Microsatellite multiplex panels for genetic studies of gray snapper (*Lutjanus griseus*) and lane snapper (*Lutjanus synagris*)

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Microsatellites are codominantly inherited nuclear-DNA markers (Wright and Bentzen, 1994) that are now commonly used to assess both stock structure and the effective population size of exploited fishes (Turner et al., 2002; Chistiakov et al., 2006; Saillant and Gold, 2006). Multiplexing is the combination of polymerase chain reaction (PCR) amplification products from multiple loci into a single lane of an electrophoretic gel (Olsen et al., 1996; Neff et al., 2000) and is accomplished either by co-amplification of multiple loci in a single reaction (Chamberlain et al., 1988) or by combination of products from multiple single-locus PCR amplifications (Olsen et al., 1996). The advantage of multiplexing microsatellites lies in the significant reduction in both personnel time (labor) and consumable supplies generally required for large genotyping projects (Neff et al., 2000; Renshaw et al., 2006).

In this note, we report the development of multiplex panels of microsatellites that will facilitate population-level genetic studies of both gray (*Lutjanus griseus*) and lane (*L. synagris*) snappers. The overexploitation of Gulf red snapper (*L. campechanus*) in U.S. waters and the increasing restrictions on both commercial and recreational red snapper catches (Gillig et al., 2001) have led to increased fishing pressure on other snapper species (Fischer et al., 2005), including both gray (Burton, 2001; Fischer et al., 2005) and lane snappers (GMFMC[1]). Although neither species has yet been classified as "overfished" or subject to "overfishing," the increased exploitation of the two species could jeopardize these snapper resources in the future. In this study, we optimized multiplex panels for gray and lane snappers from among microsatellite markers designed originally for red snapper by Gold et al. (2001) and vermillion snapper (*Rhomboplites aurorubens*) by Bagley and Geller (1998).

Materials and methods

Samples of gray and lane snappers were obtained off the west coast of Florida during April of 2004. Fin clips and pieces of liver were preserved in 95% ethanol, brought to the laboratory, and stored at room temperature. Genomic DNA was extracted by using an alkaline-lysis method (Saillant et al., 2002) and stored at –20°C.

Microsatellites were first evaluated in single-locus (simplex) reactions in order to determine the size range and ease of scoring of PCR products in each species. PCR amplifications were performed in 11.5-μL volumes comprising 1.5 μL of DNA (approximately 50 ng), 1 μL of 10× reaction buffer [50 mm KCl, 10 mm Tris, 1% Triton-X 100], 0.75 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), 200 μm of each dNTP, 1 mm $MgCl_2$, and various quantities of PCR primers. One PCR primer of each pair was labeled with one of three fluorescent dyes from set D (Applied Biosystems, Foster City, CA): FAM, HEX, or NED. Fragment analysis was carried out on an ABI PRISM 377 automated DNA sequencer (Applied Biosystems, Foster City, CA). Allele size was estimated by using the GENESCAN-400HD [ROX] size standard (Applied Biosystems, Foster City, CA); allele size estimation was performed with GENESCAN 3.1.2 (Applied Biosystems, Foster City, CA) and allele calling was performed in GENOTYPER, version 2.5 (Applied Biosystems, Foster City, CA). Multiplex tests were performed only on those microsatellites that amplified successfully and yielded PCR products that were easy to score; loci not meeting these criteria were eliminated from subsequent analyses.

Initial multiplex PCR amplifications were performed by using the three multiplex panels designed to amplify 20 microsatellite loci in red snapper (Renshaw et al., 2006). At first, PCR primer concentrations followed those outlined in Renshaw et al. (2006), and changes were made according to the relative success of amplifications at each microsatellite within a particular multiplex. Microsatellites that failed to amplify in multiplex reactions were optimized in simplex reactions. Additional PCR

[1] GMFMC (Gulf of Mexico Fishery Management Council). 2005. Final amendment to the FMPs for: reef fish (Amendment 25) and coastal migratory pelagics (Amendment 17) for extending the charter vessel/headboat permit moratorium (including SEIS/RIR/IRFA), 111 p. Gulf of Mexico Fishery Management Council, 3018 North U.S. Highway 301, Suite 1000, Tampa, FL 33619–2272.

