

Conflict between mitochondrial DNA and meristic identification of *Pterois miles* and *Pterois volitans*: Is hybridization to blame?

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Introduction

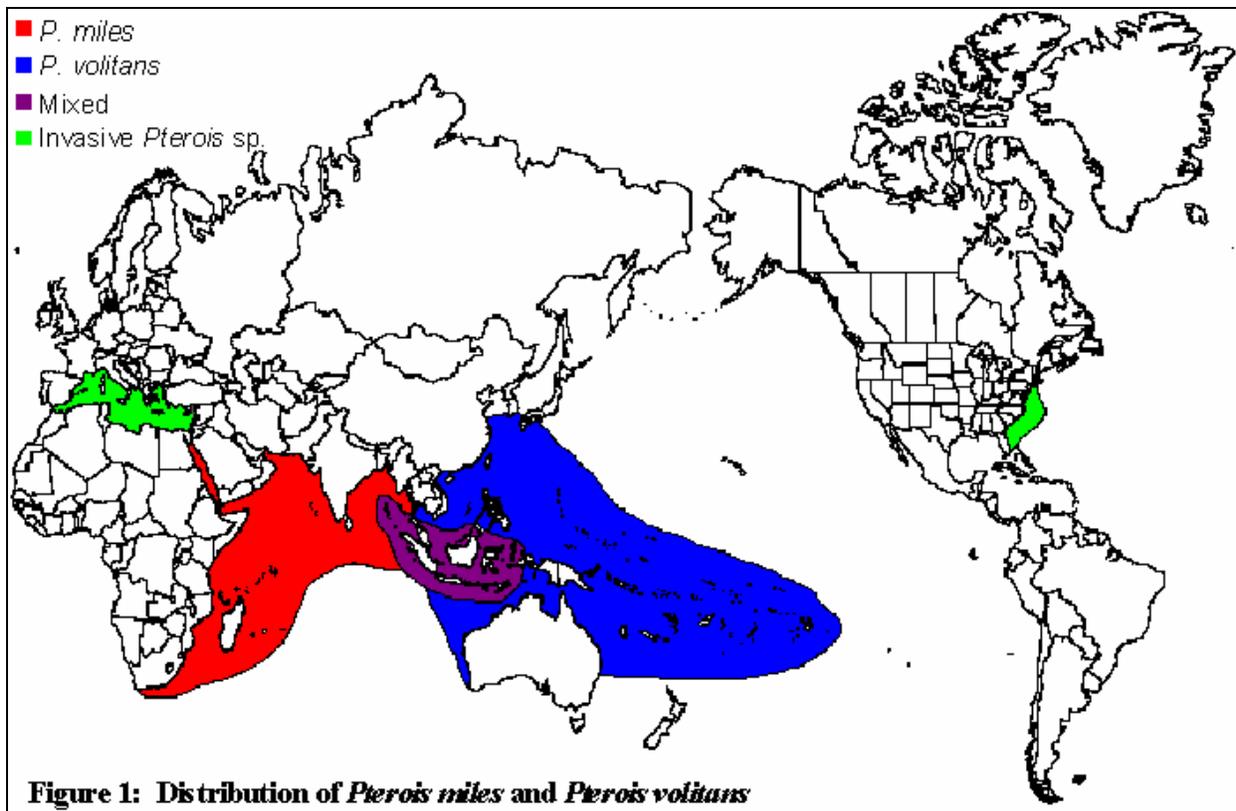
Lionfish species *Pterois miles* and *Pterois volitans* are similar in appearance with a common number of pectoral rays, venomous dorsal and anal fin spines, cycloid-shaped scales, and vertical bars that range in color from red to brown to black across their lightly colored bodies (Schultz, 1986). The two species were historically thought to be one (Beaufort and Briggs, 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).

