

GENETIC ANALYSES OF LIONFISH:  
VENOMOUS MARINE PREDATORS INVASIVE TO THE WESTERN ATLANTIC

By

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## Abstract

Lionfish (Scorpaenidae) are upper trophic level predators that are native to the Indo-Pacific region and have recently become invasive to the western Atlantic Ocean. Since the invasion was first documented with three individuals in 2000, the number of lionfish in the Atlantic has increased significantly and spurred a series of investigations regarding their biology and potential impacts on the ecosystem. The present study (1) investigates the cause of disagreement between identifications based on meristic and mitochondrial DNA data that were detected for seven native lionfish specimens, (2) determines the number of lionfish species involved in the Atlantic invasion based on genetic data, (3) estimates the extent of the genetic bottleneck that accompanied the invasion, and (4) determines the likely source population of the invasive lionfish using mitochondrial DNA haplotypes. The lack of species-specific variation found in fifteen nuclear DNA loci that were examined and the absence of species-specific banding phenotypes resulting from randomly amplified polymorphic DNA (RAPD) analysis leaves the conflict of the meristic and mitochondrial DNA data unresolved. The mitochondrial cytochrome *b* data, however, indicate that *P. volitans* along with a small number of *P. miles* are present in the Atlantic Ocean. The genetic bottleneck that accompanied the invasion resulted in the Atlantic population of *Pterois volitans* displaying only 10.7% of the genetic diversity found in the native range. The cytochrome *b* data were also used to identify Indonesia as the most likely area from which the founders of the Atlantic population of *P. volitans* came.

## **Acknowledgements**

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Most of all, thanks to Wilson Freshwater for his guidance, patience, sense of humor, and for finding a place in his lab for an over-achieving undergraduate who ultimately wanted to study animals—worst of all marine mammals. I learned more than I expected and have a great foundation for graduate studies and beyond....

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## Project Background

Lionfish belong to the family Scorpaenidae, which is classified into 3 subfamilies, 25 genera, and 199 species. Scorpaenids are primarily marine fishes and are found in all tropical and temperate seas (Nelson 1994). They are easily recognizable with ridges and spines on the head region, and many display aposematic coloration to warn potential predators of their venomous dorsal, anal, and pelvic spines. The genus *Pterois* consists of nine species including *Pterois miles* and *Pterois volitans*—the subjects of this research. *Pterois miles* and *P. volitans* are similar in appearance with a common number of pectoral rays, venomous dorsal, pelvic, and anal spines, cycloid-shaped scales, and vertical bars that range in color from reddish-brown to black across their otherwise pale bodies (Schultz 1986). The two species were historically thought to be one (Beaufort and DeBriggs 1962; Randall 1983), but more recent treatments have identified *P. miles* and *P. volitans* as distinct species based on a number of meristic and morphometric differences (Schultz 1986) (Table 1).

*Table 1.* Meristic and morphometric differences between *Pterois miles* and *P. volitans*.

	<i>Pterois miles</i>	<i>Pterois volitans</i>
<b>Dorsal Fin Rays</b>	10	11
<b>Anal Fin Rays</b>	6	7
<b>Pectoral Fin Size</b>	Shorter	Longer
<b>Spot Size</b>	Smaller	Larger

*Pterois volitans* has relatively longer pectoral fins, larger spots on the dorsal fin, and most notably an additional ray in the dorsal and anal fins as compared to *P. miles*. Meristic counts, such as the number of fin rays, however, are not always reliable for the identification of fishes, as variation can occur within a species due to environmental factors such as water temperature (Hubbs 1922; Lindsey 1954). Despite more recent studies suggesting a polygenic basis for these traits in some fishes (Schroder 1965; Billerbeck et al. 1997), there is

still doubt in the ability of meristic characteristics to provide indisputable identifications of specimens. Kochzius et al. (2003) were able to distinguish between specimens identified as *P. miles* and *P. volitans* using mitochondrial cytochrome *b* and 16S rDNA sequences.

However, the present study found disagreement between the meristic and mitochondrial cytochrome *b* identifications of some specimens from Indonesia, where the distributions of *P. miles* and *P. volitans* overlap (see Part I Results).

The divided geographic distribution of *P. miles* and *P. volitans* is believed to be a principal catalyst for the variance that differentiates them (Kochzius et al. 2003). *Pterois miles* ranges throughout the Indian Ocean from South Africa to the Red Sea and east to Indonesia, while the range of *P. volitans* extends along the west coast of Australia, north around Indonesia to southern Japan and east to the Pitcairn Group (Schultz 1986) (Figure 1).

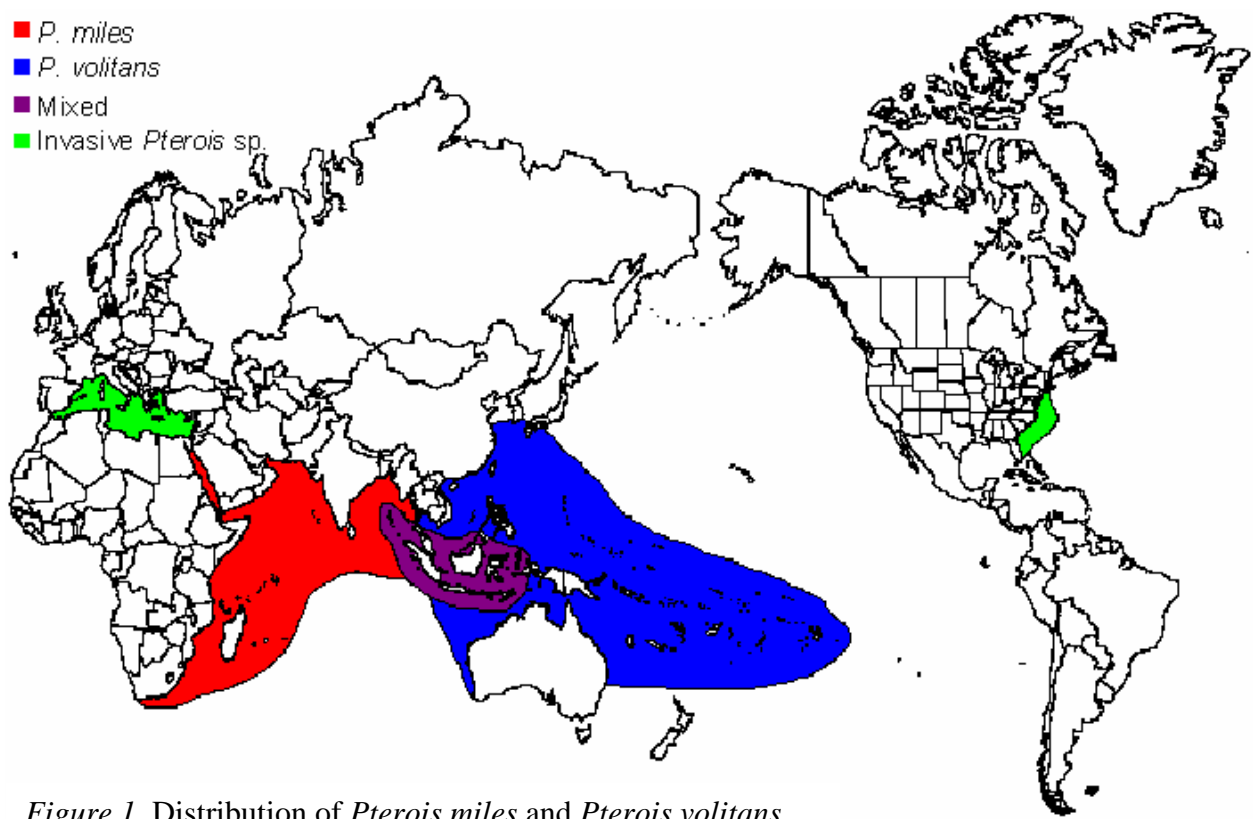


Figure 1. Distribution of *Pterois miles* and *Pterois volitans*.

