrial DNA***

Fixed allelic differences between *Cephalopholis fulva* and *Paranthias furcifer* were found at all three mitochondrial gene regions. The ATPase 6 region, approximately 650 base pairs long, was screened with six enzymes, one of which, *Dde* I, showed fixed differences between *C. fulva*, *P. furcifer*, and *C. cruentata* (Table 4). Digestion of the ATPase 6 region of *C. fulva* with *Dde* I resulted in two fragments, (haplotype A), *P.*

**Figure 7:** RFLP fragment patterns of *Cephalopholis fulva, Paranthias furcifer, C. cruentata* and putative hybrids for the three mitochondrial DNA regions, ATPase 6 (digested with *Dde* I), 12S/16S (digested with *Rsa* I and *Ban* II), and ND4 (digested with *Bst*O I, *Hpa* II, *Mbo* I, and *Rsa* I)

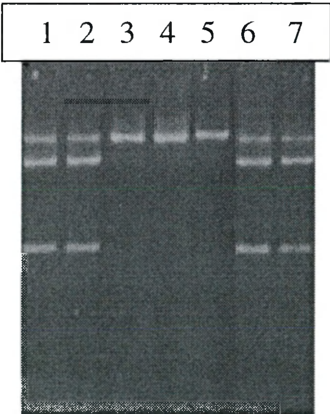
1. *C. fulva*
2. *C. fulva*
3. *P. furcifer*
4. *P. furcifer*
5. *C. cruentata*
6. hybrid
7. hybrid

ATPase 6 *Dde* I

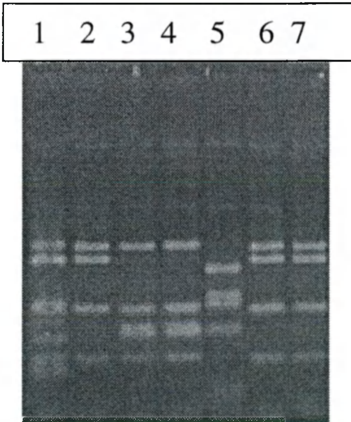



12S/16S

*Ban* II



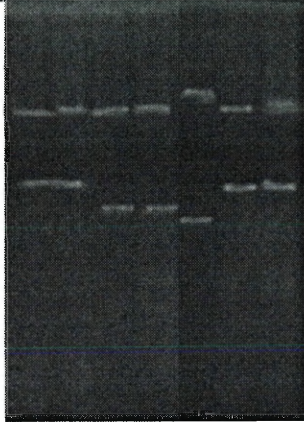

*Rsa* I



ND4

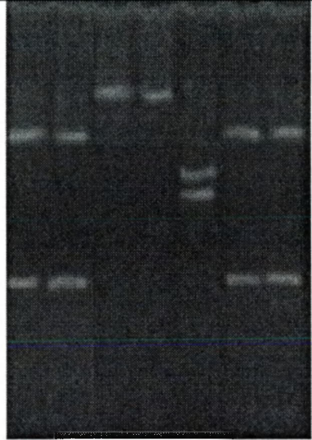
*Bst*O I

1 2 3 4 5 6 7



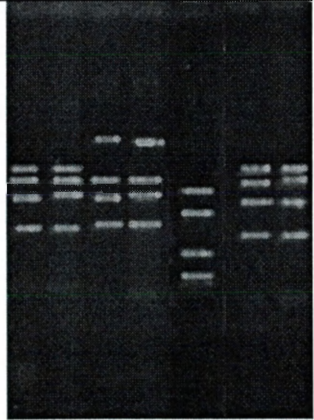
*Hpa* II

1 2 3 4 5 6 7



*Mbo* I

1 2 3 4 5 6 7



*Rsa* I

1 2 3 4 5 6 7

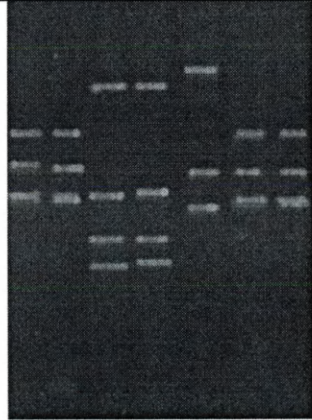


**Table 10:** Restriction fragment lengths of the second intron in the S7 ribosomal protein region, digested with *Dra* I. The asterisk* indicates bands that were inferred so fragments would sum to the total (uncut) fragment size.

**S7 Ribosomal Protein Intron**

*Dra* I

| A | D | E |
|---|---|---|
| - | 1200 | - |
| - | - | - |
| - | - | - |
| 575 | - | 575 |
| 550 | - | - |
| - | - | 525 |
| 75* | - | 75* |
| - | - | 25* |

**Table 11:** Restriction fragment lengths of the ATPase 6 region, digested with *Dde* I. The asterisk* marks bands that are too small to be viewed and have been inferred based on total fragment size.

**ATPase 6**
*Dde* I

| A | B | C |
|---|---|---|
| - | - | 650 |
| 600 | - | - |
| - | 360 | - |
| - | 150 | - |
| - | 90 | - |
| 50* | 50* | - |

*furcifer* individuals had 4 fragments, (haplotype B), and *C.cruentata* remained uncut by the enzyme, (haplotype C; Figure 7, Table 11). Only one fragment was shared between *C. fulva* and *P. furcifer*.

The 12S/16S region, approximately 1600 base pairs long, was screened with seven enzymes (Table 4). Two enzymes, *Ban* II and *Rsa* I, showed fixed differences between *Cephalopholis fulva*, *Paranthias furcifer,* and *C. cruentata*. *Ban* II digestions produced only two fragment patterns. *C. fulva* individuals remained uncut (haplotype A), while *P. furcifer* and *C. cruentata* produced two fragments (haplotype B; Figure 7, Table 12). *Rsa* I digestions produced three fragment patterns in these species. *Cephalopholis fulva* individuals had four fragments (haplotype A), *P. furcifer* had five fragments (haplotype B), and *C. cruentata* had six fragments (haplotype C). All three haplotypes shared one fragment, and haplotypes A and B shared a second fragment.

The ND4 region, approximately 1900 base pairs long, was screened with nine restriction enzymes four of which showed fixed differences between the species: *Bst*O I, *Hpa* II, *Mbo* I, and *Rsa* I (Table 4). *Bst*O I digestions produced three fragment patterns in these species. *Cephalopholis fulva* individuals had three fragments (haplotype A), *Paranthias furcifer* had three fragments (haplotype B), and *C. cruentata* had two fragments (haplotype C; Figure 7, Table 13). Haplotypes A and B shared one fragment, and haplotypes B and C shared one fragment. *Hpa* II digestions produced three different fragment patterns. *Cephalopholis fulva* individuals had two fragments (haplotype A), all but one *P. furcifer* were uncut by the enzyme (haplotype C), and one *P. furcifer* and all *C. cruentata* had two fragments (haplotype D). There were no shared fragments between

**Table 12:** Restriction fragment lengths of the 12S/16S ribosomal RNA region digested with *Rsa* I and *Ban* II. The asterisk* indicates a band that was interpreted to be two bands of the same size due to intensity and width.

**12S/16S**

*Rsa* I

| A | B | C |
|---|---|---|
| 600 | 600 | - |
| 500 | - | - |
| - | - | 450 |
| - | - | 375 |
| 300 | 300 | 300 |
| - | - | 275 |
| | 250 | |
| | 250 | |
| 200 | 200 | - |
| - | - | 100 |
| - | - | 100* |

*Ban* II

| A | B |
|---|---|
| 1600 | - |
| - | 1100 |
| - | 500 |

**Table 13:** Restriction fragment lengths of the ND4 region, digested with *Bst*O I, *Hpa* II, *Mbo* I, and *Rsa* I. The asterisk* marks bands that were too small to be viewed and were inferred based on total fragment size.

