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MOLECULAR EVIDENCE THAT THE LIONFISHES *PTEROIS MILES* AND *PTEROIS VOLITANS* ARE DISTINCT SPECIES

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Abstract: The lionfish species *Pterois miles* and *P. volitans* are popular aquarium fishes that have gained recent notoriety as invasive species along the east coast of the United States and the Bahamas. The two species can usually be identified using dorsal and anal fin ray counts as well as geographic origin, but neither meristics nor geography are always definitive, and their taxonomic status as separate species has been questioned. Analyses of two mitochondria-encoded cytochrome *b* sequence data sets resolved specimens of *P. miles* and *P. volitans* in distinct monophyletic clades. There was also a >4% difference in the maximum intraspecific and minimum interspecific sequence divergences between specimens of the two species. These results are comparable to those of other analyzed *Pterois* and *Dendrochirus* sister species, and support their recognition as separate species. The cytochrome *b* analyses also show that *Dendrochirus* and *Pterois* are not reciprocally monophyletic as currently circumscribed, and that a comprehensive study is needed to resolve the taxonomy of Pteroinae genera.

Key Words: *Dendrochirus*; invasive species; lionfish; mitochondrial control region; molecular systematics; *Pterois*; *Pterois miles*; *Pterois volitans*; Pteroinae; Scorpaenidae.

INTRODUCTION

Lionfishes are Scorpionfishes in the genera *Dendrochirus* (Swainson 1839), *Ebosia* (Jordan & Starks 1904), *Parapterois* (Bleeker 1876), and *Pterois* (Oken 1817), and belong to the family Scorpaenidae. The best-known members of this group are *Pterois miles* (Bennett 1828) and *P. volitans* (Linnaeus 1758), because of their popularity as aquarium fishes. *Pterois miles* has been treated as a synonym of *P. volitans* in the past (e.g., De Beaufort and Briggs 1962; Dor 1984), however, Schultz (1986) described meristic and morphometric differences between the two species. His study found that specimens of *P. volitans* usually have 11 dorsal and 7 anal fin rays while *P. miles* specimens usually have 10 dorsal and 6 anal fin rays, and that *P. volitans* has significantly larger pectoral fins and larger spots on the soft vertical fins (Schultz 1986). The native geographic range of *P. miles* extends from South Africa north and east along the coasts of southern Asia to Indonesia in the Indian Ocean. *Pterois volitans* is found in the tropical Pacific from the Pitcairn group in the east to Indonesia in the west, and along the western coast of Australia in the Indian Ocean (Froese and Pauly 2008). The two species are sympatric in western Indonesia (Allen and Mohammed 2003).

Specimens can usually be identified using dorsal and anal fin ray counts as well as geographic location, however, the meristic counts are not definitive (Tables 1 and 2 in Schultz 1986) and the geographic origin is not diagnostic. As an example, in the same publication where Bennett describes *P. miles*, he also illustrates and reports, with typical counts, *P. volitans* from Sri Lanka (Bennett 1828). Identification by geographic location is also impossible when the species in question have been introduced beyond their native ranges, a situation that exists for both *P. miles* and *P. volitans*. *Pterois miles* moved into the Mediterranean Sea through the Suez Canal (Golani and Sonin 1992), and both species are invasive in the western Atlantic (Whitfield et al. 2002; Hamner et al. 2007). Specimens with counts typical of both species as well as mixed meristics – *P. volitans* dorsal fin ray count and *P. miles* anal fin ray count, and vice versa – have been collected (Whitfield unpubl. data, Morris unpubl. data, Freshwater unpubl. data). The difficult identification of these two invasive *Pterois* species makes them ideal candidates for molecular assisted identification.

DNA sequence data has been used extensively for molecular assisted identifications in cases, or at life stages, when morphology is inadequate (e.g., Schlei et al. 2008; Vandersea et al. 2008). Aligned DNA sequences may be analyzed to generate phylogenetic trees and the identification of unknown specimens made by observing their resolved positions relative to known species within the trees. Comparisons of pairwise sequence divergences may also be used if baseline data has established that there is no overlap between the range of intra- and interspecific sequence divergences of congeneric species. Mitochondrial DNA loci have been used extensively for phylogenetic analyses and species identifications in vertebrate taxa. Their utility for the study of closely related taxa is enhanced by their relatively rapid rates of evolution, very low rate of recombination, maternal

Table 1. GenBank accession numbers and sources of cytochrome *b* sequences for *Pterois*, *Dendrochirus* and the *Scorpaena* outgroup species included in the analyzed data sets.