Tectonic activity that formed the Indo-Malayan Archipelago and limited flow between the Pacific and Indian Oceans coincides with the divergence time of *P. miles* and *P. volitans*, estimated to have occurred 2.4 to 8.3 million years ago (Kochzius et al. 2003). Furthermore, these events are linked to the speciation of fishes in the genus *Dascyllus* (McCafferty et al. 2002) and the phylogeographic population structure of other Indo-West Pacific organisms including tropical starfish, *Linckia laevigata*, (Williams and Benzie 1998) and black tiger prawn, *Penaeus monodon* (Duda and Palumbi 1999).

More recently, both *P. miles* and *P. volitans* have invaded areas beyond their native ranges (Figure 1). *Pterois miles* used the Suez Canal to enter the Mediterranean Sea as a Lessepsian immigrant (Kochzuis et al 2003), and *P. volitans*, potentially along with *P. miles*, has become established in the Atlantic Ocean along the east coast of the United States. A biological invasion is defined as the arrival, survival, successful reproduction, and dispersal of a species into an ecosystem where it did not previously exist (Carlton 1989). Biological invasions by marine fishes are rare and the effects have not been well studied in the past. The lionfish invasion in the Atlantic was first documented in the year 2000 with the capture of two lionfish at two locations along the coast of North Carolina and one lionfish caught near Bermuda (Whitfield et al. 2002); however, several sightings in Florida were reported in the mid 1990s (Hare and Whitfield 2003). Their survival and reproduction has resulted in multiple lionfish present at over 64 known sites in North Carolina (P.E. Whitfield, personal communication) and surveys have confirmed the continuous presence of lionfish in abundances similar to native grouper species (Serranidae) from Florida to North Carolina (Whitfield et al. submitted). Florida is hypothesized to be the initial site of the lionfish introduction, from where pelagic egg masses and early larval forms were carried north by

currents (Whitfield et al. 2002). The northern boundary for over-wintering is Cape Hatteras, NC, because lionfish stop feeding when the temperature drops to 16°C (60°F). However, each year juveniles can be transported via the Gulf Stream as far north as New York where they can survive during the warmer seasons before they are killed when the water temperature drops below 16°C (Hare and Whitfield 2003; Kimball et al. 2004). When this temperature threshold is considered along with the oceanic current patterns and the vast distance between the native range and the east coast of the United States, it is unlikely that the invasion occurred as a result of natural dispersal. Additionally, adult lionfish are relatively stationary and tend to remain around a single site on a reef (Fishelson 1997). The cause of the invasion is currently believed to be the result of accidental or intentional releases from aquaria (Whitfield et al. 2002; Semmens et al. 2004). This is supported by the popularity of lionfish in the aquarium trade and a known accidental release from an aquarium in Biscayne Bay, Florida in 1992 (Courtenay 1995).

The ecological impacts of lionfish in the Atlantic are currently unknown; however they pose potential problems for the native fauna that could potentially escalate to have detrimental impacts on local fisheries. In their native range, lionfish are upper trophic level predators on reefs and rocky outcrops that extend to depths of 150 feet. They use an ambush strategy to feed on small fishes and invertebrates by cornering them with outstretched pectoral fins (Fishelson 1975 and 1997; Whitfield et al. 2002). In the Atlantic Ocean, lionfish have invaded similar habitats, which are already stressed from over-fishing and climate change (Huntsman et al. 1999; Parker and Dixon 1998). Native Atlantic fishes may become easy prey since they are naïve to the lionfish's predatory strategy. Economically important fisheries could suffer if juveniles fall victim to this naïveté and are consumed at



rates that do not allow for replacement. Lionfish could also potentially out-compete species, such as groupers (Serranidae) that occupy a similar niche and are already over-fished (Whitfield et al. submitted). Lionfish have few, if any, predators in their native environment, so it is unlikely that they will have any in the Atlantic. The only known case of predation on lionfish was documented by Bernadsky and Goulet (1991) who discovered a partially digested *Pterois miles* in the stomach of an adult cornetfish (*Fistularia commersonii*). The lionfish was swallowed tail first, allowing the cornetfish to avoid puncture by the venomous spines during ingestion. However, predatory fish in the Atlantic are unfamiliar with the lionfish's venomous spines and may attempt to prey upon them. The lionfish's venom is lethal to most fishes (Allen and Eschmeyer 1973), so if these attempted predators do not learn to avoid lionfish quickly, then their numbers could be reduced, posing another potential risk to the ecological balance in the Atlantic Ocean.

The present study includes four objectives related to the investigation of the invasive population of lionfish in the western Atlantic Ocean: (1) to investigate the cause of disagreement between identifications based on meristic and mitochondrial DNA data that were detected for some lionfish specimens; (2) to determine the number of lionfish species involved in the Atlantic invasion based on genetic data; (3) to estimate the extent of the genetic bottleneck that accompanied the invasion; and (4) to determine the source population of the invasive lionfish using mitochondrial DNA haplotypes.

**Part I:** *Conflict between meristic and mitochondrial DNA identifications of Pterois miles and P. volitans*

**Introduction**

This section will consider the investigation of the conflicting identifications as *Pterois miles* and *P. volitans* by meristic counts and mitochondrial DNA displayed by seven specimens. There are four processes that could explain the disagreement of meristic and mitochondrial identifications: hybridization, lineage sorting, unfixed meristic characteristics, or the classification of *P. miles* and *P. volitans* as a single species. The identification of specimens based on nuclear DNA will provide insight into which is the cause. For any of the possibilities, the mitochondrial DNA will always indicate the maternal species, as it is directly inherited from the mother. If hybridization is to blame for the disagreement, the nuclear DNA—inherited from both parents—would produce a meristic phenotype that identifies the specimen as the paternal species and disagrees with the mitochondrial DNA identification. The likelihood of this occurrence increases if the female hybrid offspring back-cross with males of the paternal species, further diluting the nuclear DNA of their offspring with alleles of the paternal species (Figure 2). A nuclear DNA identification that disagrees with the mitochondrial DNA identification could also be the result of lineage sorting. This is a process in which a single species containing a variety of alleles is divided by speciation, and by chance each of the resulting species contains different alleles and/or different proportions of alleles (Figure 3). When a dominant allele of one species is present in a small portion of the other species, it can appear that species-specific alleles were transmitted by hybridization, when in fact the shared alleles are remnants from a time when the two species were part of the same parent population.