**ND4**

*Bst*OI

| A | B | C |
|---|---|---|
| - | - | 1450 |
| 1250 | 1250 | - |
| 625 | - | - |
| - | 450 | 450 |
| - | 200 | - |
| 25* | - | - |

*Hpa* II

| A | C | D |
|---|---|---|
| - | 1900 | - |
| 1400 | - | - |
| - | - | 1000 |
| - | - | 900 |
| 500 | - | - |

*Mbo* I

| A | B | C |
|---|---|---|
| 700 | - | - |
| - | 550 | - |
| - | - | 525 |
| 500 | 500 | - |
| 400 | 400 | 400 |
| 300 | 300 | 300 |
| - | - | 275 |
| - | - | 250 |
| - | 150 | - |
| | | 80 |
| | | 70 |

*Rsa* I

| A | B | C | D |
|---|---|---|---|
| 1025 | - | - | - |
| - | - | - | 900 |
| - | 610 | - | - |
| 500 | 500 | 500 | - |
| - | - | - | 425 |
| - | 400 | 400 | - |
| - | 390 | - | - |
| 375 | - | - | - |
| - | - | 350 | - |
| - | - | - | 325 |
| - | - | 300 | - |
| - | - | 300 | - |
| - | - | - | 250 |
| - | - | 50* | - |

any of the haplotypes resulting from digestion with this enzyme. *Mbo* I digestions also produced three fragment patterns in these species. *Cephalopholis fulva* individuals had five fragments (haplotype B), *P. furcifer* individuals had four fragments (haplotype A), and *C. cruentata* had seven fragments (haplotype C). All haplotypes shared two fragments and A and B shared a third fragment. *Rsa* I digestions of the ND4 region produced four fragment patterns in these species. Fifty-six *C. fulva* individuals had four fragments (haplotype B), one *C. fulva* had six fragments (haplotype C), all *P. furcifer* individuals had four fragments (haplotype D), and *C. cruentata* had three fragments (haplotype A). Haplotypes A, B and C shared one fragment, and B and C shared a second fragment.

Each restriction pattern was given a haplotype designation (letter) and composite haplotypes for the mitochondrial DNA locus are listed in Table 14. At all three mitochondrial regions, composite haplotypes were unique to a species. All 15 putative hybrids in the study had a composite haplotype matching that of *Cephalopholis fulva*, indicating that it was the maternal parent for all hybrid individuals. Three *Epinephelus guttatus* specimens were screened at the ND4 and ATPase 6 regions, however the composite haplotype was unique and therefore this species was not included in the study as a putative parent species.

### *Genetic Distance*

Nucleotide sequence divergence and Nei's (1978) unbiased genetic distance were estimated between *Cephalopholis fulva, C. cruentata* and *Paranthias furcifer* for the mitochondrial and allozyme data respectively (Table 15). The mitochondrial genomes of

**Table 14:** Composite haplotypes of *Cephalopholis fulva, Paranthias furcifer,* and *C. cruentata* for the mitochondrial DNA data. Haplotypes are listed in the following order:

ATPase 6– *Dde* I, ND4 – *Bst*O I, *Hpa* II, *Mbo* II, *Rsa* I, 12S/16S – *Rsa* I, *Ban* II

| **Species** | **N** | **Composite Haplotype** |
|---|---|---|
| *Cephalopholis fulva* | 56 | AAABBAA |
| | 1 | AAABCAA |
| *Paranthias furcifer* | 36 | BBCADBB |
| | 1 | BBDADBB |
| *C. cruentata* | 5 | CCDCACB |
| Putative hybrids | 15 | AAABBAA |

**Table 15:** Nucleotide sequence divergence (Nei and Li 1979) between *Cephalopholis fulva, Paranthias furcifer,* and *C. cruentata* for the mitochondrial DNA and the unbiased genetic distance (Nei 1978) between *C. fulva* and *P. furcifer* for the allozyme data.

**Mitochondrial DNA:**

| | *C. fulva* | *P. furcifer* | *C. cruentata* |
|---|---|---|---|
| *C. fulva* | - | | |
| *P. furcifer* | 0.036 | - | |
| *C. cruentata* | 0.052 | 0.042 | |

**Allozyme Data:**

| | *C. fulva* | *P. furcifer* |
|---|---|---|
| *C. fulva* | - | |
| *P. furcifer* | 0.356 | |

*Cephalopholis fulva* and *P. furcifer* were more closely related to each other,(p = 0.036) than *C. cruentata* was to either *C. fulva,* (p = 0.052) or *P. furcifer*, (p = 0.042). Nei's (1978) unbiased genetic distance between *C. fulva* and *P. furcifer* was estimated from the allozyme data to be 0.356.

## Discussion

### *Morphology*

The morphological data are consistent with the hypothesis of hybridization between *Cephalopholis fulva* and *Paranthias furcifer*. Measurements of *Cephalopholis fulva, Paranthias furcifer,* and the putative hybrids were within or close to the ranges reported by C.L. Smith (1966, 1971). In Smith's (1966) study, forty-one of forty-five hybrid characters were found to be intermediate to those of the putative parent species, while in this study, thirty-seven of forty-four hybrid characters were intermediate between *C. fulva* and *P. furcifer*. These characters included scale counts, head measurements, body measurements and some of the fin spine lengths. Other measurements, such as the anal spine II length, depressed anal length and the tip of the lower jaw to the gular notch were within the range of one or the other species, but not intermediate. For one measurement, the tip of the snout to the dorsal origin, the range of the putative hybrids was outside the ranges of both parent species.

For six characters, ranges of the hybrid individuals were not intermediate between the ranges of the putative parent species. The hybrid ranges extended beyond the ranges of the parent species for the following characters; caudal peduncle scales, caudal peduncle depth, depressed dorsal length, pectoral length, dorsal spine I length, and anal spine III length. The extended ranges for both the caudal peduncle depth and caudal peduncle scales were the result of a single individual that appeared to have a slightly

wider caudal peduncle that the other hybrids. This individual was also the cause for the extended range in pectoral length, the dorsal spine I length, and the anal spine III length. Overall, the majority of morphological characters was suggestive of hybridization between *C. fulva* and *P. furcifer.*

The principal component analysis proved to be an excellent statistical method to define parent species and identify hybrid individuals. In theory, $F_1$ hybrids should be morphologically intermediate to the parent species and have low variation within characters among themselves. Backcross individuals, due to random sorting of chromosomes, should have higher variation within intermediate characters, and could fall anywhere in the range of the pure parent species (Anderson 1949). In a principal component plot, a backcross hybrid's score would be expected to be closer to the parent species to which the hybrid backcrossed, whereas an $F_1$ hybrid's characters would be expected to be in the center, closer to an average of the scores of the parent species. An $F_2$ hybrid could have a principal score anywhere within that of either parent species.

G. R. Smith (1973) showed the usefulness of principal component analysis in hybridization studies. Using morphological data, both hybrids and potential backcross individuals were identified with the plot of the first two principal components. Smith was also able to eliminate pure individuals of unusual size or shape that may have been mistakenly classified as hybrids. A plot of the first two principal components in the present study (Figure 4) shows that *Cephalopholis fulva* and *Paranthias furcifer* were well segregated based on morphological characters. The intermediate placement of the putative hybrid individuals was consistent with the hypothesis that they were hybrids of *P. furcifer* and *C. fulva.* Variation within the hybrid group was no larger than that of the

pure parent species and no putative hybrids fell outside the intermediate area on the principle component plot, including the putative hybrid individual that had extended ranges on some of the measurements mentioned above. This analysis does not prove an absence of backcross hybrids, it is possible that they exist and cannot be distinguished from $F_1$ or $F_2$ hybrids using the morphological data.

### *Genetic Data*

#### *Allozyme Analysis*

The results of the allozyme analysis supported hybridization between *Cephalopholis fulva* and *Paranthias furcifer* in Bermuda based on heterozygosity at the four distinguishing loci. *Cephalopholis cruentata* was eliminated as a putative parent because it was homozygous for a unique allele at the *ICDH-S** locus not found in any putative hybrid (Table 7). Seven of the nine hybrids studied were heterozygous at all four diagnostic allozyme loci, consistent with expected profiles for $F_1$ hybrid individuals. Two individuals, however, were homozygous at one diagnostic locus each. The first individual, "hybrid #1", was homozygous at the *LDH-A** locus for the **75* allele. All twenty-eight *P. furcifer* were homozygous for this same allele, suggesting that hybrid 1 was the product of a hybrid backcrossing to *P. furcifer* or two $F_1$ hybrids crossing. The other hybrid individual, "hybrid #7", was homozygous at the *FH** locus for the **90* allele. All 40 sampled *C. fulva* were homozygous for this allele, indicating that it was the product of a hybrid backcrossing to *C. fulva* or $F_1$ hybrids crossing. An $F_2$ individual can only be distinguished from a backcross hybrid by homozygosity at two or more diagnostic loci, each for a different parent species. Because this was not the case, it was impossible

to distinguish $F_2$ hybrids and backcross hybrid individuals and herein the two individuals described above were referred to as post-$F_1$ hybrids. Because all members of the putative parent samples were homozygous at all loci for diagnostic alleles, there was no evidence of introgression between *C. fulva* and *P. furcifer*.