Pterois volitans have an additional ray in their dorsal and anal fins, longer pectoral fins, and larger spots on their dorsal fin as

	<i>Pterois miles</i>	<i>Pterois volitans</i>
Dorsal Fin Rays	10	11
Anal Fin Rays	6	7
Pectoral Fin Size	Shorter	Longer
Spot size	Smaller	Larger
Distribution	Indian Ocean, S. Africa to Red Sea, East to Sumatra	West Pacific Ocean from Japan to Australia, off W Australia, South Pacific to Pitcairn Group

compared to *P. miles*. Kochzius et al. (2003) were also able to distinguish between specimens identified as *P. miles* and *P. volitans* using mitochondrial cytochrome *b* and 16S rDNA sequences.

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 Sumatra, while *P. volitans* ranges in the western Pacific Ocean from Japan to Australia, along the west coast of Australia, and in the southern Pacific Ocean to the Pitcairn Group (Figure 1). An overlap in their distributions occurs around the Indo-Malayan Archipelago (Schultz, 1986). 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 mya (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* has taken up residence in the Atlantic Ocean along the east coast of the United States, potentially as a result of aquaria releases (Whitfield, et al., 2002) (Figure 1).

The study of invasive species is essential due to the detrimental impacts they can have on the affected ecosystem, as well as the people and industries that depend on that ecosystem. This

is demonstrated by the invasive zebra mussels in the Great Lakes, on which approximately \$1,205,000 is spent annually by affected municipalities and industries to control their presence (<http://www.anstaskforce.gov/ansimpact.htm>).

The invasion of lionfishes in the Atlantic Ocean has spurred the examination of many aspects regarding their biology including genetic analyses. The accurate identification of lionfish as *P. miles* and *P. volitans* is important for the examination of the invasive population(s) in the Atlantic waters along the eastern coast of the United States. It is necessary to know if one or both species compose the invasive population(s) so that their origin, impacts, and appropriate interventions can be determined. Meristic counts 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 meristics to provide accurate identifications of specimens. Therefore, the use of genetic data is essential for making accurate distinctions between *P. miles* and *P. volitans*.

The cytochrome *b* mitochondrial marker, previously used by Kochzius et al. (2003), was shown to disagree with meristic identifications of several lionfish specimens from the aquarium trade (Hamner and Freshwater, unpublished data). This discovery led to the current investigation of hybridization between *P. miles* and *P. volitans*. The objective of this study was to develop a sequence-based nuclear DNA marker for *P. miles* and *P. volitans* that could be compared to the cytochrome *b* mitochondrial marker and meristic identifications.

Methods

Total genomic DNA was extracted from lionfish muscle tissue samples using a Puregene Kit (Gentra Systems, Minneapolis, MN) or a modification of the method described by Sambrook

and Russell (2000). A standard method for the polymerase chain reaction (PCR) (Freshwater, et al., 2000) was used to amplify 14 nuclear loci from a small subset of fishes from the aquarium trade, using previously developed primers (Chow and Hazama, 1998; Touriya et al., 2003; Lessa and Applebaum, 1993; Quattro and Jones, 1999; Quattro et al., 2001) (Table 2). Products of successful amplifications were sequenced using a Big Dye Kit and protocol (Applied Biosystems, Foster City, CA), and sequence reactions were run on an ABI 3100 Genetic Analyzer (DNA Analysis Core Facility, CMS). Sequences were analyzed using computer programs Sequencher (Gene Codes Corp., Ann Arbor, MI) and MacClade (v.4, Maddison & Maddison, 2000).

Results

None of the 14 nuclear DNA loci that were investigated as possible markers to distinguish between *P. miles* and *P. volitans* showed species specific mutations (Table 2).

No product resulted from the PCR amplification of AldB and CK. Multiple regions of the DNA were amplified by the primers for Act-2, Cam-3, ChymB-6,

CKA7, LDH (Figure 2), and Ops-1 resulting in multiple

bands of DNA fragments after gel electrophoresis of the PCR product. No usable sequence data was obtained from Cam-3, ChymB-6, CKA7, LDH, or Ops-1. Multiple DNA fragments from Act-2 amplifications were isolated and sequenced; however, the sequences were conserved between *P. miles* and *P. volitans*, showing no differences. The PCR of LDHA6, SSU, ITS, and Mlc-3 each resulted in a single band of DNA fragments; however, the sequences were also



Figure 2. Multiple bands of DNA fragments that resulted from the PCR and gel electrophoresis of LDH. SM stands for size marker; 1-8 indicate eight lionfish samples.