Species	Source	Accession No.	Data Set	
			'long' (891 bp)	'short' (360 bp)
P. antennata	This study	FJ607313-16	+	+
	Kochzius et al. 2003	AJ429417-18		+
P. miles	Hamner et al. 2007	EF209664-76	+	+
	Kochzius et al. 2003	AJ429419-26		+
P. mombasae	Kochzius et al. 2003	AJ429427-8		+
P. radiata	This study	FJ607317-18	+	+
	Kochzius et al. 2003	AJ429429-30		+
P. volitans	Hamner et al. 2007	DQ482583-607	+	+
	Kochzius et al. 2003	AJ429431-3		+
D. biocellatus	This study	FJ607319-20	+	+
D. brachypterus	This study	FJ607321-23	+	+
	Kochzius et al. 2003	AJ429412-4		+
D. zebra	This study	FJ607324-26	+	+
	Kochzius et al. 2003	AJ429415-6		+
S. elongata	FishTrace[1]	EF456020	+	+

[1] Gonzalez-Sevilla et al. FishTrace: Genetic catalogue, biological reference collections and online database of European marine fishes (www.fishtrace.org).

inheritance, and reduced effective population size (Avise et al. 1983; Moritz et al. 1987; Piganeau et al. 2004).

Kochzius et al. (2003) analyzed partial mitochondria-encoded cytochrome *b* and 16S rDNA sequence data for native range specimens of *P. miles*, *P. volitans* and five other *Pterois* and *Dendrochirus* species. The results showed that their samples of *P. miles* and *P. volitans* were clearly separate, but it was impossible to determine if this separation was a product of *P. miles* and *P. volitans* representing distinct species or a product of sampling distant geographic populations of a single species. Reciprocal monophyly in the latter case may not reflect species status as demonstrated for the butterfly fishes *Dascyllus albisella* and *D. trimaculatus* (Bernardi and Crane 1999; McCafferty et al. 2002). Hamner et al. (2007) sequenced a larger portion of cytochrome *b* from the western Atlantic invasive lionfish population and also additional native range specimens of *P. miles* and *P. volitans*, and concluded that *P. miles* and *P. volitans* are distinct species and that both are present within the western Atlantic. However, the reasoning behind these conclusions was not explicitly discussed. We analyzed the sequence data of Hamner et al. (2007), Kochzius et al. (2003) and that newly generated from additional specimens of *Pterois* and *Dendrochirus* in order to better assess the taxonomic status of *P. miles* and *P. volitans*.

METHODS

Muscle tissue samples of *Pterois* and *Dendrochirus* species from their native range in the Indian and Pacific oceans were obtained for fish purchased from LiveAquaria.com (www.liveaquaria.com). Muscle tissue samples from lionfishes caught in the Atlantic Ocean were obtained as part of 2004–2006 lionfish surveys off the North Carolina coast. 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). Extractions were further purified using a QIAquick PCR Purification Kit and protocol (Qiagen Inc., Valencia, CA). Molecular delineation of *P. miles* and *P. volitans* was addressed by phylogenetic analyses of partial mitochondria-encoded cytochrome *b* DNA sequences. The locus was amplified using HotStar Taq (Qiagen Inc., Valencia, CA, USA) or GoTaq (Promega, Maddison, WI, USA)

Table 2. Uncorrected pairwise distances (%) between 891 base pairs of cytochrome *b* sequences from specimens of *Pterois* and *Dendrochirus* species.