These results show the utility of allozyme electrophoresis in the detection of hybridization. Based on four nuclear loci, hybridization between *Cephalopholis fulva* and *Paranthias furcifer* could be established and, not only could post-$F_1$ individuals be identified, but the direction to which probable backcrossing occurred was also revealed.

*Nuclear Intron Analysis*

The nuclear DNA data also support hybridization between *Cephalopholis fulva* and *Paranthias furcifer*. *Cephalopholis fulva* and *P. furcifer* were each homozygous for different alleles at the actin intron. *Cephalopholis cruentata* individuals were homozygous for the same allele as *P. furcifer*, however it was already eliminated as a putative parent using the allozyme data. All fifteen putative hybrids sampled were heterozygous for the two alleles fixed in *C. fulva* and *P. furcifer*.

Interpretation of the second S7 intron data was more complex. Within *Cephalopholis fulva* the intron was polymorphic among individuals. Fifty *C. fulva* were homozygous for allele A, three were homozygous for allele E, and four were heterozygous for alleles A and E. All 37 *Paranthias furcifer* and five *C. cruentata* were homozygous for the D allele. The 15 sampled hybrids reflected the variation observed within the species *C. fulva*. Fourteen were heterozygous for the A and D alleles, while one was heterozygous for the E and D alleles.

The nuclear intron data is consistent with the allozyme data and supports the hypothesis of hybridization between *Cephalopholis fulva* and *Paranthias furcifer*. Since all hybrid individuals were heterozygous, post-$F_1$ hybridization was not detected at these loci. This does not mean, however, that the intron data conflicts with the allozyme data, only that allelic patterns that would indicate post-$F_1$ hybridization were not observed at these nuclear DNA loci. Alleles present at both nuclear DNA loci were unique between parent species and there was no indication of introgression between these species.

*Mitochondrial DNA*

The mtDNA analysis did not dispute conclusion of the nuclear genetic analysis, that hybridization has occurred between *Cephalopholis fulva* and *Paranthias furcifer*. The mtDNA data clearly show that *C. fulva* was the maternal parent for all hybrids, including the two post-$F_1$ hybrids, indicating a gender bias in hybridization. All sampled *C. fulva* had a unique composite haplotype that was identical to the composite haplotypes of all 15 sampled hybrids (Table 14). As with the allozyme data the parent species, *C. fulva* and *P. furcifer,* were fixed for different mtDNA haplotypes. At this level of analysis, there was no indication of introgression between *C. fulva* and *P. furcifer*.

Overall, the genetic analysis of all but two of the 15 putative hybrids suggested that they were $F_1$ hybrids, specifically the results indicated that they were the product of interbreeding between a female *Cephalopholis fulva* and a male *Paranthias furcifer* as all hybrids carried *C. fulva* mtDNA. The two post-$F_1$ hybrids identified in the allozyme analysis also had *C. fulva* type mtDNA. For hybrid #1 this indicates that a female $F_1$ hybrid with a *C. fulva* mother and a *P. furcifer* father may have backcrossed with a pure *P. furcifer*. Hybrid #7, on the other hand, may have been the product of backcrossing to *C.*

*fulva*. Therefore, the $F_1$ hybrid could have been a female with a *C. fulva* mother, or a male reproducing with a *C. fulva* female. It is possible that both post-$F_1$ hybrids are the product of $F_1$ X $F_1$ mating, the female having *C. fulva* mtDNA. In either case, the data indicate that all initial successful crosses were between a *C. fulva* female and a *P. furcifer* male.

### *Cumulative Data*

The morphological and genetic data were consistent, suggesting that all putative hybrid individuals should to be classified as $F_1$or post-$F_1$ hybrid individuals (Table 16). Morphological data indicate a definite separation for *Cephalopholis fulva* and *Paranthias furcifer,* and show that the hybrid individuals were intermediate between the two species supporting hybridization. The morphological analysis did not indicate that any of the hybrid individuals were post-$F_1$ hybrids, but their presence was not refuted either. The hybrid individual with extreme morphological measurements was concluded to be an $F_1$ hybrid based on the genetic analysis. The nuclear data (allozyme and intron) indicate that at least seven of the putative hybrid individuals are $F_1$ hybrids. The allozyme data indicated that two putative hybrids were post-$F_1$ hybrid individuals and that if they are backcrosses, it is occurring indiscriminate of species. The possibility that either of these two individuals are $F_2$ individuals, could not be refuted. Six of the hybrids were not analyzed with allozymes due to sample age, and available intron data did not distinguish them as $F_1$ or post-$F_1$ hybrids. They were concluded to be $F_1$ hybrids based on available data. The mtDNA data clearly show that in all cases, the maternal parent of the $F_1$ hybrids was *C. fulva*. The combined molecular data also showed that, while there is post-

**Table 16:** Classification of all putative hybrid individuals as $F_1$ or post-$F_1$ individuals based on morphological and genetic data.

| Sample ID | Classification | Data |
|---|---|---|
| H1 | post-$F_1$ | Allozyme, mtDNA, nDNA |
| H2 | $F_1$ | mtDNA, nDNA |
| H3 | $F_1$ | mtDNA, nDNA |
| H4 | $F_1$ | mtDNA, nDNA |
| H5 | $F_1$ | mtDNA, nDNA |
| H6 | $F_1$ | mtDNA, nDNA |
| H7 | post-$F_1$ | Morphology, Allozyme, mtDNA, nDNA |
| H8 | $F_1$ | Morphology, Allozyme, mtDNA, nDNA |
| H9 | $F_1$ | Morphology, Allozyme, mtDNA, nDNA |
| H10 | $F_1$ | Morphology, Allozyme, mtDNA, nDNA |
| H11 | $F_1$ | Morphology, Allozyme, mtDNA, nDNA |
| H12 | $F_1$ | Morphology, Allozyme, mtDNA, nDNA |
| H13 | $F_1$ | Morphology, Allozyme, mtDNA, nDNA |
| H14 | $F_1$ | Morphology, Allozyme, mtDNA, nDNA |
| H15 | $F_1$ | Morphology, Allozyme, mtDNA, nDNA |

$F_1$ hybridization, introgression has not occurred in the 94 pure parent individuals sampled.

### *Hypotheses*

Based on the combined morphological and molecular analyses, it was concluded that hybridization has occurred between *Cephalopholis fulva* and *Paranthias furcifer*. Therefore, hypothesis 1, that hybridization has occurred, could not be rejected. Based on the mtDNA data, I was able to show that all $F_1$ hybrids were the product of a female *C. fulva* and a male *P. furcifer* and was able to reject hypothesis 2, that hybridization was not gender biased. The allozyme data clearly showed post-$F_1$ hybridization in the sample and hypothesis 3, that post-$F_1$ hybridization was absent, was rejected. None of the evidence presented indicated introgression between *C. fulva* and *P. furcifer* in Bermuda and hypothesis 4, the absence of introgression, could not be rejected.

### *Hybridization in Bermuda*

#### *Reproduction*

Hybridization between *Cephalopholis fulva* and *Paranthias furcifer* is known from only certain localities in the tropical Atlantic, despite broad overlap in the geographical ranges of each species. Biological information on both species is limited. It is known that *C. fulva* are protogynous hermaphrodites like most other epinepheline serranids, and spawn at various times of the year depending on locality and water temperature. For example, in Bermuda, *C. fulva* spawns from May to early August (Thompson and Munro 1978), while in Puerto Rico it spawns between October and April.

*Paranthias furcifer* on the other hand, is gonochoristic (Posada 1996). It spawns in Bermuda from May to August (Thompson and Munroe 1978) and in Puerto Rico through November and December (Posada 1996).

In Bermuda, these two species' spawning periods overlap, allowing heterospecific gametes to come in contact. This suggests that the reproductive isolating mechanism between species may be spawning season and location. This is supported by the presence of at least two hybrids caught in Jamaica, where the two species also have overlapping spawning times (Thompson and Munro 1978). By identifying those regions where spawning times of these species overlap, it may be possible to predict where to find *C. fulva* X *P. furcifer* hybrids.

Information about spawning in groupers is limited. Groupers in general, aggregate in small groups or pairs, depending on the species, and rise from their present depth to spawn further up in the water column. Larger aggregations of breeding individuals with absence of pairing have been reported for *Paranthias furcifer* (Posada 1996), *Epinephelus striatus* (Guitart Manday and Fernandez 1966), and *E. guttatus* (Colin et al. 1987). *Cephalopholis fulva* has been reported to have harem spawning behavior, with a single male spawning with several females (Heemstra and Randall 1993). Some groupers, including members of *Epinephelus* and *Plectropomus,* have demonstrated elaborate courting behavior (Thresher 1984). For example, *Plectropomus lepardus* males exhibit courting colors while patrolling the reef for females, then swim alongside them at a 90° rotation to signal interest in spawning.

