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Technical note

Microsatellite multiplex panels for genetic studies of three species of marine fishes: red drum (*Sciaenops ocellatus*), red snapper (*Lutjanus campechanus*), and cobia (*Rachycentron canadum*)

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Abstract

Multiplex panels of nuclear-encoded microsatellites were developed for three species of marine fishes of interest to both public and private aquaculture ventures: red drum (*Sciaenops ocellatus*), red snapper (*Lutjanus campechanus*), and cobia (*Rachycentron canadum*). The multiplex panels will be useful in a variety of applications, including strain and hybrid identification, parentage assignment, pedigree reconstruction, estimating genetic diversity and/or inbreeding, mapping of quantitative trait loci, and marker-assisted selection. The panels also will be useful in studies of stock structure of 'wild' populations. Comparison of costs for expendable supplies revealed that four- and eight-panel multiplexes reduced expenditures four- and eight-fold, respectively, relative to single microsatellite gels. Personnel time also was reduced significantly.
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Keywords: Microsatellites; Multiplexes; Aquaculture; Red drum; Red snapper; Cobia

1. Introduction

Microsatellites are hypervariable, nuclear-encoded genetic markers that are widely used in a variety of aquaculture applications, including strain and hybrid identification, parentage assignment, pedigree reconstruction, estimating genetic diversity and/or inbreeding, mapping of quantitative trait loci, and marker-assisted selection (Liu and Cordes, 2004). The large number of microsatellites needed for many of these applications can often generate high costs due to materials and personnel time. Major cost reduction, however, can be achieved through multiplexing, the combination of polymerase chain reaction (PCR) amplification products from multiple microsatellites into a single lane of an electrophoretic gel (Olsen et al., 1996; Neff et al., 2000). Multiplexing can be accomplished through either co-amplification of multiple microsatellites in a single PCR cocktail (Chamberlain et al., 1988) or combination of PCR products from multiple, single amplification reactions (Olsen et al., 1996). A blend of the two approaches can optimize sample throughput (Devey et al., 2002; Paterson et al., 2004). Despite its cost-effectiveness multiplexing is often not employed, apparently because of a general apprehension that it increases complexity of microsatellite genotyping (Neff et al., 2000).

In this technical report, we describe simple and straightforward protocols for multiplexing microsatellites

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in each of three species of marine fishes of interest to public and private (commercial) aquaculture: red drum (*Sciaenops ocellatus*), red snapper (*Lutjanus campechanus*), and cobia (*Rachycentron canadum*). Briefly, red drum is an estuarine-dependent sciaenid found primarily along the Atlantic and Gulf coasts of the southern United States (U.S.). Culture, including breeding, of red drum is relatively advanced (Lee and Ostrowski, 2001). Extensive use by resource management agencies of hatchery-raised red drum to enhance 'wild' stocks occurs in several southern states (Grimes, 1998; Smith et al., 2001), especially Texas (McEachron et al., 1995). Commercial (private) aquaculture of red drum also occurs in the southern U.S. (Lee and Ostrowski, 2001), and in China, Israel, and Taiwan as well (Lutz, 1999; Hong and Zhang, 2003). Culture of red snapper, a structure- or reef-associated lutjanid found primarily along the continental shelf of the Gulf of Mexico (Hoese and Moore, 1977), is far less advanced. Wild-caught adults have been successfully strip-spawned in captivity following hormone inducement (Lee and Ostrowski, 2001; Riley et al., 2004), and efforts to optimize aquaculture protocols to mass produce fingerlings for stock enhancement and commercial aquaculture are underway (Chigbu et al., 2002; Riley et al., 2004). In addition, the grow-out and market potential for red snapper make it ideal for offshore cage culture (Bridger and Costa-Pierce, 2002). Cobia is a nearly cosmopolitan, pelagic species found in tropical and subtropical waters (Shaffer and Nakamura, 1989). Like red snapper, cobia is relatively new to aquaculture, although in Taiwan cobia is now a very popular and profitable farmed marine fish (Liao et al., 2004). Recent success in captive spawning and progeny production in the U.S. (Dodd, 2001) has generated considerable interest in commercial aquaculture of cobia (Lee and Ostrowski, 2001; Smith et al., 2001).