	P. volitans	*P. miles*	*P. atennata*	*P. radiata*	*D. biocellatus*	*D. brachypterus*	*D. zebra*
P. volitans	0.11–2.13						
P. miles	6.35–7.74	0.11–0.79					
P. atennata	14.37–15.32	15.15–16.47	0.67–2.45				
P. radiata	15.60–16.50	15.60–16.05	5.95–6.82	0.79			
D. biocellatus	17.96–18.74	17.28–17.90	16.84–17.76	16.95–17.40	0.11		
D. brachypterus	15.71–16.72	15.94–16.50	15.94–16.60	17.28–17.62	19.75–19.98	0.22–0.45	
D. zebra	14.37–15.65	15.04–16.69	11.45–12.81	12.80–13.71	19.08–19.53	18.07–18.24	0.26–1.04

DNA polymerases with the reaction mixture and thermocycling protocol outlined in Freshwater et al. (2000). Oligonucleotide primers for cytochrome *b* included the previously published 'cytb L' and 'cytb H' (Schmidt and Gold, 1993), and one designed specifically for lionfishes by Hamner et al. (2007), R1063: 5′-TAA TGA A(CT)G G(AG)T G(GC)G AGA CA-3′. Amplification products were sequenced in both directions using the BigDye v.3 sequencing kit and protocol (Applied Biosystems, Foster City, CA, USA), and individual sequence reactions were combined and edited using Sequencher (Gene Codes Corp., Ann Arbor, MI, USA). Data sets including the newly generated sequences plus those available in GenBank (Table 1) were compiled and aligned in MacClade (Maddison and Maddison 2000).

Two different DNA sequence alignments were analyzed. The first ('long') included 891 base pairs of cytochrome *b* sequence from 14 specimens of *Pterois* and *Dendrochirus* generated during this study plus the Hamner et al. (2007) *P. miles* and *P. volitans* haplotype sequences (50 total sequences). The second data file ('short') included 360 base pairs of cytochrome *b* sequence from these same specimens plus the Kochzius et al. (2003) *Pterois* and *Dendrochirus* haplotype sequences (68 total sequences). Analyses of the 'long' data file included distance and parsimony methods as implemented in PAUP* (Swofford 2002) and Bayesian analyses using MrBayes (Ronquist and Huelsenbeck 2003). Modeltest (v.3.6, Posada and Crandall 1998) was used to determine that a Tamura-Nei plus invariant sites model was the best fit for sequence evolution, and this model was applied to correct distances during neighbor-joining distance tree construction. The neighbor-joining distance analysis was subjected to 5000 replications of bootstrap resampling. A parsimony bootstrap analysis with 500 replications of simple sequence addition and tree bisection reconnection branch swapping was performed, and Bayesian clade probability values were determined by an analysis run under a codon model with four chains for 317,000 generations, sampling every 50 generations and using a 1347 tree burnin value. The 'short' data file was submitted to distance and Bayesian analyses. The distance analysis consisted of neighbor-joining tree building with Tamura-Nei corrected distances and 5000 bootstrap replications. Bayesian clade probability values were based on an analysis run under a codon model with four chains for 336,000 generations, sampling every 100 generations, and using a 1000 tree burnin value.

RESULTS AND DISCUSSION

All tree-building methods resulted in the same basic topology when the 'long' data set was analyzed (Fig. 1). *Pterois miles* and *P. volitans* haplotypes were resolved as strongly supported but separate sister clades. Specimens of *P. miles* from Africa and western Indonesia were in the same clade and some shared the same haplotype. Likewise, specimens of *P. volitans* from both western Indonesia and the Philippines were in the same clade and sometimes also shared the same haplotypes. Sympatric specimens identified as *P. miles* or *P. volitans* from western Indonesia were never resolved in the same clade. Other analyzed specimens of *Pterois* and *Dendrochirus* species were distributed in three strongly supported lineages. The three specimens of *D. brachypterus* and the two specimens of *D. biocellatus* terminated two of the three lineages. The third lineage includes three clades corresponding to the species *P. antennata*, *P. radiata*, and *D. zebra*; with *P. antennata* and *P. radiata* resolved as sister species. Similar to specimens of *P. miles* and *P. volitans*, specimens of *P. antennata* from Tahiti were resolved in the same clade as specimens from Africa, and distinct from Tahiti *P. radiata* specimens with which they are sympatric.