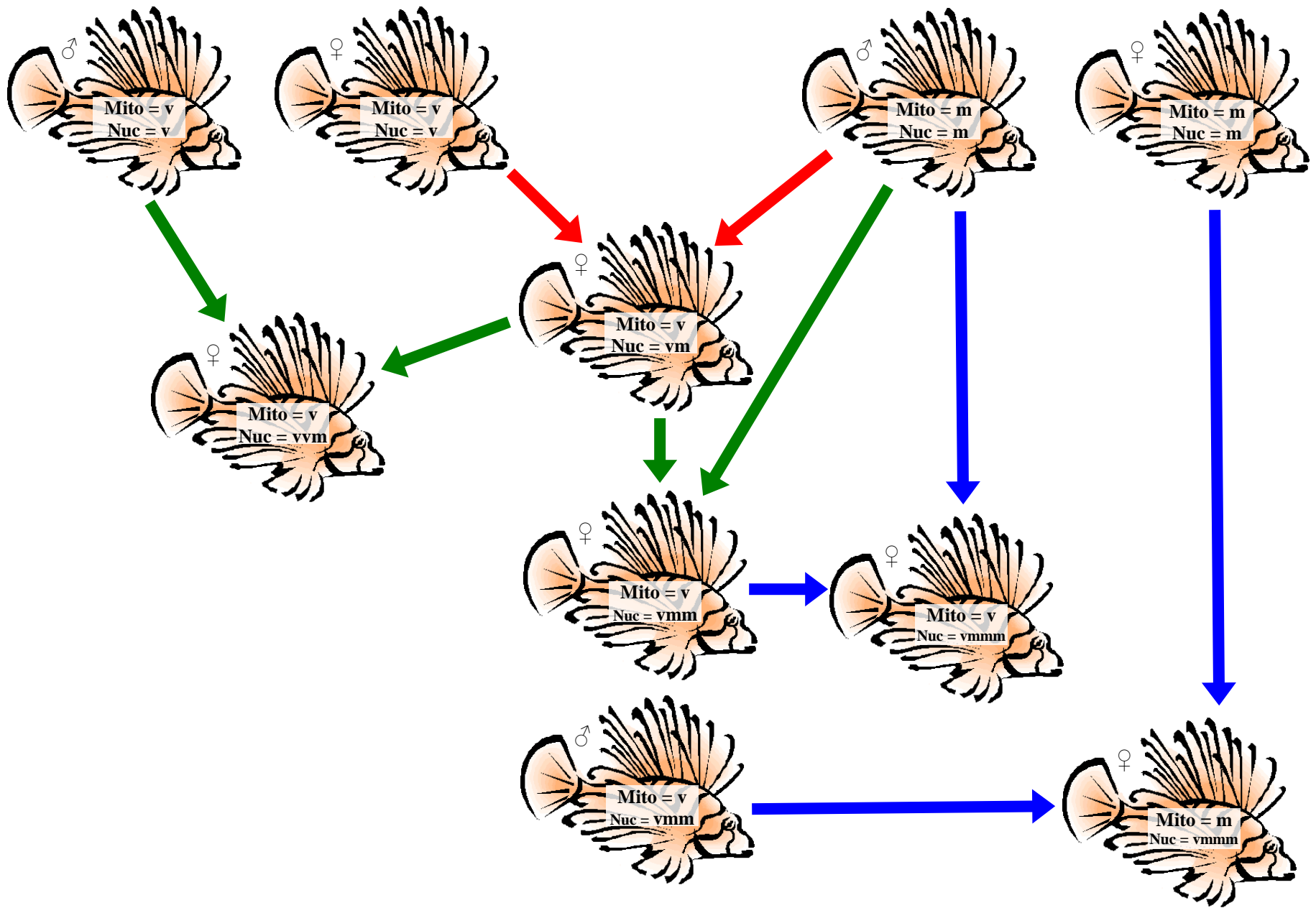
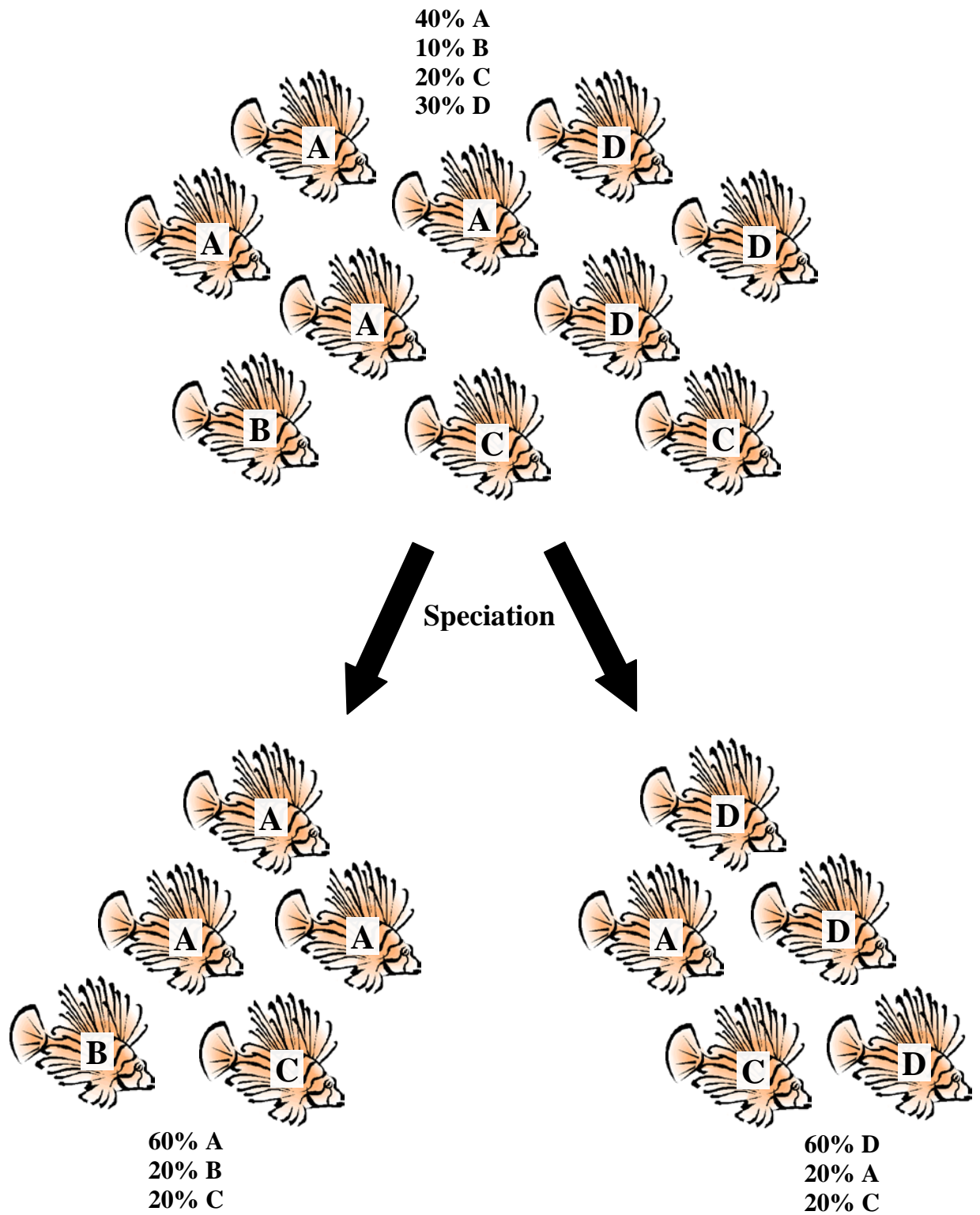


Figure 2. The mechanism of hybridization facilitating the transmission of mitochondrial DNA haplotypes between *Pterois miles* and *P. volitans*. (Mito = mitochondrial DNA, Nuc = nuclear DNA, m = *P. miles*, v = *P. volitans*)



*Figure 3.* The mechanism of lineage sorting, which can explain the presence of alleles in two species that diverged from a single parent population. The letters A, B, C, and D represent alleles.

However, if the nuclear DNA identification matches that of the mitochondrial DNA, then it may be the case that the meristic characters are not fixed for *P. miles* and *P. volitans*, and therefore, should not be used to identify specimens. It could also be the result of *P. miles* and *P. volitans* representing extremes along a continuum of variation within a single species, as opposed to comprising two distinct species. Consequently, if the nuclear DNA

identification matches

that of the meristic

characters, it will be

concluded that either

hybridization or

lineage sorting has

occurred; however, if

it matches that of the

mitochondrial DNA,

then it will be

concluded that the

meristic characters

are not fixed for the

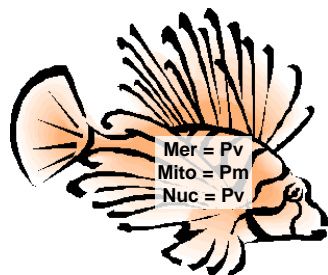
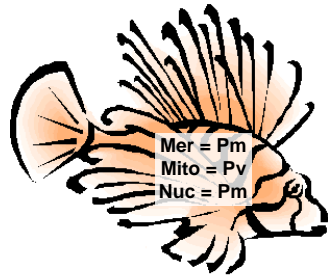
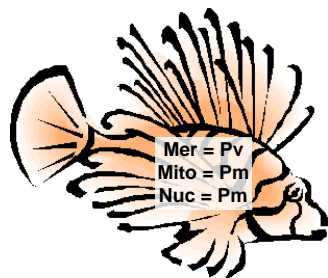
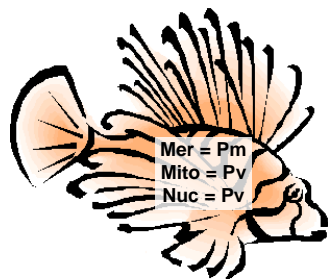
two species or that *P.*

*miles* and *P. volitans*

should actually be

classified as a single

species (Figure 4).