The reproductive habits of *Cephalopholis fulva* and *Paranthias furcifer* are relatively unknown, so I can only speculate on how these two species have hybridized. It

is thought that groupers spawn over the shelf so the ebb tide will transport eggs quickly offshore, and it seems that both gamete longevity and dilution will affect the fertilization success in *C. fulva* and *P. furcifer.*

For some species of fish, eggs hatch within 40 hours of spawning (Smith 1971), so fertilization must occur relatively soon after release of gametes. Pennington (1985) showed the dependence of echinoid fertilization on group spawning and that fertilization success depended on sperm dilution as a result of current speed. There was also a 50% decrease in fertilization success when males and females were two meters apart, success quickly deteriorating with distance even in the presence of multiple males. Marconato and Shapiro (1996) showed a similar need for proximity for fertilization in the bucktooth parrotfish, *Sparisoma radians*. Based on average number of sperm and eggs released in each spawn, the necessary concentration for fertilization was only maintained for a few moments.

Peterson et al. (1992) on the other hand, showed that fertilization success in labroids depended mostly on sperm longevity and that 75% of the fertilization occurred in the first 15 seconds. Teleost sperm is generally short lived, and the labroid sperm in the study were unable to fertilize eggs after the first 15 seconds. The eggs are believed to have a longer viability, but probably not more than a few minutes, although information is limited. For example, bluehead wrasse, *Thalassoma bifasciatum*, eggs show decreased fertilization success only sixty seconds after spawning (Peterson et al. 1992). Considering gamete viability and the currents at the location of spawning, it is believed that the fish would have to be within a few meters of each other to successful interbreed.

Hybridization between *Cephalopholis fulva* and *Paranthias furcifer* could be a chance meeting of gametes, which happen to be spawned a the same time and in the same general location. This is consistent with the observation of Burnett-Herkes (1975), who observed indirect evidence of spawning groups of both species in the same location in Bermuda. If the species do pair spawn, it is possible that *C. fulva* females are pairing with *P. furcifer* males, although it would be hindered by any species specific courting behavior.

*Gender Bias*

The mtDNA data indicate that $F_1$ hybrids are produced from *Cephalopholis fulva* females crossing with *Paranthias furcifer* males. There is no known reason why hybridization is gender biased. There may be a decline in one species prompting the need for a mate of another species. *Paranthias furcifer* numbers have declined in the last twenty years due to commercial fishing in Bermuda (Luckhurst 1996) although there is no evidence that numbers are so low that they are reproductively limited.

Sex ratio may also be a factor in gender bias of hybridization. *Cephalopholis fulva* is a protogynous hermaphrodite and consequently there would be a larger number of small females and a smaller number of large males. *Paranthias furcifer* is gonochoristic and sex ratio is not necessarily affected by age of the fish. Therefore, the large number of female *C. fulva* could be promoting hybridization with male *P. furcifer.*

There could also be a biochemical block of fertilization of *P. furcifer* eggs by *C. fulva* sperm. While the eggs of *C. fulva* are compatible with the sperm of *P. furcifer,* the reverse may not be possible. It has been shown in oysters that *Crassostrea gigas* males and *C. sikamea* females successfully hybridize, but *C. gigas* eggs cannot be naturally

fertilized by *C. sikamea* sperm (Banks et al. 1994). It is believed that there may be a biochemical block that prevents the *C. sikamea* sperm from successfully fertilizing the *C. gigas* egg.

A likely explanation for gender bias in hybridization is the presence of sneaker males. It is possible that *P. furcifer* males, excited by nearby massive *C. fulva* spawning, will sneak into the harem and deposit sperm and quickly swim out or be chased off by the *C. fulva* male. This act of sneaking by *P. furcifer* males could result in the occasional production of *P. furcifer* X *C. fulva* hybrids with *C. fulva* mothers.

*Extent of Hybridization*

Based on the number of putative hybrids collected in Bermuda over the last two years, hybridization between *Cephalopholis fulva* and *Paranthias furcifer* seems to be a relatively rare event. Although post-$F_1$ hybridization has occurred, the number of individuals is low. There may be some selective disadvantage preventing further hybridization because it does not appear to have progressed beyond an occasional event. Regardless, there does not seem to be extensive enough hybridization to be concerned with the integrity of the participating species.

*Reproductive Status*

The reproductive status of the putative hybrids is unknown, although one ripe female (John Graves, personal communication) and spent male and female fish of hybrid descent have been found through macroscopic examination (Brian Luckhurst, personal communication). Since *Cephalopholis fulva* is a hermaphrodite and *Paranthias furcifer* is gonochoristic, it raises the question of what type of reproductive system the hybrids have. This question has not been pursued here, but should be considered in future work. Based

on the occurrence of post-$F_1$ hybrids, it can at least be concluded that $F_1$ hybrids are capable of producing viable offspring based on the existence of post-$F_1$ hybrids.

*Ecology*

The ecological requirements of the putative hybrid are also a topic for speculation. There is little information on the habitat of the *Cephalopholis fulva* and *Paranthias furcifer* hybrids, although the specimens used here were caught in the vicinity of both species with handlines and lobster traps. In theory, hybrids should be best able to compete in a habitat that is intermediate to that of its putative parents (Anderson 1949). Since one parent is a bottom dweller and another parent is a schooling water-column fish, it is conceivable that the hybrids can be found wherever the ranges of the species overlap.

The type of food the hybrids are able to catch is also of interest from an ecological standpoint. The wide gaping mouth of *Cephalopholis fulva* allows it to gulp whole fish, while the mouth of *Paranthias furcifer* is small and only capable of capturing small prey such as zooplankton. The hybrids have mouth measurements that are intermediate between those of the parent species, as expected, but that does not elucidate how the hybrids are able to eat. Their mouths may be too small to gulp whole prey as their *C. fulva* parents do, however the large mouth may not permit capture of the same zooplankton that *P. furcifer* eat. Perhaps the hybrids eat something in between, large zooplankton or other planktonic invertebrates. In a review of the feeding behavior of grouper, Dodrill et al. (1993) reported that *C. fulva* off North Carolina fed half on crustatceans and half on fish, while *P. furcifer* fed on almost entirely crustaceans. It is reasonable to assume that the hybrids will feed on mostly crustaceans, intermediate to the

diets of its parents. However, a stomach content analysis of the $F_1$ and post-$F_1$ hybrids would be necessary to expand these speculations.

### *Intergeneric Hybridization*

Intergeneric hybridization is generally considered rare. It is believed that the ability to hybridize is an indication of evolutionary relatedness (Hubbs 1955) and that divergent taxa should have lost the ability to interbreed through the evolution of reproductive isolating barriers (Sibley 1957). However, in fishes this is not necessarily the case. There are several examples of intergeneric hybridization in fishes, most notably in the cyprinids. For example, the redside shiner, *Richardsonius balteatus,* is known to hybridize with members of three other genera: *Mylocheilus caurinus* (Aspinwall et al. 1993a), *Acrocheilus alutaceus,* and *Rhinichthys osculus* (Smith 1973). In these cases, hybridization appears to be a result of overlap of spawning time, location and behavior (Aspinwall and McPhail 1995).

Although two species from different genera successfully interbreed, their differences could prevent their offspring from being able to effectively compete and reproduce. However, in the cyprinids mentioned above, the $F_1$ hybrids are indeed viable and in some cases fertile, although backcross hybrids were less able to compete (Smith 1973). Since the reproductive organs of the *Cephalopholis fulva* and *Paranthias furcifer* hybrids have not been examined, there is no indication of hybrid inferiority. However, due to the presence of post-$F_1$ hybrids, it can be concluded that at least some of the $F_1$ hybrids are fertile.

Intergeneric hybridization between two such ecologically different species as *Cephalopholis fulva* and *Paranthias furcifer* has also been noted in the lutjanids. In his overview of Cuban reef fishes, Poey (1860) described an intergeneric snapper hybrid, which he named *Lutjanus ambiguus*, a cross between *L. synagris* and *Ocyurus chrysurus* (Loftus 1992). A total of about 25 putative hybrids has been caught off Cuba since Poey's specimens. Both Loftus (1992) and Domeier and Clarke (1992) presented reviews of this instance of hybridization. The hybridizing species are remarkably similar to *C. fulva* and *P. furcifer*. *Lutjanus synagris*, the lane snapper, lives in deep water and feeds on fish and large invertebrates (Böhlke and Chaplin 1968). *Ocyurus chrysurus*, on the other hand, is much like *P. furcifer*, its tail is more deeply forked than other snappers, it lives in the middle of the water column in aggregations, and feeds on small fish and crustaceans (Böhlke and Chaplin 1968). In fact, Smith (1971) referred to *P. furcifer* as having a similar taxonomic placement among the groupers, that *O. chrysurus* has among the snappers.