These relationships are also reflected in the pairwise sequence divergences calculated for the analyzed specimens (Table 2). Intraspecific divergence values for *P. miles* specimens from Africa and western Indonesia, and *P. volitans* specimens from western Indonesia and the Philippines, ranged from 0.11% to 2.13% and 0.11% to 0.79% respectively. Interspecific divergence values for *P. miles* and *P. volitans* specimens ranged from 6.35% to 7.74% despite some of these specimens being sympatric. Similar intraspecific and interspecific sequence divergences are found in comparisons of Africa and Tahiti *P. antennata* and Tahiti *P. radiata* specimens (Table 2). There is >4% difference between the maximum intraspecific and minimum interspecific divergences for these two sister species comparisons.

Distance and Bayesian analyses of the 'short' data set also resulted in similar trees (Fig. 2). The separation of *P. miles* and *P. volitans* in strongly supported sister clades was also resolved when the Kochzius et al. (2003) generated haplotype sequences from additional Indian and Pacific Ocean specimens were included. Specimens of *P. miles* from Africa, the Red Sea, Sri Lanka and western Indonesia formed a clade clearly distinct from that which contains specimens of *P. volitans* from western Indonesia, the Philippines, and Taiwan. Other examples of geographically distant specimens forming tight clades are seen in the additional *Pterois and Dendrochirus* species included in the analyses. Specimens of *D. zebra* from Taiwan and the 'Indo-Pacific' were resolved together in a strongly supported clade. The source of the 'Indo-Pacific' specimens suggested that fish of Indonesian origin were usually given this geographic identifier (LiveAquaria.com, pers. comm.) although the exact provenance of these specimens could