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Table 1

Technical details on amplification of microsatellite loci in gray snapper (*Lutjanus griseus*) and lane snapper (*L. synagris*). Included in the table are the multiplex polymerase chain reaction (PCR) panel, primer quantities, and fluorescent dye labels (Set D; Applied Biosystems, Foster City, CA). PCR protocols, including appropriate annealing temperatures, correspond to those outlined in Renshaw et al. (2006). Primer sequences for *Lca* and *Prs* microsatellites are given in Gold et al. (2001); those for *Ra* microsatellites are given in Bagley and Geller (1998).

Species	Panel	Microsatellite	Quantity (pmol)	ABI dye	PCR protocol
Lutjanus griseus	1	*Lca* 20	5	FAM	Touchdown II
		Lca 43	4	FAM	56°
		Prs 260	1.5	FAM	53°
		Ra 1	3.5	HEX	50°
	2	*Prs* 137	7	FAM	Touchdown II
		Prs 275	6	FAM	54°
		Prs 328	0.8	FAM	52°
		Ra 6	2	NED	50°
	3	*Lca* 22	2.5	FAM	Touchdown I
		Lca 91	8	FAM	56°–50.5°
		Lca 107	6.5	HEX	50°
	simplex	*Prs* 221	5	HEX	Same as panel 2
	simplex	*Prs* 240	5	HEX	Same as panel 3
	simplex	*Ra* 7	5	NED	Same as panel 3
Lutjanus synagris	1	*Lca* 20	5	FAM	Touchdown II
		Prs 248	5	NED	52°
		Prs 260	1.5	FAM	50°
		Prs 303	1.5	NED	48°
		Ra 1	7.5	NED	
		Ra 4	5	HEX	
	2	*Prs* 275	4	FAM	Touchdown II
		Prs 328	1	FAM	52°
		Ra 2	3.5	FAM	50°
		Ra 6	3	NED	48°
	3	*Lca* 22	1	FAM	Touchdown II
		Lca 91	8	FAM	52°
		Prs 240	9	HEX	50°
		Prs 333	3	HEX	48°
		Ra 7	5	NED	

primers (*Ra* 1, *Ra* 2, and *Ra* 4) designed by Bagley and Geller (1998) for vermillion snapper microsatellites were tested in simplex reactions as described above and labeled with one fluorescent dye to allow integration into one of the three multiplex panels.

Multiplex PCR amplifications initially followed the Touchdown I and II protocols outlined in Renshaw et al. (2006). Touchdown PCR protocols enable the amplification of multiple loci with different optimal annealing temperatures. The former (Touchdown I) protocol involved a one-half degree drop in annealing temperature with each subsequent cycle, for a total of 12 cycles, followed by 30 cycles at a bottom annealing temperature that was 6°C below the initial annealing temperature; the latter (Touchdown II) protocol featured a three-step drop in annealing temperature with seven cycles at an initial annealing temperature, followed by seven cycles at a lower annealing temperature, followed by 28 cycles at a bottom annealing temperature that was 4–6°C below the initial annealing temperature. Changes were made to optimize multiplex panels individually for each species. These changes included 1) removal of various primers and addition of others, and 2) modification of annealing temperatures and species-specific differences in primer concentrations.

Once multiplex or simplex protocols were developed for the two species, DNA from 28 individuals of each species was assayed across all optimized loci. Genetic variability of microsatellites in each species was measured by the number of alleles, expected heterozygosity (gene diversity), and observed heterozygosity. Fisher's exact test was used to evaluate loci for significant departures from Hardy-Weinberg equilibrium expectations. Three common causes for genotyping errors—null alleles, large allele dropout, and stuttering—were assessed with MICRO-CHECKER (Van Oosterhout et al., 2004).

Table 2

Summary data for microsatellites amplified from 28 gray snapper (*Lutjanus griseus*) and 28 lane snapper (*L. synagris*). Data for each microsatellite include number of alleles (N_A), size range (in base pairs) of detected alleles, expected (H_E) and observed (H_O) heterozygosity, and probability (P_{HW}) of conformity to Hardy-Weinberg equilibrium expectations. *Lca* and *Prs* microsatellites were developed initially for red snapper (*L. campechanus*) (Gold et al., 2001), and *Ra* microsatellites were developed initially for vermillion snapper (*Rhombloplites aurorubens*) (Bagley and Geller, 1998).