**Nuclear and mitochondrial DNA identities agree**

Explanations:

- Meristic and morphological characters are not fixed for *Pterois miles* and *P. volitans*
- *Pterois miles* and *P. volitans* represent the extremes of a continuum of variation within a single species

**Nuclear and meristic identities agree**

Explanations:

- Hybridization
- Lineage sorting

Figure 4. Potential explanations for the meristic (Mer) and mitochondrial DNA (Mito) identifications as *Pterois miles* (Pm) and *P. volitans* (Pv) depending on nuclear DNA (Nuc) identifications.

This first objective was approached using two methods for identifying a nuclear marker: direct sequence comparison and randomly amplified polymorphic DNAs (RAPDs). The first method directly compares the DNA sequence of a known locus to look for fixed differences between taxa. RAPDs, on the other hand, amplify multiple loci from genomic DNA by the polymerase chain reaction (PCR) using short primers of about 10 base pairs in length (Welsh and McClelland 1990). Mutations at primer sites and indels (insertions/deletions) between primer sites cause variations in the number and size of amplified fragments, which are separated for visual analysis using agarose gels (O'Brien and Freshwater 1999). If fragment length variation is fixed between species, it can act as a nuclear marker to distinguish them.

## **Methods**

Muscle tissue samples along with meristic identities as *P. miles* or *P. volitans* based on dorsal and anal fin ray counts for 28 lionfish were provided by Paula Whitfield from the NOAA laboratory in Beaufort, North Carolina. Total genomic DNA was extracted using a Puregene Kit (Gentra Systems, Minneapolis, MN) or a modification of the method described by Sambrook and Russell (2000). The extractions were further purified using a QIAquick PCR Purification Kit and protocol (Qiagen Inc., Valencia, CA). The amplification and sequencing of DNA loci were attempted according to Freshwater et al. (2000 and 2005). A variety of thermocycling programs were used for amplification depending on the primers used for the target the locus (Tables 2 a and b). Sequences were aligned in MacClade (Maddison and Maddison 2000) where species-specific differences—if they existed—would be easily identified by eye. The mitochondrially encoded cytochrome *b* gene was amplified and sequenced from each sample using previously published primers and one designed

*Tables 2a and b. Thermocycling protocols used for the amplification of DNA loci.*

a)	SCN-45		SCN-50		
	Step	T (°C)	Time (s)	T (°C)	Time (s)
	1. Initial Denature & Enzyme Activation	95.0	900	95.0	870
	2. Denature	94.0	30	94.0	30
	3. Primer Anneal	45.0	30	50.0	30
	4. Slow Ramp	+0.2°C/s to 72.0°		+0.2°C/s to 72.0°	
	5. Polymerization	72.0	90	72.0	90
	6. Cycle Repeat	34 cycles to step 2		29 cycles to step 2	
	7. Polymerization	72.0	300	72.0	300
	Loci Amplified	ITS, SSU		Act 2, Cam3, ChymB6, cyt <i>b</i> , LDH, Mlc, Ops	

b)	BECK-1		BECK-2		BECK-3		
	Step	T (°C)	Time (s)	T (°C)	Time (s)	T (°C)	Time (s)
	1. Initial Denature & Enzyme Activation	95.0	870	95.0	870	95.0	870
	2. Denature	94.0	30	94.0	30	94.0	30
	3. Primer Anneal	70.0-0.5°/cycle	30	65.0-0.5°/cycle	30	60.0-0.5°/cycle	30
	4. Polymerization	72.0	30	72.0	30	72.0	60
	5. Cycle Repeat	19 cycles to step 2		19 cycles to step 2		19 cycles to step 2	
	6 Denature	94.0	25	94.0	25	94.0	25
	7. Primer Anneal	60.0	30	55.0	30	50.0	30
	8. Polymerization	72.0	30	72.0	30	72.0	60
	9. Cycle repeat	19 times to step 6		19 cycles to step 6		19 cycles to step 6	
	10. Polymerization	72.0	300	72.0	300	72.0	300
	Loci Amplified	Ops-1, Cam-3, ChymB6, CKA7, LDHA6, AldB		Act 2, Mlc-3, RP1, RP2		Act 2, Cam3, ChymB6, Ops, RP1, RP2, RAG	

specifically for lionfish (Table 3). The sequences were used to construct a neighbor-joining tree that also included other species from the subfamily Pteroinae using PAUP\* (Swofford 2002). Fifteen nuclear DNA loci were amplified, and if successful, sequenced from a small subset of lionfish using previously developed primers (Table 3).

RAPD reactions were set up according to the recipe of O'Brien and Freshwater (1999) and run using a thermocycling program modified from Gallego and Martínez (1997). Reactions set up using full strength,  $1/5$ ,  $1/10$ ,  $1/20$ ,  $1/50$ ,  $1/100$ , and  $1/200$  dilutions of the same DNA template from a lionfish caught off North Carolina yielded amplifications of equal quality for several primers, so additional primers were tested using  $1/50$  dilutions to conserve

Table 3. Loci investigated as genetic markers to distinguish *Pterois miles* and *P. volitans*. Single/multiple bands refers to the PCR amplification product after gel electrophoresis.

Locus	Genome	Gene	Intron	Result	Primers
Act-2	nuclear	actin	2	multiple bands sequence conserved	F: GCATAACCCTCGTAGATGGGCAC <sup>1</sup> F21: GCACATTATGGGTACACCCAT <sup>1</sup> R: ATCTGGCACCACACCTTCTACAA <sup>1</sup> R220: CTCGAACATGATCTGAGTCATC <sup>1</sup> R307: CACTCGCAGCTCGTTGTAG <sup>1</sup>
AldB	nuclear	aldolase B	G	no amplification product	F: TGCGCCCAGTACAAGAAGGACGGTTG <sup>2</sup> F2: CTCAAGATCTCGGACGGCTG <sup>2</sup> Ald2: CCCATCAGGGAGAATTTTCAGGCTCCACAA <sup>3</sup>
Cam-3	nuclear	calmodulin	3	multiple bands no sequence	F: TGACGGAGCTCTGCAGCACTGAC <sup>1</sup> R: GTGAGGAGGAGCTCCGTGAGGC <sup>1</sup>
ChymB-6	nuclear	chymotrypsin B	6	multiple bands no sequence	F: GCATGAGGGCTGTGACTCGGG <sup>1</sup> R: ATCGTGTCCGAGGCTGACTGCAA <sup>1</sup>
CK	nuclear	creatine kinase	-	no amplification product	F: CC5GG5CA(CT)CC5T(AT)(CT) AT(ACT) ATG <sup>4</sup> R: A(AG)(CT)TT5AC5CC(AG)TC5AC5AC <sup>4</sup>
CKA7	nuclear	creatine kinase	7	multiple bands no sequence	F1: CCCAAGTT(CT)GAGGAGATCCTGAC <sup>4</sup> R1: CCGTGCACGACCAGCTGCACCTG <sup>4</sup> R2: CAGTCGGTC(AG)GC(AG)TTGGAGATGTC <sup>4</sup>
cyt <i>b</i>	mitochondrial	cytochrome <i>b</i>	-	single band species marker	L: GTGACTTGAAAAACCACCGTT <sup>5</sup> F52: ATCGCAAATAATGCCCT(AG)GT <sup>6</sup> F381: CTAGTTATGATAACCGCTTTC <sup>6</sup> H: CAACGATCTCCGGTTTACAAGAC <sup>5</sup> R1063: TAATGAA(CT)GG(AG)TG(GC)GAGACA <sup>6</sup>