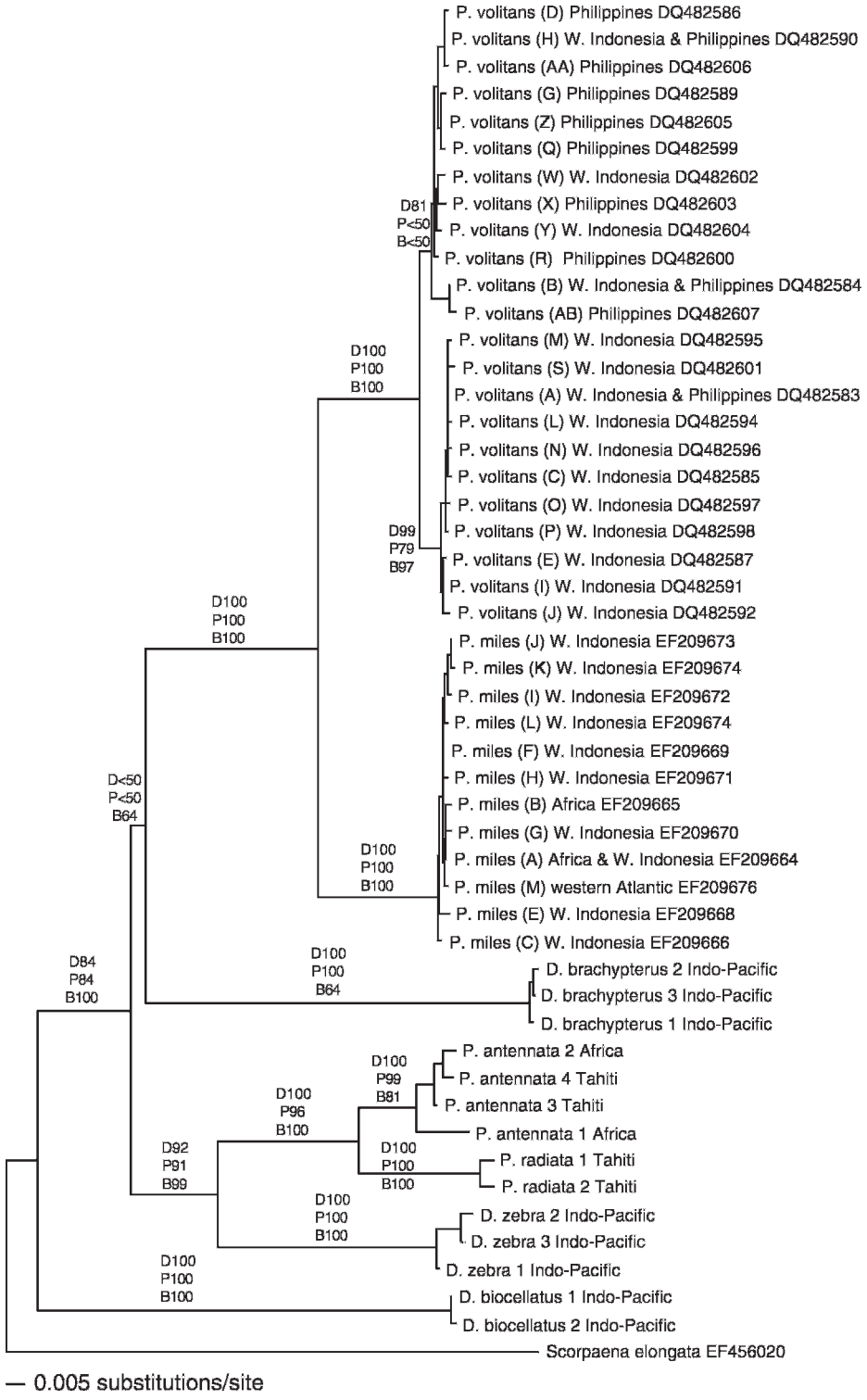

FIG. 1. Neighbor-joining distance tree resulting from analyses of partial cytochrome *b* sequences (891 base pairs) for 50 taxa. Bootstrap support (distance = D and parsimony = P), and Bayesian clade probability (B) values are shown above interspecific branches.

not be determined. Haplotype sequences generated for Tahiti *P. radiata* specimens formed a clade with the Kochzius et al. (2003) haplotypes from Red Sea *P. radiata*. Sequenced specimens of *P. antennata* from Africa and Tahiti are also resolved in a clade, however, the clade was only distantly related to that formed by the Kochzius et al. (2003) African *P. antennata* sequences. These latter sequences were resolved sister to a closely related clade containing two *P. mombasae* sequences. The *P. antennata* specimens we sequenced