Loftus (1992) examined the natural intergeneric snapper hybrids using morphological characters to confirm hybridization. He also theorized that the species were capable of hybridization based on overlap of spawning time and habitat. At around the same time, Domeier and Clarke (1992) artificially crossed *Lutjanus synagris* and *Ocyurus chrysurus*, proving that the specimens Poey and Loftus examined were indeed intergeneric hybrids of these two species. Unfortunately there has been little investigation into the ecology or reproductive biology of the snapper hybrids, and therefore no parallel conclusions can be drawn for the *Cephalopholis fulva* X *Paranthias furcifer* hybrids.

Despite differences in morphology and ecology, data here and in the literature show that viable $F_1$ hybrids can be produced across genera, as is the case with *Cephalopholis fulva* and *Paranthias furcifer*. The apparent intermediacy of these hybrids is maintained despite the broad differences in the parent species. The limited number of post-$F_1$ hybrids and the absence of introgression indicate that there is some post-mating reproductive isolation and the species will most likely retain their integrity for the present.

*Generic Placement of P. furcifer*

In the above case of snapper hybridization, both Loftus (1992) and Domeier and Clarke (1992) surmised that *Ocyurus chrysurus* should not be in its own genus, but included in *Lutjanus,* based on morphology and its ability to hybridize. More conclusive evidence was presented by Chow and Walsh (1992) in their revision of the phylogenetic relationship of western Atlantic snappers. They demonstrated, based on Nei's (1978) genetic distance calculated from allozyme data, that *Ocyurus chrysurus* was close enough to *Lutjanus* species be considered congeneric and they recommended this revision. Nei's (1978) genetic distances between *O. chrysurus* and five *Lutjanus* species ranged from 0.584 to 0.975, while distances within *Lutjanus* ranged from 0.428 to 0.898. In this case, it appears that the species was misplaced due to its unique morphological features.

The family Serranidae contains five subfamilies: Serraninae, Anthinae, Niphoninae, Grammistinae, and Epinephelinae (the groupers) (Heemstra and Randall 1993). Epinephelinae contains fifteen genera, including *Cephalopholis*, *Paranthias*, and *Epinephelus*, the largest grouper genus. The placement of *Paranthias furcifer* in Serranidae is very similar to that of *Ocyurus* in Lutjanidae. It is the only species of *Paranthias* in the Atlantic, and based on morphology, Smith (1971) speculated that

*Paranthias* diverged early from the other epinepheline serranids, prior to the divergence of *Epinephelus* and *Cephalopholis*. However, Smith (1966) did mention in his review of hybridization with *Cephalopholis fulva*, that perhaps *Paranthias* was not as distant as morphology suggested.

Here, estimated sequence divergence suggests that *Paranthias furcifer* does not belong in a separate genus from *Cephalopholis.* The mitochondrial DNA sequence divergence data indicate that *P. furcifer* is closer to, or at least no farther from, *C. fulva* than *C. cruentata,* and *C. cruentata* was equidistant from both species. The between *C. fulva* and *P. furcifer* was low compared to other congeneric species. For example, whole mitochondrial DNA RFLP analysis by Graves et al. (1990) for three species of *Paralabrax* produced estimates of sequence divergence from 0.069 to 0.142 with standard errors between 0.019 to 0.027. The divergence calculated from the three mitochondrial regions between *C. fulva* and *P. furcifer* was a little bit larger, 0.036.

Nei's (1978) unbiased genetic distance based on the allozyme data, 0.356 is somewhat high, compared to that of the same three *Paralabrax* species. Graves et al., (1990) reported distances from 0.165 to 0.304, calculated from 43 allozyme loci. However, the unbiased genetic distance, (Chow and Walsh 1992) reported within the *Lutjanus* ranged from 0.428 to 0.898 based on 25 allozyme loci, indicating that the value calculated for *Cephalopholis fulva* and *Paranthias furcifer* is not unreasonably high for species within the same genus.

I would not recommend taxonomic revision of *Paranthias furcifer* at this time: however, the instance of hybridization and the estimated genetic distance between *Cephalopholis fulva* and *P. furcifer* indicates that a full scale phylogenetic study is

necessary. The study would have to include more species of *Cephalopholis,* other members of the subfamily Epinephelinae, and the Pacific species of *Paranthias, P. colonus*.

### *Future Research*

Future research in this case of intergeneric hybridization should focus on the ecological niche of *Cephalopholis fulva* X *Paranthias furcifer* hybrids. It would be interesting to understand how the hybrids are able to compete and reproduce. A stomach content analysis would be necessary to understand the ecological habits of the hybrids and how well they are competing for food. An investigation of the reproduction of both *C. fulva* and *P. furcifer* is necessary to further speculate on the mode of hybridization between these two species. A full scale study of the reproductive habits of *C. fulva* and *P. furcifer* throughout their overlapping ranges would provide information on the geographic extent of hybridization. Finally, as noted previously, a study of the phylogeny of the groupers in the family Serranidae is necessary to assess the appropriate taxonomic placement of *P. furcifer.*

## Conclusions

Using morphological and molecular evidence, it can be concluded that *Cephalopholis fulva* and *Paranthias furcifer* are hybridizing in Bermuda. Hybrids are able to survive to adulthood and reproduce, as indicated by the presence of two post-$F_1$ hybrid individuals. Mitochondrial DNA data indicated that *C. fulva* is the maternal parent of all $F_1$ hybrids. At loci that were fixed differently between *C. fulva* and *P. furcifer*, there was no indication of introgression between these two species. Based on the estimated genetic distance between *C. fulva* and *P. furcifer* it appears this may not be a case of intergeneric hybridization, but of intrageneric hybridization and generic misplacement. Overall, this study has shown the utility of morphological and molecular analyses in detecting hybridization.

## Appendix A: Recipes for Histochemical Stains for Allozyme Electrophoresis

**Alcohol Dehydrogenase**
40ml 0.2M Tris-HCl pH 8.0
0.5ml amyl alcohol (2-pentanol)
5ml 95% ethanol
1ml 10mg/ml NAD
1ml 5mg/ml NBT
1ml 5mg/ml PMS

Tris HCl and amyl alcohol were mixed thoroughly prior to adding the remaining reagents.

**Esterase**
50ml Phosphate Buffer (9.07g $KH_2PO_4$; 4.73g $Na_2HPO_4$ per 1 liter)
1ml $\alpha$-napthyl Acetate
0.03g Fast Blue RR Salt

**Fumarase**
50ml 0.2M Tris/HCl pH 8.0
0.05g Fumaric Acid
0.5ml 10mg/ml NAD
30U Malic Dehydrogenase
0.5ml 5mg/ml NBT
0.5ml 5mg/ml MTT
0.5ml 5mg/ml PMS

**General Protein**
50ml Fixing Solution (5 methanol: 5 $H_2O$: 1 glacial acetic acid)
1.5ml 30mg/ml Brilliant Blue R

Gel was fixed at room temperature and washed with fixing solution until background was pale.

**Glucose-6-Phosphate Dehydrogenase**
50ml .2M Tris-HCl pH 8.0
1ml 0.1M $MgCl_2$
0.3g D-glucose-6-phosphate
1ml 5mg/ml NADP
1ml 5mg/ml MTT
0.5ml 5mg/ml PMS

**Glucosephosphate Isomerase**
30ml 0.2M Tris/HCl pH 8.0
10ml 0.1M $MgCl_2$
0.025g D-fructose-6-phosphate

10 U Glucose-6-Phosphate Dehydrogenase
1ml 10mg/ml NAD
1ml 5mg/ml NBT
0.5ml 5mg/ml PMS

**Isocitrate Dehydrogenase**
50ml 0.2M Tris-HCl pH 8.0
3ml 0.1M $MgCl_2$
3ml 0.1M DL-isocitric acid
1ml 10mg/ml NADP
0.5ml 5mg/ml NBT
0.5ml 5mg/ml PMS

**Lactate Dehydrogenase**
50ml 0.2M Tris-HCl pH 8.0
9ml 0.5M Lithium Lactate pH 8.0 (DL Lactic Acid adjusted to pH 8.0 with LiOH)
1ml 10mg/ml NAD
0.5ml 5mg/ml NBT
0.5ml 5mg/ml PMS

**Malate Dehydrogenase**
40ml 0.2M Tris-HCl pH 8.0
5ml 2M DL-Malic acid
1ml 10mg/ml NAD
1ml 5mg/ml NBT
0.5ml 5mg/ml PMS

**Peptidases**
10ml 0.2M Tris-HCl pH 8.0
0.005g substrate (Leu-Gly-Gly, Leu-Tyr, Leu-Pro)
0.002g L-Amino Acid Oxide (snake venom)
0.001g Peroxidase
0.01g o-Dianisidine Dihydrochloride

**Xanthine Dehydrogenase**
50ml 0.2M Tris-HCl pH 8.0
0.025 Hypoxanthine
1ml 10mg/ml NAD
1ml 5mg/ml NBT
1ml 5mg/ml MTT
0.5ml 5mg/ml PMS

NAD - Nicotinamide Adenine Dinucleotide; NBT - Nitro Blue Tetrazolium; PMS - Phenazine methylsulfate; MTT - tetrazolium salt

**Appendix B:** Description of morphological measurements and counts made on putative hybrids, *Cephalopholis fulva*, and *Paranthias furcifer* (reproduced from Smith 1971, with minor alterations). Letters in parantheses correspond to measurements and counts in Appendix C.