Locus	Genome	Gene	Intron	Result	Primers
ITS	nuclear	internal transcribed spacer and 5.8S	-	single band sequence conserved	5:GGAAGTAAAAGTCGTAACAAGG <sup>7</sup> 4: TCCTCCGCTTATTGATATGC <sup>7</sup>
LDH	nuclear	lactate dehydrogenase	-	multiple bands no sequence	F: AA(CT)GT5AA(CT)AT(ACT)TT(CT)AA(AG)TT <sup>4</sup> R: A(AG)(CT)TC(CT)TT(CT)TG5A(CT)5CCCCA <sup>4</sup>
LDHA6	nuclear	lactate dehydrogenase	6	single band sequence conserved	F1: TACACTTCCTGGGC(GC) AT(CT)GG(GCT)ATG <sup>4</sup> F2: G(CT)GGACAGCATC(GC)(AT)(GT)AAGAAC(AC)TGC <sup>4</sup> R: GCT(GC)AGGAA(GC)ACCTC(AG)TCCTTCAC <sup>4</sup>
Mlc-3	nuclear	myosin light chain	3	single band no sequence	F: AGTAATGACGTCGCAGATGTTCT <sup>1</sup> R: CGACAGGTTCACTCTCGAGGAG <sup>1</sup>
Ops-1	nuclear	opsin	1	multiple bands no sequence	F: GCTCATGGGCTGCAGACCACAA <sup>1</sup> R: CCTGCTCAACCTGGCCATGGC <sup>1</sup>
RAG1	nuclear		-	single band mutations not species-specific	1F: AGCTGTAGTCAGTAYCACAARATG <sup>8</sup> 9R: GTGTAGAGCCATGRTGYTT <sup>8</sup>
RP1	nuclear	S7 ribosomal protein	1	single band mutations not species specific	F: TGGCCTCTTCCTTGGCCGT <sup>9</sup> R: AACTCGTCTGGCTTTTCGCC <sup>9</sup>
RP2	nuclear	S7 ribosomal protein	2	single band mutations not species specific	F: AGCGCCAAAATAGTGAAGCC <sup>9</sup> R: GCCTTCAGGTCAGAGTTCAT <sup>9</sup>
SSU	nuclear	ribosomal rRNA small subunit	-	single band sequence conserved	G01: CACCTGGTTGATCCTGCCAG <sup>10</sup> G07: ATCCTTCTGCAGGTTACCTAC <sup>10</sup>

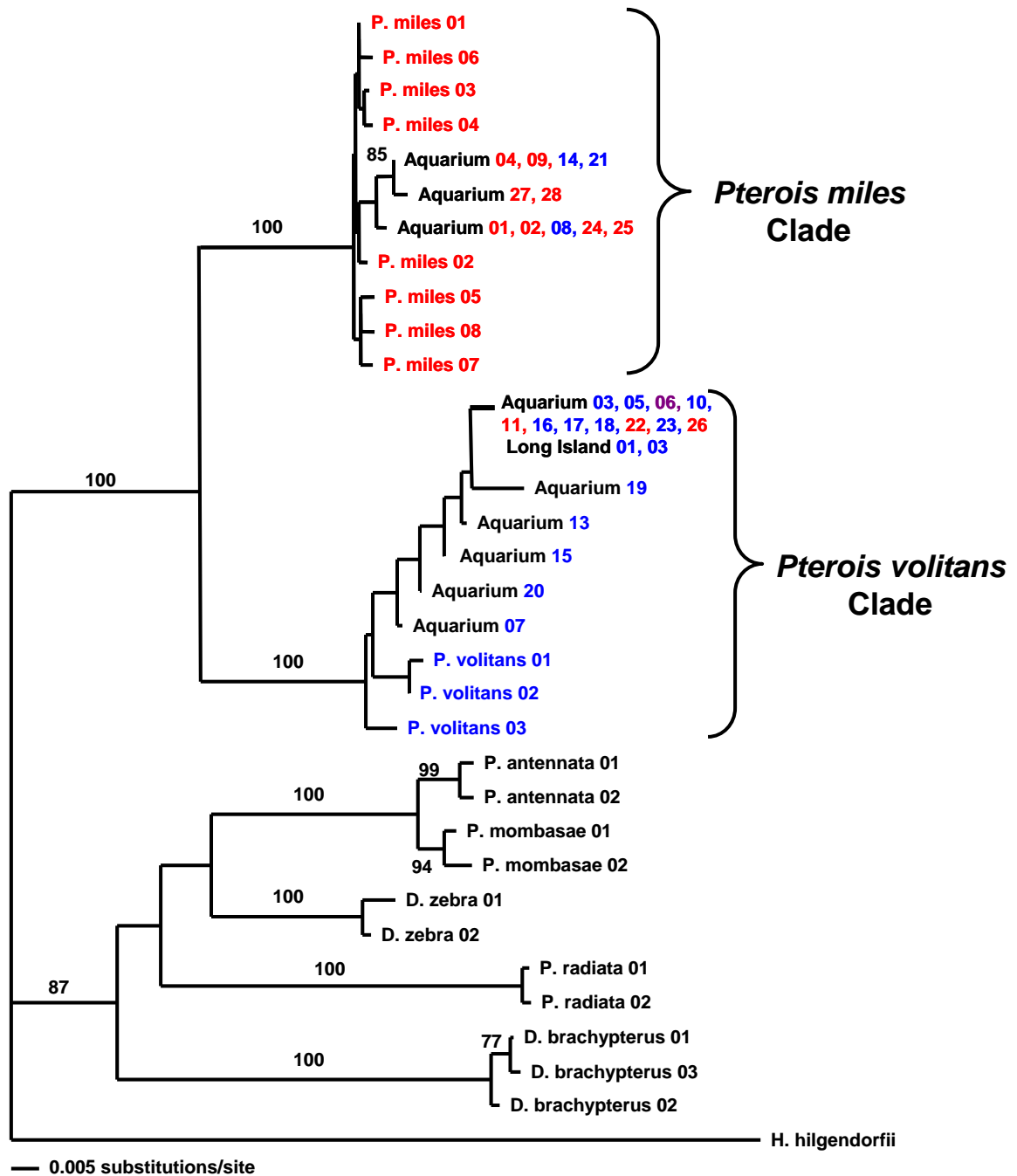
<sup>1</sup>Touriya et al. 2003, <sup>2</sup>Quattro et al. 2001, <sup>3</sup>Lessa and Applebaum 1993, <sup>4</sup>Quattro and Jones 1999, <sup>5</sup>Schmidt and Gold 1993, <sup>6</sup>Hamner and Freshwater, unpublished, <sup>7</sup>White et al. 1990, <sup>8</sup>Quenouille et al. 2004, <sup>9</sup>Chow and Hazama 1998, <sup>10</sup>Saunders and Kraft 1994

DNA template. Reactions were set up using the operon primer sets OP-B, OP-G, and OP-X (Operon Technologies Inc., Alameda, CA) to test for amplification success. Primers resulting in potentially useful bands that warranted further investigation for use as nuclear markers were included in reactions with eight *P. miles* and eight *P. volitans* to test for species-specific banding patterns. The RAPD amplification products were run on 2% agarose gels to separate the bands of DNA fragments by length for comparison. Pictures of the gels were scanned and enlarged in Adobe Photoshop (Adobe Systems Inc., San Jose, CA) to enhance the ability to score bands as present or absent. Three of the primers were fluorescently tagged allowing the amplification products to be run on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) to provide better resolution of fragment bands and a more objective scoring method. A data matrix coding each band as present or absent was constructed to provide a banding phenotype for each sample.

## **Results**

The neighbor-joining tree constructed using the cytochrome *b* mitochondrial DNA locus from species of the subfamily Pteroinae displayed distinct clades for *Pterois miles* and *P. volitans* (Figure 5).

None of the 15 nuclear DNA loci that were investigated as possible markers to distinguish between *Pterois miles* and *P. volitans* showed species specific mutations (Table 3). The PCR amplification of AldB and CK resulted in no product. Conversely, multiple regions of the DNA were amplified by the primers for Act-2, Cam-3, ChymB-6, CKA7, LDH, and Ops-1 (Figure 6a). No usable sequence data were obtained from Cam-3, ChymB-6, CKA7, LDH, or Ops-1. Several appropriately sized DNA fragments from Act-2 amplifications were isolated and sequenced; however, the sequences were conserved between



*Figure 5.* A neighbor-joining tree constructed using the cytochrome *b* mitochondrial DNA locus of species in the subfamily Pteroinae. Distinct clades are formed for *Pterois miles* and *Pterois volitans*; however, Aquarium 08, 14, and 21 clade with *P. miles*, but display the meristic characteristics of *P. volitans*, while the opposite is true for Aquarium 06, 11, 22, and 26. Bootstrap values >50 are shown on the branches. All samples designated an Aquarium number are from Indonesia; *P. volitans* 01-03 are from Taiwan; and *P. miles* 01-08 are from the Red Sea, Kenya, Sri Lanka, and the Java Sea. All sequences not designated by an Aquarium number were obtained from GenBank.