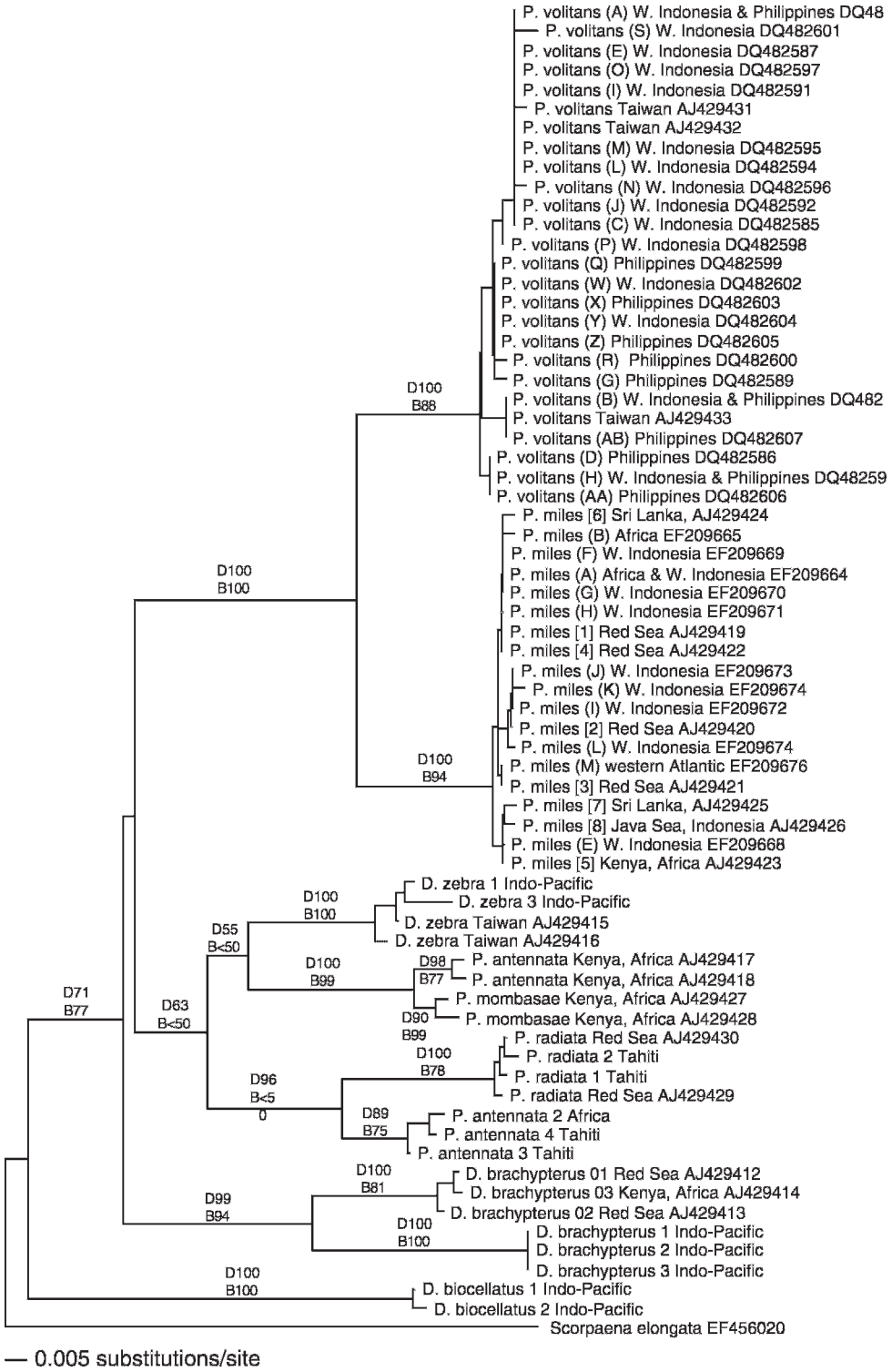

FIG. 2. Neighbor-joining distance tree resulting from analyses of partial cytochrome *b* sequences (360 base pairs) for 68 taxa. Distance bootstrap support (D) and Bayesian clade probability (B) values are shown above interspecific branches.

were purchased from an aquarium dealer and fit the meristic and color pattern characteristics reported for the species: XII dorsal spines; 11–12 dorsal rays; III anal spines; 6–7 anal rays, and 16–17 pectoral fin rays (De Beaufort and Briggs 1962; Eschmeyer 1986). Specimens identified as *P. antennata* and *P. mombasae* in Kochzius et al. (2003) were also purchased from aquarium dealers, however their meristic and color pattern characteristics were unknown. It may be that these latter specimens represented one species rather than two, as *P. antennata*

Table 3. Uncorrected pairwise distances (%) between 360 base pairs of cytochrome *b* sequences from specimens of *Pterois* and *Dendrochirus* species.

	P. volitans	*P. miles*	*P. atennata*	*P. 'atennata'*	*P. mombasae*	*P. radiata*	*D. biocellatus*	*D. brachypterus*	*D. zebra*
P. volitans	0.00–1.67								
P. miles	5.83–7.78	0.00–1.11							
P. atennata	12.78–14.44	13.89–15.56	0.56–0.83						
P. 'atennata'	14.44–15.32	15.00–15.60	8.33–9.19	0.56					
P. mombasae	14.17–15.28	14.72–15.56	8.33–9.17	1.94–2.23	0.83				
P. radiata	15.28–16.94	14.44–15.56	4.72–6.11	10.83–11.42	10.28–11.39	0.28–0.56			
D. biocellatus	17.78–19.17	16.94–18.06	15.00–15.83	15.88–16.39	15.28–15.83	14.44–15.28	0.278		
D. brachypterus	14.17–17.78	14.72–16.11	12.78–15.83	12.22–15.04	11.94–14.72	13.89–16.67	16.67–19.72	0.00–8.33[1]	
D. zebra	12.39–14.72	12.96–15.28	9.01–11.11	7.35–9.44	7.04–8.89	11.27–13.61	16.34–17.50	12.96–16.94	0.28–1.39

[1] Africa and Red Sea specimens divergences are 0.28–0.83%, 'Indo-Pacific' specimens divergences are 0.00%, and divergences between Africa/Red Sea and 'Indo-Pacific' specimens are 7.78–8.33%.

and *P. mombasae* are morphologically very similar. Conversely the different position of *P. antennata* specimens in the tree may indicate the presence of cryptic species under this name. Presence of cryptic species may also be indicated by the separation of specimens identified as *D. brachypterus* into two divergent clades. Kochzius et al. (2003) specimens from Africa and the Red Sea are resolved in one, while the three 'Indo-Pacific' specimens we sequenced form another. While the divergence between these clades may reflect the geographic separation of the included specimens, it is more than that seen between some even more widely separated geographic specimens of *P. miles*, *P. antennata*, *P. radiata*, and *D. zebra*.