**Counts**

| | |
|---|---|
| Dorsal Rays (DSLRYS) | All dorsal spines and rays were counted. |
| Anal Soft Rays (ASRYS) | All soft rays in the anal fin were enumerated, rays that are branched to the base were counted as two. |
| Pectoral Rays (PCTRYS) | All pectoral elements were counted, and branched rays were counted as two. |
| Gill Rakers (GLRKRS) | Gill rakers were counted on the first arch right side of the fish. All countable elements were included. |
| Scales Above the Lateral Line (SCALL) | Scales were counted obliquely from the highest portion of the lateral line to the dorsal fin, not including lateral line scales. Counts were made three times and the lowest number was recorded. |
| Scales Below the Lateral Line (SCBLL) | Scales were enumerated from the posterior edge of the anus obliquely backward and upward to the lateral line. Lateral |

line scales were not included.

| | |
|---|---|
| Transverse Scale Rows (TSCRWS) | Scale rows were counted in a straight line from the posterior supracleithrum to the mid-base of the caudal fin. |
| Caudal Peduncle Scales (CLPDSC) | Scales were counted in a zig zag pattern around the least depth of the caudal peduncle. The count was made three times and the lowest number was recorded. |

**Measurements**

| | |
|---|---|
| Head Length (HDLTH) | Measured from the tip of the snout to the most posterior portion of the opercular flap. |
| Head Width (HDWTH) | Measured just anterior to the posterior edge of the preopercle. |
| Head Depth (HDDTH) | Measured just posterior to the anterior edge of the preopercle. |
| Snout Length (SNLTH) | Measured from the tip of the snout to the anterior edge of the orbit. |

| | |
|---|---|
| Suborbital Width (SOWTH) | Measured as the least width from crease above the maxilla to the ventral edge of the orbit. |
| Interorbital Width (IOWTH) | The least distance between the orbits. |
| Orbit Length (OBTLTH) | The greatest diameter of the orbit. |
| Postorbital Head Length (PHDLTH) | Measured from the posterior edge of the orbit to the most posterior edge of the operculum. |
| Maxillary length (MAXLTH) | Distance from the anterior extremity of the upper jaw to the midpoint of the posterior end of the maxilla. |
| Lower Jaw Length (LJLTH) | Distance from the anterior extremity of the lower jaw to the midpoint of the posterior end of the maxilla. |
| Distance from Snout to Angle of Preopercle (SNPOCL) | Distance from the tip of the snout to the posterior edge of the preopercle below the notch. |
| Maxillary Width (MAXWTH) | Greatest width of the maxilla, without the supramaxilla. |

| | |
|---|---|
| The Distance from Tip of Lower Jaw to Gular Notch (LJGN) | The distance between anterior extremity of the lower jaw to the anterior end of the gular notch. |
| Body Width (BDYWTH) | Measured as the greatest width of the body posterior to the shoulder girdle. |
| Body Depth (BDYDTH) | Measured as the greatest depth of the body taken just anterior of the base of the pelvic fins. |
| Caudal Peduncle Depth (CPDTH) | Measured as the least depth of the caudal peducle. |
| Tip of Snout to Dorsal Origin Distance (SNTDRSL) | The distance from the base of the first dorsal spine to the tip of the snout. |
| Tip of Snout to Pectoral Base Distance (SNTPCT) | The distance from the tip of the snout to the base of the anterior most spine of the pectoral fin. |
| Tip of the Lower Jaw to | The distance from the tip of the lower jaw to the base of the |

| | |
|---|---|
| Pelvic Base Distance (LJPVC) | outermost pelvic spine. |
| Dorsal Base Length (DBLTH) | The distance from the anterior edge of the base of the first dorsal spine to the posterior edge of the base of the last dorsal fin ray. |
| Depressed Dorsal Length (DDLTH) | The distance from the anterior edge of the base of the first dorsal spine to the posteriormost tip of the the dorsal soft rays when depressed. |
| Anal Base Length (ABLTH) | The distance from the anterior edge of the base of the first anal spine to the posterior edge of the base of the last soft ray. |
| Depressed Anal Length (DALTH) | The distance from the anterior edge of the base of the first anal spine to the posteriormost tip of the soft rays. |
| End of Dorsal to Caudal Base Distance (DLCLB) | The distance from the base of the last dorsal soft ray to the midbase of the caudal fin. |
| Length of Caudal Peduncle (CPLTH) | The distance from the base of the last anal fin ray to the midbase of the caudal peduncle. |

| | |
|---|---|
| Pectoral Length (PCTLTH) | The distance from the midpoint of the pectoral base to the tip of the longest ray. |
| Pelvic Length (PVCLTH) | The distance from the midpoint of the pelvic base to the tip of the longest ray. |
| Dorsal Spine I Length (DSI) | The distance from the anterior edge of the base of the spine to the tip. |
| Dorsal Spine III Length (DSIII) | see above |
| Dorsal Spine IX Length (DSIX) | see above |
| Anal Spine I Length (ASI) | The distance from the anterior edge of the base of the spine to the tip. |
| Anal Spine II Length (ASII) | see above |

| | |
|---|---|
| Anal Spine III Length (ASIII) | see above |
| Distance from Caudal Base to Tip of Upper Rays (CBSURY) | The distance from the midbase of the caudal fin to the tip of the uppermost rays. |
| Distance from Caudal Base to Tip of Middle Rays (CBSMRY) | The distance from the midbase of the caudal fin to the tip of the middle rays. |
| Distance from Caudal Base to Tip of Lower Rays (CBSLRY) | The distance from the midbase of the caudal fin to the tip of the lower rays. |

**Appendix C:** The Results of the morphological study on *Cephalopholis fulva*, *Paranthias furcifer,* and putative hybrid individuals.

Values are presented as 1000 X the fraction of the standard length. Catalogue numbers are for specimens in the US National Museum Collection.