*P. miles* and *P. volitans*, showing no differences. The PCR of LDHA6, SSU, ITS, and Mlc-3 each resulted in a

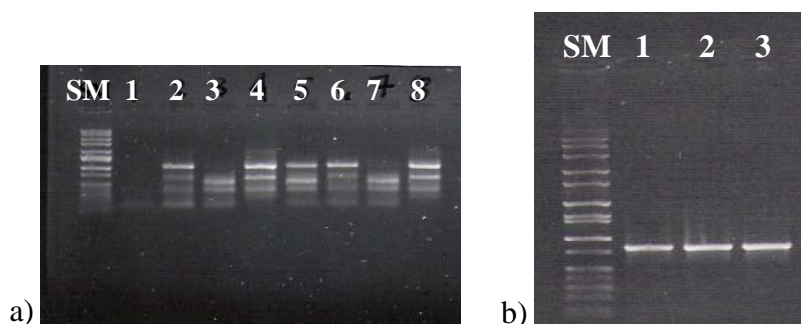


Figure 6. a) Multiple bands of DNA fragments that resulted from the PCR and gel electrophoresis of LDH. b) Single bands of DNA fragments resulting from the PCR and gel electrophoresis of RP1. (SM = size marker; numbers = lionfish samples)

single band of DNA fragments; however, the sequences were also conserved between the two species. Additionally, RP1, RP2, and RAG1 each amplified a single band of DNA fragments (Figure 6b) and provided sequences that displayed variations, but the differences were not species specific.

RAPDs proved to be no better at distinguishing between *P. miles* and *P. volitans*. Of the 60 primers tested, 43 resulted in an amplification product for lionfish; however, only 11 of them showed clear, reproducible, and potentially useful bands warranting further investigation for use as a nuclear marker (Figure 7 and Table 4). Two of these primers did not amplify consistently, one showed no

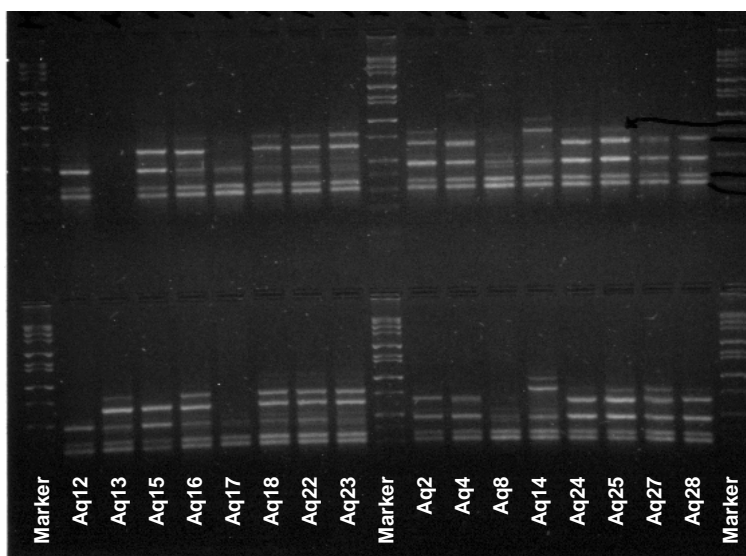


Figure 7. Randomly amplified polymorphic DNA (RAPD) banding phenotypes of eight *Pterois miles* (right) and eight *P. volitans* (left) amplified with primer X17. The top and bottom rows are duplicate reactions set up to test for reproducibility.

variability, five showed low individual variability of banding phenotypes with no interspecific differences, and three showed high individual variability of banding phenotypes with no interspecific differences.

*Table 4.* Randomly amplified polymorphic DNA (RAPD) primers investigated as markers to distinguish *Pterois miles* and *P. volitans*, and the results obtained from amplification. (\*fluorescently tagged and run on ABI 3100 Genetic Analyzer)

<b>Primer</b>	<b>Result</b>
B6	No species specific differences, low variability
B7	No species specific differences, high variability
B14	No variability
G3	No species specific differences, low variability
G9	Inconsistent amplification
G11	No species specific differences, low variability
G17*	No species specific differences, high variability
G18*	No species specific differences, high variability
X11	No species specific differences, low variability
X17	No species specific differences, low variability
X19*	Inconsistent amplification

## **Discussion**

A nuclear DNA based marker for the identification of *Pterois miles* and *P. volitans* was not developed because no species-specific variations were found for any of the 15 nuclear DNA loci that were examined or for the banding phenotypes obtained by RAPDs.

Large evolutionary separations between lionfish and the species used to design the loci-specific primers could explain the inability to generate amplification products or the amplification of multiple DNA fragments for lionfish. If there are mismatches between the primer and DNA template sequences, the primers may not anneal to the template DNA, failing to initiate replication, and therefore, yielding no PCR product. On the other hand, multiple DNA fragments of different sizes can amplify if the primers anneal to multiple regions of the DNA that vary by a few base pairs.

The majority of nuclear DNA loci that were able to be sequenced were found to be conserved despite the use of non-coding gene introns. The close phylogenetic relationship of *P. miles* and *P. volitans* may not have allowed the evolutionary time required for mutations to occur and become fixed to distinguish the two species. The greater amount of variation

found in the mitochondrial DNA can be explained by a smaller effective population size for mitochondrial DNA as compared to nuclear DNA. The mitochondrial genome is only inherited maternally, cutting the effective population size in half. It is halved again because there is only one copy of the mitochondrial genome per mitochondria, while the presence of homologous chromosomes in the nucleus provides two copies of each locus in the nuclear genome. This results in an effective population size for mitochondrial DNA that is one-fourth of the effective population size of nuclear loci (Avice 2004). Consequently, mutations in mitochondrial DNA are more likely to become fixed in a population because only half of the breeding population is contributing mitochondrial DNA and there is only one copy of the loci, preventing them from being hidden by an alternate allele. Furthermore, mitochondria lack DNA repair mechanisms that are present in the nucleus, directly increasing the rate of mutation fixation in the mitochondrial DNA (Avice 2004).

If a nuclear DNA marker can not be identified, the conflict between the meristic and mitochondrial DNA identifications can also be approached by questioning the classification of *P. miles* and *P. volitans* as two distinct species. Mitochondrial DNA collected from lionfish at regular intervals between the extremes of the native range of *P. miles* and *P. volitans* can be used to observe patterns of haplotype frequency and distribution. If a gradual spectrum of change in haplotype frequency is observed from one extreme to the other, then it is likely that *P. miles* and *P. volitans* are one species that varies in appearance from the typical characteristics of *P. miles* to those of *P. volitans*. A key area on which to focus for this analysis is the area around Indonesia where the distributions of the two overlap. Even if a gradual change is found to occur in the distributions as they approach Indonesia, if the *P. miles* and *P. volitans* that coexist in the area of overlap display distinct genetic differences

that suggest the absence of gene flow, then the hypothesis of a single species with a gradual spectrum of change must be rejected. The present data suggest such a rejection, but are insufficient to form a solid conclusion. Two distinct clades for *P. miles* and *P. volitans* form when specimens of *P. miles* and *P. volitans* from Indonesia are examined along with *P. volitans* from Taiwan and *P. miles* from Kenya, the Red Sea, Sri Lanka, and the Java Sea (Figure 5). However, more samples from both Indonesia and other locations throughout the native range are needed before a conclusion can be made. If the distinction of *P. miles* and *P. volitans* as two species is supported by the collection of more data, it still leaves hybridization, lineage sorting and unfixed meristic characteristics to be investigated.