Species	Microsatellite	N_A	Size range	H_E/H_O	P_{HW}
Lutjanus griseus	*Lca* 20	4	216–222	0.707/0.643	0.721
	Lca 22	5	242–252	0.593/0.500	0.050
	Lca 43	2	176–188	0.195/0.214	1.000
	Lca 91	2	134–136	0.036/0.036	1.000
	Lca 107	6	112–144	0.771/0.750	0.266
	Prs 137	7	127–141	0.794/0.679	0.075
	Prs 221	4	235–245	0.566/0.714	0.448
	Prs 240	4	210–220	0.642/0.679	0.963
	Prs 260	9	105–129	0.847/1.000	0.060
	Prs 275	5	156–168	0.548/0.571	0.343
	Prs 328	3	202–206	0.527/0.464	0.842
	Ra 1	7	149–171	0.594/0.571	0.081
	Ra 6	15	113–155	0.926/0.679	0.000
	Ra 7	3	154–158	0.200/0.143	0.127
Lutjanus synagris	*Lca* 20	12	234–262	0.810/0.571	0.009
	Lca 22	8	234–256	0.712/0.679	0.257
	Lca 91	7	139–153	0.601/0.679	0.263
	Prs 240	5	192–202	0.592/0.538	0.484
	Prs 248	17	224–260	0.938/0.893	0.402
	Prs 260	4	92–120	0.292/0.321	1.000
	Prs 275	2	154–156	0.036/0.036	1.000
	Prs 303	4	135–141	0.690/0.643	0.724
	Prs 328	5	198–214	0.631/0.571	0.499
	Prs 333	12	152–180	0.870/0.821	0.327
	Ra 1	9	141–163	0.806/0.893	0.587
	Ra 2	6	82–100	0.704/0.821	0.972
	Ra 4	9	59–99	0.787/0.821	0.660
	Ra 6	4	118–124	0.384/0.464	0.770
	Ra 7	4	177–187	0.612/0.536	0.578

Results and discussion

A total of 14 microsatellites (11 in three multiplex panels and 3 in simplex reactions) were optimized for gray snappers, and 15 microsatellites (in three multiplex panels) were optimized for lane snappers. The multiplex and simplex formats are presented in Table 1 and include for each microsatellite the fluorescent label (Applied Biosystems, Foster City, CA), primer quantity, PCR protocol, and optimized annealing temperatures. Genotype summary data from 28 individuals (in both species) are given in Table 2; data for each microsatellite include number of alleles, size range of alleles detected, expected and observed heterozygosity, and probability of conformity to Hardy-Weinberg equilibrium expectations.

For gray snappers, the number of alleles per microsatellite ranged from two (*Lca* 43 and *Lca* 91) to 15 (*Ra* 6), and expected and observed heterozygosity per microsatellite ranged from 0.036 (*Lca* 91) to 0.926 (*Ra* 6) and from 0.036 (*Lca* 91) to 1.000 (*Prs* 260), respectively. Only one microsatellite (*Ra* 6) deviated significantly ($P<0.05$) from Hardy-Weinberg equilibrium expectations after sequential Bonferroni correction (Rice, 1989). Analysis with MICRO-CHECKER (Van Oosterhout et al., 2004) indicated the possible occurrence of null alleles at *Ra* 6.

For lane snappers, the number of alleles per microsatellite ranged from two (*Prs* 275) to 17 (*Prs* 248), and expected and observed heterozygosity per microsatellite ranged from 0.036 (*Prs* 275) to 0.938 (*Prs* 248) and from 0.036 (*Prs* 275) to 0.893 (*Prs* 248 and *Ra* 1), respectively. None of the 15 microsatellites deviated significantly from Hardy-Weinberg equilibrium expectations after sequential Bonferroni correction (Rice, 1989). Analysis with MICRO-CHECKER indicated the possibility of null alleles at one microsatellite (*Lca* 20) where the probability

of deviation from Hardy-Weinberg equilibrium expectations was significant (P=0.009) before Bonferroni correction. One locus, *Lca* 91, exhibited a one base-pair shift (rather than the two base-pair shifts expected from dinucleotide repeats) in samples of lane snapper; this difference in shift could generate scoring problems in future genotyping efforts.

The costs of large genotyping projects can be substantially reduced through the combination of multiple loci in single PCR amplifications (Renshaw et al., 2006). The reduction in costs could enable researchers to increase sample sizes, possibly allowing for the inclusion of additional year classes for temporal studies or could extend a proposed sampling range to include more of the geographic distribution of a species. The multiplex panels and simplex reactions developed in the present study will provide critical tools for future population-level genetic studies designed to identify fishery management units and to monitor changes in genetic effective population size for both gray and lane snappers.

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