| Species | Specimen | Collection | DSLRYS | ASRYS | PCTRYS | GLRKRS | SCALL | SCBLL | TSCRWS | CLPDSC | HDLTH | HDWTH | HDDTH | SNLTH | SOWTH |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *C. fulva* | 88717 | USNM | IX, 15 | 9 | 34 | 24 | 8 | 23 | 70 | 44 | 396 | 173 | 268 | 101 | 52 |
| *C. fulva* | 53134 | USNM (b) | IX, 16 | 9 | 34 | 24 | 8 | 24 | 68 | 49 | 418 | 227 | 270 | 119 | 49 |
| *C. fulva* | 53134 | USNM (s) | IX, 15 | 8 | 34 | 25 | 6 | 27 | 64 | 40 | 378 | 202 | 253 | 86 | 44 |
| *C. fulva* | 133689 | USNM | IX, 15 | 9 | 34 | 25 | 8 | 24 | 69 | 45 | 384 | 189 | 271 | 92 | 44 |
| *C. fulva* | 99-1 | vims | IX, 16 | 9 | 34 | 24 | 8 | 26 | 73 | 48 | 422 | 184 | 250 | 107 | 47 |
| *C. fulva* | 99-2 | vims | IX, 16 | 9 | 34 | 22 | 8 | 22 | 75 | 44 | 395 | 208 | 260 | 122 | 48 |
| *C. fulva* | 99-3 | vims | IX, 15 | 9 | 35 | 22 | 8 | 27 | 68 | 48 | 427 | 210 | 248 | 118 | 46 |
| *C. fulva* | 99-4 | vims | IX, 16 | 10 | 34 | 27 | 7 | 27 | 70 | 48 | 425 | 201 | 288 | 115 | 49 |
| *C. fulva* | 99-5 | vims | IX, 16 | 9 | 33 | 28 | 8 | 27 | 84 | 45 | 402 | 201 | 270 | 112 | 44 |
| *C. fulva* | 320539 | USNM | IX, 16 | 10 | 32 | 24 | 8 | 27 | 68 | 45 | 374 | 200 | 281 | 102 | 48 |
| *P. furcifer* | PF 102 | vims PF102 | IX, 18 | 9 | 38 | 39 | 14 | 30 | 92 | 45 | 262 | 137 | 183 | 70 | 24 |
| *P. furcifer* | PF 103 | vims PF103 | IX, 19 | 9 | 39 | 35 | 10 | 28 | 89 | 43 | 274 | 141 | 188 | 79 | 24 |
| *P. furcifer* | PF104 | vims PF104 | IX, 19 | 9 | 38 | 38 | 10 | 26 | 96 | 44 | 259 | 130 | 193 | 67 | 22 |
| *P. furcifer* | 107108 | USNM | IX, 19 | 10 | 39 | 33 | 14 | 31 | 93 | 47 | 282 | 140 | 222 | 71 | 21 |
| *P. furcifer* | 358541 | USNM | IX, 19 | 9 | 38 | 36 | 10 | 30 | 94 | 46 | 297 | 162 | 205 | 71 | 21 |
| *P. furcifer* | 33255 | USNM | IX, 19 | 9 | 39 | 32 | 13 | 28 | 85 | 46 | 301 | 142 | 241 | 68 | 21 |
| *P. furcifer* | 12540 | USNM | IX, 19 | 10 | 39 | 38 | 13 | 30 | 88 | 45 | 280 | 134 | 186 | 71 | 20 |
| *P. furcifer* | 65605-8244 | USNM | IX, 19 | 11 | 38 | 36 | 12 | 31 | 92 | 45 | 272 | 139 | 231 | 67 | 20 |
| *P. furcifer* | 65605-8246 | USNM | IX, 19 | 10 | 36 | 35 | 11 | 32 | 89 | 47 | 282 | 145 | 232 | 55 | 20 |
| *P. furcifer* | 65605-8246 | USNM | IX, 18 | 10 | 38 | 35 | 11 | 32 | 90 | 48 | 291 | 147 | 237 | 53 | 22 |
| Put hybrid | 99-6 | vims 99-6 | IX, 17 | 9 | 35 | 29 | 11 | 29 | 91 | 50 | 353 | 174 | 237 | 111 | 35 |
| Put hybrid | H7 | vims H7 | IX,17 | 9 | 35 | 31 | 11 | 29 | 84 | 47 | 335 | 165 | 228 | 89 | 32 |
| Put hybrid | H8 | vims H8 | IX, 17 | 9 | 36 | 35 | 10 | 29 | 83 | 47 | 320 | 156 | 226 | 79 | 33 |
| Put hybrid | H9 | vims H9 | IX,17 | 9 | 36 | 32 | 10 | 28 | 83 | 46 | 326 | 189 | 250 | 96 | 32 |
| Put hybrid | H10 | vims H10 | IX, 17 | 9 | 36 | 32 | 10 | 29 | 82 | 47 | 327 | 151 | 218 | 91 | 33 |
| Put hybrid | H11 | vims H11 | IX, 18 | 9 | 36 | 31 | 10 | 26 | 82 | 44 | 325 | 150 | 214 | 99 | 33 |
| Put hybrid | H12 | vims H12 | IX, 17 | 10 | 35 | 31 | 10 | 28 | 85 | 45 | 335 | 158 | 212 | 85 | 33 |
| Put hybrid | H13 | vims H13 | IX,17 | 10 | 37 | 30 | 10 | 29 | 84 | 47 | 353 | 159 | 250 | 104 | 33 |
| Put hybrid | H15 | vims H15 | IX, 17 | 9 | 36 | 32 | 11 | 27 | 91 | 51 | 349 | 154 | 245 | 91 | 29 |

**Appendix C:** Continued.

| Species | Specimen | IOWTH | OBTLTH | PHDLTH | MAXLTH | LJLTH | SNPOCL | MAXWTH | LJGN | BDYWTH | BDYDTH | CPDTH | SNTDRSL | SNTPCT |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *C. fulva* | 88717 | 70 | 65 | 234 | 177 | 196 | 279 | 49 | 125 | 154 | 325 | 130 | 407 | 363 |
| *C. fulva* | 53134 | 67 | 64 | 233 | 182 | 185 | 279 | 46 | 158 | 172 | 363 | 135 | 421 | 390 |
| *C. fulva* | 53134 | 64 | 70 | 226 | 166 | 177 | 257 | 42 | 119 | 167 | 342 | 131 | 386 | 374 |
| *C. fulva* | 133689 | 66 | 73 | 227 | 178 | 176 | 281 | 45 | 136 | 179 | 355 | 135 | 394 | 366 |
| *C. fulva* | 99-1 | 70 | 64 | 243 | 178 | 196 | 291 | 43 | 145 | 172 | 358 | 131 | 418 | 386 |
| *C. fulva* | 99-2 | 71 | 62 | 217 | 182 | 179 | 273 | 56 | 181 | 177 | 331 | 139 | 396 | 287 |
| *C. fulva* | 99-3 | 78 | 66 | 230 | 190 | 200 | 302 | 54 | 136 | 184 | 362 | 128 | 406 | 376 |
| *C. fulva* | 99-4 | 73 | 75 | 238 | 194 | 186 | 306 | 50 | 170 | 178 | 374 | 138 | 422 | 415 |
| *C. fulva* | 99-5 | 72 | 76 | 226 | 188 | 185 | 283 | 48 | 156 | 170 | 370 | 137 | 409 | 378 |
| *C. fulva* | 320539 | 78 | 66 | 242 | 183 | 183 | 273 | 43 | 119 | 223 | 385 | 139 | 412 | 372 |
| *P. furcifer* | PF 102 | 77 | 52 | 162 | 96 | 101 | 191 | 38 | 73 | 161 | 315 | 106 | 324 | 265 |
| *P. furcifer* | PF 103 | 76 | 52 | 158 | 102 | 103 | 182 | 37 | 104 | 162 | 306 | 105 | 325 | 277 |
| *P. furcifer* | PF104 | 79 | 53 | 156 | 98 | 98 | 180 | 36 | 69 | 149 | 290 | 122 | 323 | 269 |
| *P. furcifer* | 107108 | 77 | 89 | 156 | 103 | 103 | 189 | 28 | 75 | 142 | 298 | 102 | 323 | 276 |
| *P. furcifer* | 358541 | 80 | 69 | 160 | 99 | 105 | 188 | 30 | 121 | 161 | 317 | 112 | 325 | 280 |
| *P. furcifer* | 33255 | 82 | 66 | 167 | 105 | 108 | 200 | 35 | 100 | 140 | 306 | 111 | 361 | 288 |
| *P. furcifer* | 12540 | 79 | 62 | 146 | 109 | 102 | 176 | 37 | 99 | 138 | 282 | 109 | 325 | 293 |
| *P. furcifer* | 65605-8244 | 82 | 61 | 156 | 109 | 111 | 183 | 32 | 79 | 150 | 340 | 112 | 321 | 270 |
| *P. furcifer* | 65605-8246 | 79 | 64 | 159 | 106 | 106 | 191 | 36 | 79 | 157 | 347 | 116 | 337 | 267 |
| *P. furcifer* | 65605-8246 | 89 | 78 | 171 | 115 | 114 | 200 | 34 | 86 | 142 | 354 | 116 | 327 | 280 |
| Put hybrid | 99-6 | 83 | 64 | 188 | 153 | 148 | 246 | 45 | 168 | 162 | 347 | 118 | 373 | 326 |
| Put hybrid | H7 | 76 | 63 | 191 | 138 | 137 | 229 | 43 | 136 | 160 | 338 | 131 | 351 | 323 |
| Put hybrid | H8 | 73 | 61 | 185 | 141 | 139 | 229 | 37 | 99 | 155 | 302 | 131 | 358 | 314 |
| Put hybrid | H9 | 82 | 60 | 186 | 135 | 127 | 227 | 43 | 174 | 169 | 334 | 132 | 338 | 340 |
| Put hybrid | H10 | 80 | 56 | 197 | 138 | 128 | 213 | 39 | 106 | 168 | 329 | 119 | 354 | 306 |
| Put hybrid | H11 | 82 | 61 | 187 | 142 | 147 | 239 | 43 | 90 | 161 | 321 | 130 | 359 | 314 |
| Put hybrid | H12 | 82 | 67 | 194 | 145 | 144 | 219 | 42 | 93 | 169 | 328 | 130 | 334 | 293 |
| Put hybrid | H13 | 86 | 66 | 204 | 163 | 149 | 235 | 43 | 116 | 160 | 348 | 128 | 365 | 337 |
| Put hybrid | H15 | 89 | 63 | 210 | 130 | 143 | 237 | 45 | 102 | 162 | 311 | 145 | 368 | 328 |