**Part II:** *The invasive Atlantic lionfish: Who invaded, where did they come from, and what are the genetic consequences?*

**Introduction**

Doubts regarding the use of meristic counts for accurately distinguishing *Pterois miles* and *P. volitans* have not been resolved, but genetic data can be used to identify the number of species involved in the western Atlantic invasion. These genetic data can also be used to investigate the genetic bottleneck that is likely to have accompanied the invasion. A genetic bottleneck refers to a reduction in genetic diversity that occurs when a relatively small founder population establishes a new population isolated from the source population. Severe reductions in genetic diversity can act to reduce the fitness of a population leading to its extinction (Frankham and Ralls 1998); however, less severe reductions can appear to have no effect on the success of a population (Neilson and Wilson 2005; Patti and Gambi 2001), or in some cases increase its success (Tsutsui et al. 2000). Additionally, if there is well-defined population structure in the native range of lionfish, then the genetic data can be used to determine the source population of the founders that gave rise to the invasive population, similar to what was done with the yellowfin goby (*Acanthogobius flavimanus*) invasive to California (Neilson and Wilson 2005). The cytochrome *b* gene of the mitochondrial genome was chosen for this study because it has been shown to contain an appropriate amount of variability (Kochzius et al. 2003) to achieve the objectives of this section: (2) to determine the number of lionfish species involved in the Atlantic invasion based on genetic data; (3) to estimate the extent of the genetic bottleneck that accompanied the invasion using mitochondrial DNA haplotypes; (4) to determine the source population of the invasive lionfish using mitochondrial DNA haplotypes.



## Methods

Fin clips or muscle tissue samples of lionfish from their native range were provided by Paula Whitfield and James Morris from the NOAA laboratory in Beaufort, North Carolina. Muscle tissue samples from lionfish caught in the Atlantic Ocean were provided by Paula Whitfield as part of lionfish surveys funded by NOAA's Undersea Research Center (NURC). Sequences of the mitochondrial cytochrome *b* gene were obtained from 269 *Pterois miles* and *P. volitans* lionfishes according to the methods described in Part I. Three native *P. volitans* sequences were also taken from GenBank (AJ429431, AJ429432, and AJ429433; Kochzius et al. 2003), providing a total of 100 lionfish from the native range and 172 from the Atlantic Ocean. Only samples identified as *P. volitans* were included in the subsequent analyses because only 12 of the 172 Atlantic specimens analyzed were identified as *P. miles* by the cytochrome *b* data and there was insufficient representation of *P. miles* from the native range. Mitochondrial haplotypes of *Pterois volitans* from the native range and Atlantic Ocean were identified and compared. TCS (Clement et al. 2000) was used to create haplotype networks of the native and Atlantic *P. volitans* to which the geographic source of each haplotype was added. The best-fit model of evolution for the data was determined using ModelTest (Posada and Crandall 1998) and this model was used to calculate corrected distances for a neighbor-joining tree constructed using PAUP\* (Swofford 2002). The tree was bootstrapped with 2000 replications of the same tree-building method. Pairwise distance values were calculated along with the implementation of an Exact Test and Analysis of Molecular Variance (AMOVA), all of which were implemented in Arlequin (Schneider et al. 2000) to test the significance of the population structure.

## Results

Sequences of 879 base pairs of the mitochondrial cytochrome *b* locus were examined in 100 native range lionfishes and 172 invasive Atlantic lionfishes. The majority of the 172 lionfishes caught in the Atlantic Ocean are unquestionably identified as *Pterois volitans*; however, 12 have the mitochondrial DNA sequence of *P. miles* despite displaying the characteristics of *P. volitans*. Twenty-eight haplotypes, designated A to AB, were identified in the 78 native *P. volitans*, while only three of them, A, C, and E, were identified in the 160 Atlantic *P. volitans* (Table 5). The three haplotypes in the Atlantic represent only 10.7% of the genetic diversity found in the native range. The minimum-spanning network of haplotypes displays fairly well-defined clusters based on the geographic areas in the native range (Figures 8 and 9). The three haplotypes found in the Atlantic Ocean were shared exclusively by the Indonesian population, with the exception of one fish from Quezon Province

Table 5. Mitochondrial cytochrome *b* haplotypes of *Pterois volitans* from their native range and invasive to the Atlantic Ocean.

Haplotype	Indonesia	Cebu Island, Philippines	Quezon Province, Philippines	Taiwan	Atlantic	Total
A	18	-	1	-	152	171
B	2	5	3	-	-	10
C	5	-	-	-	4	9
D	-	2	4	-	-	6
E	2	-	-	-	4	6
F	2	-	-	-	-	2
G	-	3	3	-	-	6
H	1	1	1	-	-	3
I	1	-	-	-	-	1
J	1	-	-	-	-	1
K	1	-	-	-	-	1
L	1	-	-	-	-	1
M	1	-	-	-	-	1
N	1	-	-	-	-	1
O	1	-	-	-	-	1
P	1	-	-	-	-	1
Q	-	-	1	-	-	1
R	-	1	-	-	-	1
S	2	-	-	-	-	2
T	-	-	-	1	-	1
U	-	-	-	1	-	1
V	-	-	-	1	-	1
W	1	-	-	-	-	1
X	1	1	-	-	-	2
Y	1	-	-	-	-	1
Z	-	2	1	-	-	3
AA	-	1	1	-	-	2
AB	-	-	1	-	-	1
Total	43	16	16	3	160	238



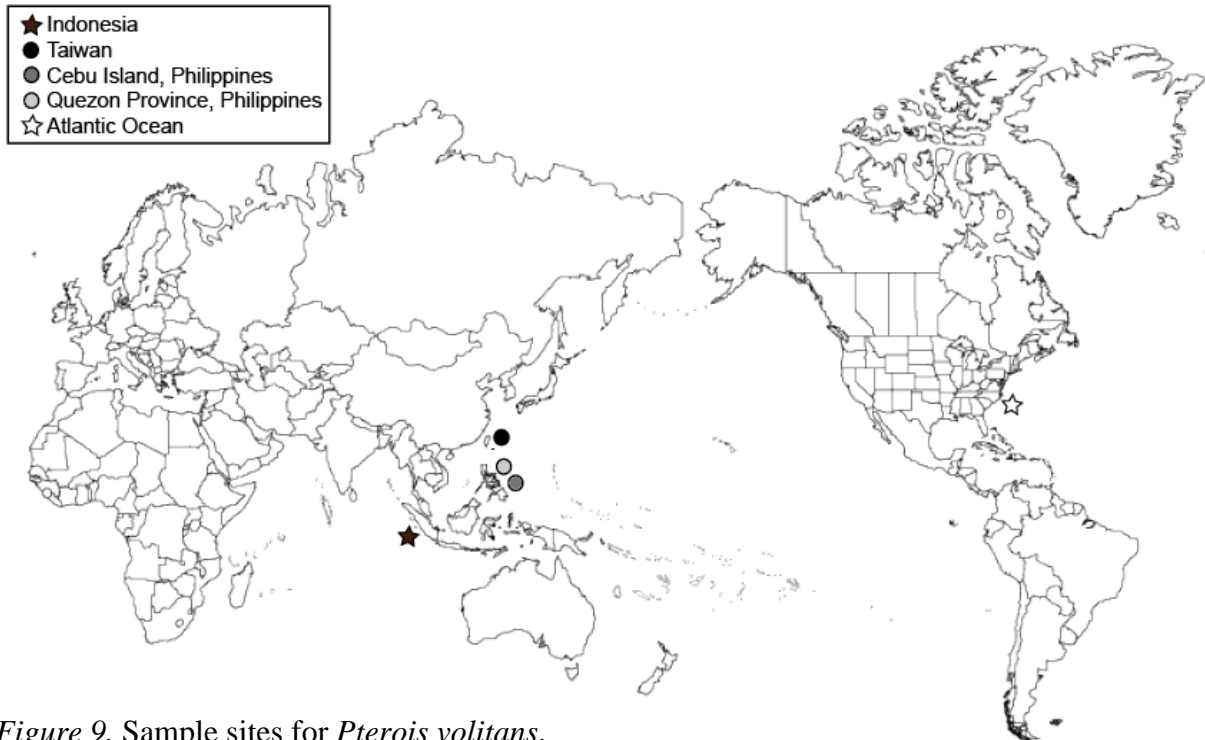


Figure 9. Sample sites for *Pterois volitans*.

in the Philippines which also shared the most common haplotype, A. Haplotypes of specimens from the same geographic region were generally more closely related in the neighbor-joining tree, as well (Figure 10). The ranges of pairwise distance values also show a trend of smaller ranges for within population comparisons than between population comparisons (Table 6). The Exact Test revealed significant differences between the Indonesian population and each of the other native populations (Table 7). Further support for the significant differentiation of lionfish populations from different geographic regions in the native range is provided by an AMOVA. The majority of the molecular variance in the native range was attributed to variation among the populations from Indonesia, the Philippines, and Taiwan (Table 8). The populations from Cebu Island and Quezon Province were grouped as the Philippines because the Exact Test found no significant difference between them (Table 7) and an independent AMOVA between them attributed all the