Pairwise sequence divergences calculated for the sister species in Fig. 2 also reveal clear differences between the maximum intraspecific and minimum interspecific values (Table 3). Intraspecific divergence values for *P. miles* specimens from Africa, the Red Sea, Sri Lanka, and western Indonesia and *P. volitans* specimens from western Indonesia, the Philippines, and Taiwan were no more than 1.67%, while interspecific comparisons were all >5%. Sequences we generated for *P. antennata* specimens from Tahiti and Africa varied by <0.9% and sequences for *P. radiata* specimens from Tahiti and the Red Sea by <0.6%, while the minimum interspecific divergence between these sister species was 4.7%. Intraspecific divergences between 'Indo-Pacific' and Taiwan *D. zebra*, Kochzius et al. (2003) African *P. antennata* and *P. mombasae*, and 'Indo-Pacific' *D. biocellatus* specimens were all <1.39%. The range of sequence divergences between the Kochzius et al. (2003) *P. antennata* and *D. brachypterus*, and the *P. antennata* and *D. brachypterus* specimens we sequenced (8.33–9.19 and 7.78–8.33 respectively) are indicative of interspecific divergences and support the presence of cryptic species suggested by the phylogenetic analyses.

The reciprocal monophyly of *P. miles* and *P. volitans* specimens in phylogenetic trees (including those that are sympatric) as well as the clear difference between the maximum intraspecific and minimum interspecific sequence divergence of specimens identified as *P. miles* and *P. volitans* provides strong support for their recognition as separate species. The break between *P. miles* and *P. volitans* specimens indicates an absence of gene flow. Whereas adult lionfish are believed to remain within a relatively narrow geographic range (Fishelson 1975, 1997), the release of *Pterois* eggs within floating mucus balls and presence of a pelagic larval stage (Fishelson 1975; Imamura and Yabe 1996) provide a wide dispersal capability (Morris et al. 2009) and consequently the potential for extensive gene flow. Population genetic analyses of *P. miles* from the Gulf of Aqaba, Red Sea, and scattered Indian Ocean sites indicate panmixia among these geographically separate populations (Kochzius and Blohm 2005). Consequently, no divergence between sympatric specimens of *P. miles* and *P. volitans* would be expected if these taxa represented the same species.

Separation of an ancestral *Pterois* species into two allopatric lineages that evolved into *P. miles* and *P. volitans* may have begun in the mid to late Miocene when tectonic activity and sea level change combined to sever surface water connections between the Indian Ocean and western Pacific (Kennett et al. 1985; Haq et al. 1987; Hodell and Vayavananda 1993). This break between ocean basins is often cited as a cause of the diversification of Indo-Pacific marine organisms including fishes (e.g., McMillan and Palumbi 1995; Chenoweth et al. 1998; McCafferty et al. 2002), and was proposed by Kochzius et al. (2003) as the origin of the *P. miles*/*P. volitans* separation.

Our results also support the conclusion of Kochzius et al. (2003) that *Dendrochirus* and *Pterois* are not distinct genera as currently delimited. Eschmeyer and Randall (1975) questioned their status, and *Dendrochirus* has sometimes been treated as a synonym of *Pterois* (e.g., Klunzinger 1870; de Beaufort and Briggs 1962). The two are distinguished by characteristics of the pectoral fin rays. There are no branched pectoral rays in *Pterois* species, while some upper pectoral rays are branched in *Dendrochirus*, and *Pterois* species have upper pectoral

rays that are free from membrane but there are no rays free from membrane in *Dendrochirus* species (Smith 1957; Eschmeyer 1986). Taxonomic adjustments are needed for these two genera, however, molecular data for many of the included species are lacking, and the necessary changes must await a comprehensive reassessment of *Dendrochirus*, *Pterois* and the other member of the subfamily Pteroinae.

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