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TECHNICAL NOTE

Characterization of 18 polymorphic microsatellite loci from invasive lionfish (*Pterois volitans* and *P. miles*)

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Abstract Lionfish (*Pterois volitans* and *Pterois miles*) are the first non-native marine reef fish to become established in the Western North Atlantic and Caribbean Sea. Next-generation sequencing techniques were employed to identify 18 polymorphic microsatellite loci for *P. volitans* and *P. miles* from waters off North Carolina, USA. Allele frequencies for all 18 loci conformed to Hardy–Weinberg expectations after correction for multiple comparisons, the number of alleles ranged from 2 to 20 (mean = 7.1), and observed heterozygosities ranged from 0.200 to 0.938 (mean $H_o = 0.636$). All 18 loci cross-amplified DNAs from representative haplotypes of both *P. volitans* and *P. miles*, and the vast majority of alleles were shared. These are the first highly polymorphic nuclear markers described for invasive lionfish and will be useful for characterizing population connectivity and monitoring the progress of the invasion on reef habitats of the Western Atlantic.

Keywords Invasive species · Lionfish microsatellites · *Pterois volitans* · *Pterois miles*

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Indo-Pacific lionfishes (Scorpaenidae) are popular aquarium fish, and well known for their ornate beauty and venomous spines. Two species of the lionfish, *Pterois volitans* and *Pterois miles,* have invaded the Western Atlantic (Whitfield et al. 2002; Freshwater et al. 2009a; Morris 2012), and are a concern to coastal managers because of their threat to fisheries resources, native fish communities, and human health (Morris and Akins 2009; Morris and Whitfield 2009). The invasive range of lionfish has expanded annually (Schofield 2010) and it is likely that it will ultimately include the entire Caribbean, Gulf of Mexico, and subtropical western Atlantic (Ahrenholz and Morris 2010; Morris and Whitfield 2009).

Mitochondrial DNA sequence analyses of lionfish revealed that both *P. volitans* and *P. miles* haplotypes are present in the Atlantic with reduced haplotype diversity compared to their native ranges, and some population structuring related to range expansion and connectivity within the Caribbean (Hamner et al. 2007; Freshwater et al. 2009a, b; Betancur et al. 2011). Highly polymorphic nuclear markers are needed to further analyze genetic diversity and population connectivity among sites in the western Atlantic. To this end, next-generation sequencing techniques were used to identify 18 polymorphic microsatellite markers from lionfish collected off the coast of North Carolina.

Genomic DNA was extracted from ethanol preserved gill tissues using the Wizard SV Genomic DNA Purification System (Promega). Non-enriched genomic DNA was subjected to next-generation sequencing on a Roche 454 GS-FLX instrument (Roche) at the Duke University Institute for Genomic Science and Policy Genome Sequencing Facility. A total of 105,334 sequence reads were generated, 69,003 were >300 bp in length and were screened for repetitive elements using Msatcommander (Faircloth

2008). A total of 5,737 repetitive sequences were found and flanking primers designed with Primer3 software (Rozen and Skaletsky 2000). Tetrameric repeats (>6) with sufficient flanking sequence information for primer design were given priority.

Genomic DNA was amplified in 20 µl polymerase chain reactions (PCRs) as follows: 2 µl DNA, 2 µl 10× PCR Buffer (200 mM Tris, pH 8.8; 500 mM KCL; 0.1 % Triton X-100, 0.2 mg/ml BSA), 1.6 µl 25 mM $MgCl_2$, 1.6 µl 2.5 mM dNTP's, 0.2 µl 10 µM Forward primer, 0.8 µl 10 µM Reverse primer, 0.8 µl 10 µM labeled (FAM, NED, PET, or VIC) T3 primer (Eurofins; Applied Biosystems), and 0.2 µl Taq DNA polymerase. PCR products were indirectly labeled using Forward primers with 5′-T3 tags (ATTAACCCTCACTAAAGGGA; not shown in Table 1) and fluorescently labeled T3 primers. Reactions were run under the following conditions:

Table 1 Polymorphic microsatellite loci from *Pterois volitans* and *Pterois miles*

Locus	Repeat	Primers	Size range (bp)	N_A	Haplotype	H_{obs}	H_{exp}	*P* value
Pvm4	$(AGAT)_{12}$	F: GGATTCTTTCAGGGCAGGTT	262–298	8	Pv	0.625	0.853	0.025
		R: ACCATGACAGCATCATGACC			Pm	0.700	0.863	0.245
Pvm7	$(AGAT)_9$	F: ACTCTTCAATCCAGCCAACG	280–310	7	Pv	0.857	0.835	0.900
		R: AATTGGGACGTTTTGAGGTG			Pm	0.846	0.828	0.384
Pvm10	$(ATCT)_7$	F: CCCCCGTTATGTGTCTTTGT	240–260	6	Pv	0.742	0.581	0.193
		R: GGGATGTGTGTGTGGAGAGA			Pm	0.929	0.659	0.019
Pvm11	$(GGAT)_9$	F: TCTATGTGCCCTGTGATGGA	212–229	6	Pv	0.733	0.549	0.103
		R: GGGCCTGAATGATCATATTGTT			Pm	0.643	0.611	0.366
Pvm12	$(ACAG)_{11}$	F: TGGTTGGGACTATGCAGACA	190–246	20	Pv	0.700	0.824	0.091
		R: CCCACACTCAATACCAGCAC			Pm	0.938	0.815	0.040
Pvm14	$(AGAT)_{12}$	F: GGATTCTTTCAGGGCAGGTT	256–302	12	Pv	0.615	0.846	0.009
		R: TTGTGACCATGACAGCATCA			Pm	0.600	0.841	0.029
Pvm15	$(ATCC)_7$	F: CATGCATCCCTTCATATTTGC	210–260	7	Pv	0.885	0.733	0.460
		R: ATATCATGCACCGCTGTCAA			Pm	0.688	0.728	0.404
Pvm17	$(GATT)_9$	F: CACAGCTCAGTCGAATCCAG	236–264	8	Pv	0.793	0.615	0.134
		R: GCAAACAGGCTGCTAAGGTC			Pm	0.875	0.631	0.012
Pvm18	$(GGAT)_9$	F: ATGGGGGTCTATGGGATTTC	221–268	5	Pv	0.645	0.508	0.160
		R: TGAGGCTGATGTTGAAGTGC			Pm	0.625	0.508	0.608
Pvm21	$(AGAT)_{11}$	F: GACAGCTGTTGTCGCCTGT	185–261	10	Pv	0.710	0.678	0.096
		R: GAGGGCTCACACACTGGATT			Pm	0.625	0.665	0.517
Pvm27	$(AAT)_{10}$	F: TTTTGGTTGCAGATCACAGC	229–297	10	Pv	0.481	0.605	0.018
		R: ACACAGCGTCTCTGGTCCAT			Pm	0.692	0.735	0.719
Pvm31	$(ACT)_9$	F: TTGGTCCTCCATTTCTGAGG	176–221	9	Pv	0.613	0.671	0.148
		R: AGCCTCACTGAGTCCACCAT			Pm	0.500	0.579	0.178
Pvm32	$(ATC)_{10}$	F: TCAATCACACCGTCAAGAGC	202–205	2	Pv	0.276	0.242	1.000
		R: CATGTATTTACACTGGACCTTTCC			Pm	0.308	0.271	1.000
Pvm37	$(AAT)_9$	F: TGAATCGTTTTGCCTCTGTCT	236–251	6	Pv	0.345	0.563	0.011
		R: CAAACATTTCCCACGTACCC			Pm	0.200	0.468	0.046
Pvm38	$(AGG)_9$	F: GCTCCGACTGTGTGTGTGTT	302–308	3	Pv	0.500	0.605	0.277
		R: GCAGGCAGGAAGACAGAGAG			Pm	0.769	0.520	0.075
Pvm41	$(GAT)_{11}$	F: CCTGCAGATGACCCTGATTT	250–262	2	Pv	0.567	0.481	0.442
		R: AGACGGAAAACAGCAGAGGA			Pm	0.714	0.519	0.280
Pvm42	$(ATC)_{11}$	F: GTGTGTCAGACGCTGAAGGA	227–236	3	Pv	0.677	0.582	0.780
		R: ACGTACAGCGGGTTAGGATG			Pm	0.467	0.559	0.797
Pvm46	$(GACTT)_9$	F: CTCTTCCCGAGGTTTCTTCC	241–262	4	Pv	0.581	0.580	0.219
		R: AGCGAATACAGCAGCACCTT			Pm	0.438	0.538	0.308