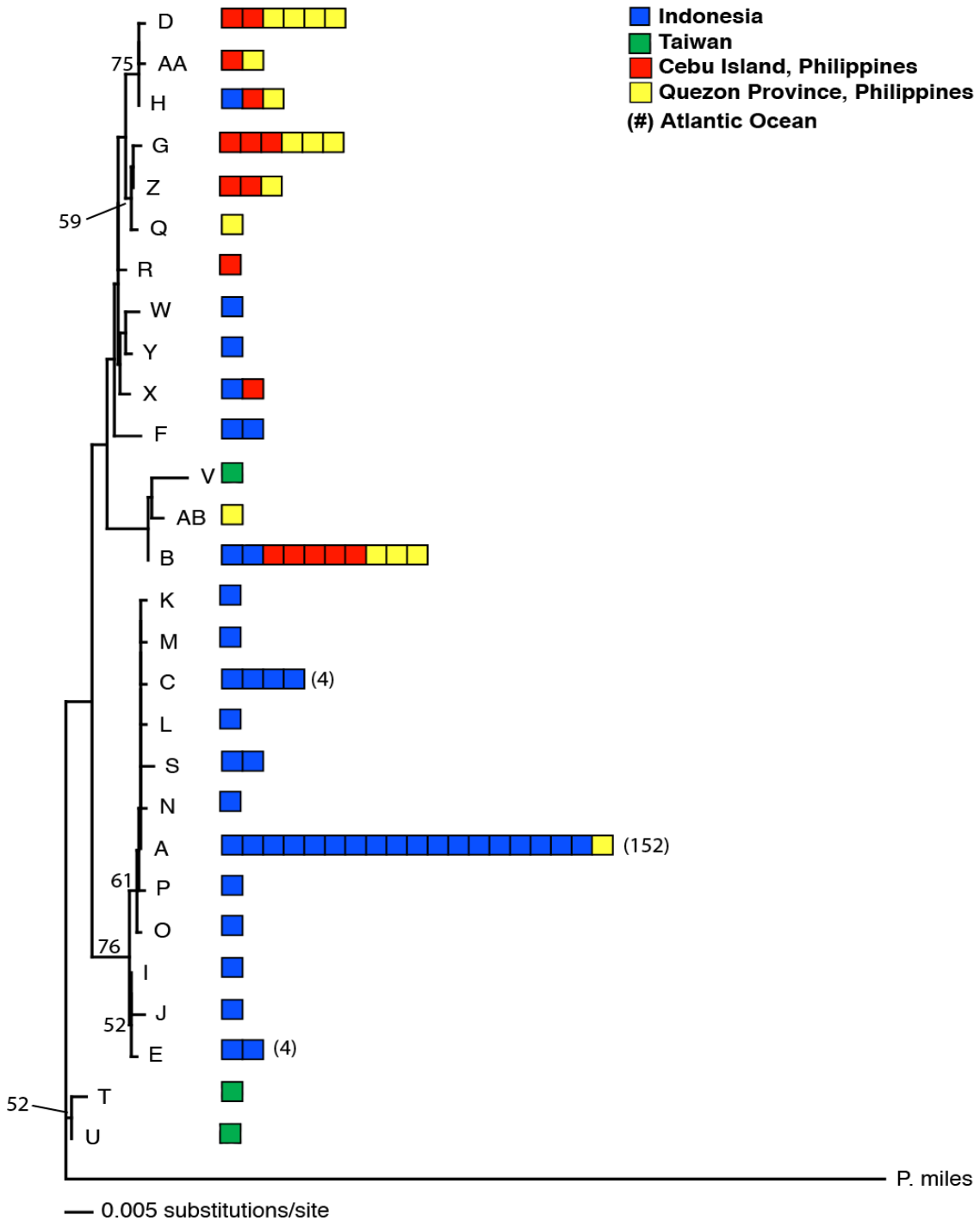


Figure 10. A neighbor-joining tree constructed with the mitochondrial cytochrome *b* haplotypes of *Pterois volitans* using distances calculated with a Tamura-Nei invariant sites model and showing bootstrap values >50. The letters represent haplotypes and each square represents one lionfish from the indicated native population. The numbers in parentheses to the right of the bars indicate the number of Atlantic *P. volitans*.

Table 6. Pairwise distance ranges for interpopulation and intrapopulation comparisons of *Pterois volitans* cytochrome *b* haplotypes.

	1	2	3	4	5
Indonesia (1)	0-17.61				
Atlantic (2)	0-17.61	0-16.47			
Cebu Island (3)	0-17.63	0-16.47	0-9.13		
Quezon Province (4)	0-19.65	0-18.51	0-17.49	0-17.49	
Taiwan (5)	0-24.90	0-22.25	0-22.25	0-22.25	2.39-14.65

Table 7. Exact Test comparing lionfish populations in the native range using  $p = 0.05$ . (+) = significant difference; (-) = no significant difference.

	1	2	3	4
Indonesia (1)				
Cebu Island (2)	+			
Quezon Province (3)	+	-		
Taiwan (4)	+	+	-	

Table 8. Hierarchical AMOVA of *Pterois volitans* mitochondrial cytochrome *b* haplotypes from sites in the native range: Indonesia, Taiwan, and the Philippines.

Source of Variation	Df	Sum of Squares	Variance	% Variance
Among Groups	2	158.270	3.83794	57.31
Among Populations within groups	1	1.039	-0.12133	-1.81
Among Individuals Within Populations	73	217.559	2.98026	44.50

molecular variance to within population variation.

Hierarchical AMOVA results also support the

association of the Atlantic lionfish with the

Indonesian lionfish. When the Atlantic population

is grouped with the Indonesian population, the vast

majority of variance is attributed to variation

among groups, however, when the Atlantic

population is grouped with the Philippines,

Taiwan, or by itself, less of the variance is

attributed to variation among groups (Table 9).

Table 9. Summary of % variance among groups calculated by hierarchical AMOVA of mitochondrial cytochrome *b* haplotypes from all possible groupings of the Atlantic *Pterois volitans* population with the native populations.

Population Grouping	% Variance Among Groups
Indonesia + Atlantic Philippines Taiwan	80.18
Indonesia Philippines + Atlantic Taiwan	-240.17
Indonesia Philippines Taiwan + Atlantic	57.45
Indonesia Philippines Taiwan Atlantic	73.50

## Discussion

Based on the analysis of haplotypes derived from the cytochrome *b* mitochondrial DNA locus, both *Pterois miles* and *P. volitans* are involved in the western Atlantic invasion, with 93% of specimens identified as *P. volitans* and 7% of them identified as *P. miles*. Indonesia has been identified as the most likely area from which the founders of the Atlantic *P. volitans* came; but to pinpoint a more specific location more samples are needed from a variety of areas around Indonesia. If the source population is pinpointed to a site that is popular for the collection of lionfish for the aquarium trade, then it will provide more support for the hypothesis of accidental or intentional aquaria releases as the mode of introduction (Whitfield et al. 2002). As is expected with invasive species (Neilson and Wilson 2005; Patti and Gambi 2001; Tsutsui et al. 2000), the lionfish in the Atlantic display a genetic bottleneck, or severe decrease in the amount of genetic variability as compared to the source population. This is demonstrated by only 3 of the 28 haplotypes in the native range (10.7%) occurring in the invasive population. Finding fewer haplotypes in the invasive population despite analyzing over twice as many invasive fish, offers strong support that the reduction in haplotype diversity is the result of a genetic bottleneck, as opposed to sampling error. The genetic bottleneck does not seem to be hindering the success of lionfish, since they are now present continuously from northern Florida to North Carolina in numbers similar to that of native grouper species, which occupy a similar ecological niche (Whitfield et al. submitted). The presence of three *P. volitans* haplotypes in the Atlantic indicates that a minimum of three female *P. volitans* were responsible for the establishment of the Atlantic population. It is likely that more females with haplotype A were involved in the initial invasion because of the large proportion of the Atlantic lionfish that have this haplotype.

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