**Appendix C:** Continued.

| Species | Specimen | LJPVC | DBLTH | DDLTH | ABLTH | DALTH | DLCLB | CPLTH | PCTLTH | PVCLTH | DSI | DSIII | DSIX | ASI | ASII | ASIII |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *C. fulva* | 88717 | 401 | 528 | 609 | 170 | 268 | 143 | 178 | 257 | 186 | 64 | 103 | 112 | 61 | 95 | 99 |
| *C. fulva* | 53134 | 446 | 549 | 636 | 170 | 285 | 141 | 175 | 279 | 192 | 65 | 129 | 97 | 52 | 97 | 78 |
| *C. fulva* | 53134 | 411 | 537 | 638 | 187 | 307 | 130 | 190 | 273 | 204 | 54 | 124 | 129 | 59 | 108 | 113 |
| *C. fulva* | 133689 | 407 | 544 | 646 | 173 | 284 | 142 | 198 | 282 | 196 | 66 | 112 | 124 | 60 | 112 | 110 |
| *C. fulva* | 99-1 | 421 | 531 | 612 | 175 | 284 | 129 | 176 | 261 | 187 | 61 | 114 | 126 | 67 | 95 | 105 |
| *C. fulva* | 99-2 | 449 | 549 | 613 | 177 | 311 | 143 | 168 | 265 | 199 | 61 | 112 | 124 | 62 | 106 | 117 |
| *C. fulva* | 99-3 | 402 | 523 | 610 | 171 | 299 | 134 | 180 | 274 | 192 | 62 | 136 | 141 | 62 | 107 | 112 |
| *C. fulva* | 99-4 | 438 | 535 | 663 | 179 | 301 | 146 | 173 | 287 | 211 | 62 | 138 | 135 | 62 | 118 | 117 |
| *C. fulva* | 99-5 | 423 | 537 | 624 | 176 | 320 | 148 | 179 | 292 | 209 | 65 | 120 | 150 | 72 | 121 | 118 |
| *C. fulva* | 320539 | 398 | 546 | 643 | 166 | 284 | 150 | 166 | 273 | 193 | 61 | 137 | 136 | 62 | 102 | 107 |
| *P. furcifer* | PF 102 | 321 | 602 | 613 | 166 | 237 | 170 | 241 | 261 | 165 | 57 | 106 | 89 | 36 | 78 | 85 |
| *P. furcifer* | PF 103 | 372 | 571 | 620 | 171 | 242 | 156 | 215 | 275 | 174 | 50 | 95 | 97 | 44 | 81 | 101 |
| *P. furcifer* | PF104 | 335 | 613 | 642 | 175 | 232 | 170 | 228 | 249 | 150 | 58 | 113 | 105 | 43 | 74 | 90 |
| *P. furcifer* | 107108 | 333 | 543 | 603 | 186 | 259 | 145 | 174 | 268 | 178 | 47 | 117 | 94 | 44 | 91 | 80 |
| *P. furcifer* | 358541 | 377 | 568 | 626 | 165 | 241 | 154 | 206 | 284 | 176 | 64 | 101 | 92 | 45 | 92 | 88 |
| *P. furcifer* | 33255 | 384 | 582 | 650 | 165 | 241 | 175 | 239 | 290 | 176 | 48 | 117 | 89 | 42 | 86 | 89 |
| *P. furcifer* | 12540 | 371 | 604 | 625 | 172 | 249 | 158 | 228 | 268 | 167 | 53 | 99 | 76 | 40 | 78 | 91 |
| *P. furcifer* | 65605-8244 | 330 | 592 | 656 | 194 | 278 | 170 | 216 | 271 | 170 | 40 | 86 | 101 | 44 | 86 | 81 |
| *P. furcifer* | 65605-8246 | 338 | 594 | 658 | 200 | 276 | 163 | 224 | 287 | 187 | 57 | 108 | 97 | 48 | 91 | 78 |
| *P. furcifer* | 65605-8246 | 347 | 565 | 658 | 204 | 276 | 163 | 219 | 288 | 184 | 56 | 107 | 104 | 45 | 98 | 86 |
| Put hybrid | 99-6 | 403 | 544 | 600 | 177 | 269 | 159 | 217 | 288 | 191 | 61 | 114 | 111 | 53 | 109 | 108 |
| Put hybrid | H7 | 389 | 542 | 615 | 192 | 273 | 161 | 194 | 285 | 183 | 68 | 116 | 123 | 52 | 102 | 112 |
| Put hybrid | H8 | 359 | 550 | 609 | 185 | 258 | 158 | 198 | 285 | 186 | 69 | 113 | 116 | 55 | 98 | 106 |
| Put hybrid | H9 | 420 | 535 | 574 | 171 | 248 | 164 | 207 | 253 | 182 | 48 | 67 | 112 | 55 | 99 | 100 |
| Put hybrid | H10 | 367 | 532 | 607 | 175 | 254 | 160 | 194 | 263 | 174 | 62 | 108 | 109 | 56 | 99 | 92 |
| Put hybrid | H11 | 362 | 552 | 627 | 177 | 260 | 154 | 206 | 275 | 182 | 57 | 90 | 109 | 43 | 93 | 98 |
| Put hybrid | H12 | 364 | 543 | 621 | 189 | 262 | 148 | 202 | 290 | 183 | 62 | 110 | 120 | 57 | 103 | 112 |
| Put hybrid | H13 | 384 | 533 | 594 | 188 | 264 | 155 | 207 | 288 | 186 | 68 | 119 | 120 | 66 | 112 | 110 |
| Put hybrid | H15 | 382 | 561 | 643 | 184 | 267 | 165 | 202 | 295 | 192 | 70 | 116 | 104 | 59 | 99 | 121 |

**Appendix C:** Continued.

| Species | Specimen | CBSURY | CBSMRY | CBSLRY | SL |
|---|---|---|---|---|---|
| *C. fulva* | 88717 | 200 | 204 | 211 | 232 |
| *C. fulva* | 53134 | 210 | 208 | 220 | 217 |
| *C. fulva* | 53134 | 197 | 229 | 201 | 176 |
| *C. fulva* | 133689 | 211 | 216 | 200 | 171 |
| *C. fulva* | 99-1 | 197 | 213 | 197 | 222 |
| *C. fulva* | 99-2 | 222 | 225 | 220 | 213 |
| *C. fulva* | 99-3 | 234 | 229 | 227 | 210 |
| *C. fulva* | 99-4 | 246 | 248 | 242 | 170 |
| *C. fulva* | 99-5 | 221 | 210 | 227 | 146 |
| *C. fulva* | 320539 | 226 | 240 | 223 | 240 |
| *P. furcifer* | PF 102 | 346 | 123 | 334 | 274 |
| *P. furcifer* | PF 103 | 352 | 126 | 329 | 259 |
| *P. furcifer* | PF104 | 334 | 134 | 308 | 274 |
| *P. furcifer* | 107108 | 373 | 124 | 338 | 218 |
| *P. furcifer* | 358541 | 329 | 125 | 311 | 237 |
| *P. furcifer* | 33255 | 344 | 122 | 321 | 207 |
| *P. furcifer* | 12540 | 321 | 122 | 311 | 185 |
| *P. furcifer* | 65605-8244 | 352 | 134 | 310 | 201 |
| *P. furcifer* | 65605-8246 | 366 | 131 | 346 | 176 |
| *P. furcifer* | 65605-8246 | 352 | 143 | 311 | 129 |
| Put hybrid | 99-6 | 228 | 183 | 285 | 189 |
| Put hybrid | H7 | 285 | 174 | 293 | 190 |
| Put hybrid | H8 | 298 | 175 | 282 | 199 |
| Put hybrid | H9 | 302 | 174 | 305 | 222 |
| Put hybrid | H10 | 268 | 169 | 297 | 237 |
| Put hybrid | H11 | 271 | 166 | 288 | 210 |
| Put hybrid | H12 | 293 | 174 | 278 | 195 |
| Put hybrid | H13 | 272 | 180 | 318 | 172 |
| Put hybrid | H15 | 279 | 192 | 308 | 181 |

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