N_A represents number alleles observed; H_{obs} and H_{exp} are observed and expected heterozygosities respectively; p value is the probability of significant deviations between observed and expected heterozygosities; Haplotype indicates *Pterois volitans* (Pv) or *Pterois miles* (Pm)

94 °C 4 min; 25 cycles of 94 °C 15 s, 62 °C 15 s, 72 °C 30 s; 8 cycles of 94 °C 15 s, 53 °C 15 s, and 72 °C 30 s; final extension at 72 °C for 5 min. All amplifications were performed using a single standard condition.

PCR products were diluted 1:3 and 2 µl mixed with 0.05 µl DNA Orange (MCLab), 0.05 µl 10 mg/ml salmon sperm DNA, and 8.95 µl water, denatured at 95 °C for 10 min, and chilled on ice. Size-fragment analysis was conducted on an ABI 3730xl DNA Analyzer (Applied Biosystems), and chromatograms scored using Genemarker v1.8 (SoftGenetics). Deviations from Hardy–Weinberg Equilibrium were calculated using Arlequin v3.2 (Excoffier and Lischer 2010). Heterozygote excess, heterozygote deficiency and linkage disequilibrium were tested with Genepop version 4.0.10 (Rousset 2008) and corrected for multiple comparisons using the sequential Bonferroni approach (Rice 1989). Presence of null alleles, stutter, and large allele dropout were assessed using MicroChecker (1,000 randomizations: Van Oosterhout et al. 2004).

Forty-eight primer pairs were screened for robust amplification using DNA from four individual lionfish samples representing both *P. volitans* and *P. miles* haplotypes. Primers showing strong polymorphic products were then used to amplify DNAs from another 74 individuals from multiple locations (North Carolina, Bahamas, and Florida). Results from 18 loci scored on a minimum of 35 individuals are presented in Table 1. The number of alleles ranged from 2 to 20 with a mean of 7.1 alleles per locus. The allele frequencies of all 18 loci conformed to Hardy–Weinberg expectations after correcting for multiple comparisons using the sequential Bonferroni method ($k = 18$; Rice 1989). MicroChecker indicated the presence of null alleles at three loci (Pvm4, Pvm14, and Pvm37), while no loci showed evidence of scoring errors due to stuttering or large allele dropout. Arlequin detected evidence for linkage disequilibrium in pairwise comparisons among loci in both *P. volitans* (22) and *P. miles* (28) populations.

The lionfish invasion provides an excellent, albeit unfortunate, natural experiment of population connectivity within the tropical and subtropical western Atlantic. Betancur et al. (2011) used mitochondrial haplotype analysis and the chronological progression of the invasion to test hypotheses of connectivity and breaks within the Caribbean. The resolving power of these haplotype data however are limited, especially for the invasive lionfish where strong initial and secondary founder effects are present (Hamner et al. 2007; Freshwater et al. 2009b; Betancur et al. 2011). These microsatellite markers will provide much greater resolution for exploring the invasion's expansion and connectivity among marine organisms.

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