Table 2. Nuclear loci investigated and not useful as genetic markers to distinguish *P. miles* and *P. volitans*. Single/multiple bands refers to the PCR product after gel electrophoresis. Bands of DNA fragments were isolated and sequenced from Act-2, Cam-3, and LDH.

Locus	Gene	Intron #	Result
Act-2	Actin	2	Multiple bands Conserved
AldB	Aldolase B	G	No PCR product
Cam-3	Calmodulin	3	Multiple bands Sequence not usable
ChymB-6	Chymotrypsin B	6	Multiple bands
CK	Creatine kinase	NA	No PCR product
CKA7	Creatine kinase	7	Multiple bands
ITS	Internal transcribed spacer and 5.8S rRNA	NA	Single band Conserved
LDH	Lactate dehydrogenase	NA	Multiple bands No sequence
LDHA6	Lactate dehydrogenase	6	Single band Conserved
Mlc-3	Myosin light chain	3	Single band Sequence not usable
Ops-1	Opsin	1	Multiple bands no sequence
RP1	S7 ribosomal protein	1	Single band Mutations not species specific
RP2	S7 ribosomal protein	2	Single band Mutations not species specific
SSU	ribosomal rRNA small subunit	NA	Single band Conserved

conserved between the two species. RP1 (Figure 3) and RP2 each amplified a single band of DNA fragments and had sequences that displayed variations, but the differences were not species specific.

Discussion

No species specific variations were found for any of the 14 nuclear loci that were examined, and therefore, none of them are useful as genetic markers to distinguish *P. miles* and *P. volitans*.

Large evolutionary separations between lionfishes and the species used to design the primers could explain the inability to amplify loci or the amplification of multiple DNA fragments for lionfishes. 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.

Despite the use of non-coding nuclear gene introns, most of the DNA loci that were able to be sequenced were found to be conserved. 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 in the two species. More variation is found in the mitochondrial DNA because it has an effective population size that is one-fourth the effective population size of nuclear DNA loci (Awise, 2004). The mitochondrial genome is only inherited from the mother, cutting the effective population size in half. It is halved again because there is only one copy of the mitochondrial genome per mitochondria, while there are two copies of the nuclear genome per

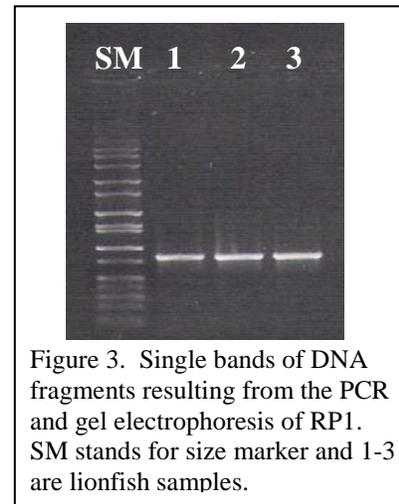


Figure 3. Single bands of DNA fragments resulting from the PCR and gel electrophoresis of RP1. SM stands for size marker and 1-3 are lionfish samples.

nucleus. This results in an effective population size for mitochondrial DNA that is one-fourth of the effective population size of nuclear loci. 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 mutations in the mitochondrial DNA (Awise, 2004).

In the future, Randomly Amplified Polymorphic DNAs (RAPDs) will be used to get a better picture of species specific variations by sampling the entire genome instead of isolating specific loci for analysis, as is done with sequence-based markers. RAPDs generally use short primers of about 10 base pairs to amplify multiple loci from genomic DNA using PCR (Welsh and McClelland, 1990). Mutations at primer sites and indels (insertions/deletions) between primer sites may cause variation in the number and size of amplified fragments, which can be separated using agarose gels (O'Brien and Freshwater, 1999). If fragment variation is fixed between species, it can be used as a nuclear marker to distinguish them.

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