PCR primer sets for microsatellites in all three species were developed previously for use in stock-structure analysis of 'wild' populations: red drum (Saillant et al., 2004); red snapper (Bagley and Geller, 1998; Gold et al., 2001), and cobia (Pruett et al., 2005; Renshaw et al., in press). Subsets of these microsatellites were used to develop protocols for the microsatellite multiplex panels described in this report.

2. Materials and multiplex protocols

An alkaline-lysis method (Saillant et al., 2002) was used to purify DNA from tissue samples (fin clips or internal organs) that had been obtained for prior studies in our laboratory. All tissues had been fixed in 95% ethanol and stored at room temperature.

The initial step in designing multiplex protocols typically involves generating PCR sequences for individual microsatellites, optimizing annealing temperatures, and determining the approximate range in allele size that might be encountered for a given microsatellite. Because these data already were available for red drum, red snapper, and cobia (Gold et al., 2001; Saillant et al., 2004; Renshaw et al., in press), the initial effort was conceptual and involved combining individual microsatellites with similar, optimal annealing temperatures and assessing whether allele-size ranges overlapped and whether alternate ABI dyes could be employed when overlap occurred. Initial experiments in each species involved testing sets of eight to ten primers in 'mega-cocktails' where equimolar (5 pmol) amounts of each primer pair were run in the same PCR reaction. Other PCR reagents (buffer, magnesium, dNTPs, *Taq* polymerase) in the 'mega-cocktails' initially followed PCR procedures outlined in Saillant et al. (2004), Gold et al. (2001), and Renshaw et al. (in press) for red drum, red snapper, and cobia, respectively. One primer (either forward or reverse) from each pair was labeled with one of three different Applied Biosystems (ABI) fluorescent dyes of set D (6-FAM, HEX, or NED). Microsatellites with non-overlapping allele-size ranges were labeled with the same fluorescent dye, whereas those with overlapping allele-size ranges were labeled with different dyes. Fragment analysis was carried out on an automated ABI-377 sequencer; fragments were sized using GENESCAN 3.1.2, and allele calling was performed using GENOTYPER® version 2.5.

Optimization of multiplex protocols began with screening of microsatellites that amplified in the 'mega-cocktails.' Primer pairs that failed to amplify were removed from the mix and further optimization focused on the remaining primer pairs; this led to six sets (hereafter multiplex panels or panels) of red drum microsatellites, three of red snapper microsatellites, and five of cobia microsatellites. Two 'touchdown' PCR protocols (I and II) were evaluated for each of the 14 panels. Touchdown protocols are used to amplify microsatellites with different, optimal annealing temperatures in the same PCR reaction via progressively reducing annealing temperature in successive annealing cycles (Rithidech et al., 1997; Fishback et al., 1999). The Touchdown I protocol featured a one-half degree (Celsius) reduction in annealing temperature at each of 12 cycles, as described by Fishback et al. (1999), followed by 30 cycles of amplification at a temperature (hereafter, 'bottom' temperature) six degrees below the starting annealing temperature. Initial evaluation started at the highest (optimal) annealing temperature for a

Table 1
Multiplex panels for red drum (*Sciaenops ocellatus*) developed from 31 microsatellite primers

Panel	Microsatellite	Quantity	ABI dye	Range in allele size	Touchdown protocol
1	*Soc*412	4.3	HEX	102–168	Touchdown I
	*Soc*416	3.2	NED	141–181	55°–49.5°
	*Soc*417	0.7	6-FAM	86–112	49°
	*Soc*423	0.6	6-FAM	172–208	
	*Soc*428	2.7	HEX	172–242	
	*Soc*445	35	6-FAM	134–166	
2	*Soc*60	2.2	6-FAM	151–163	Touchdown I
	*Soc*140	0.5	NED	132–144	55°–49.5°
	*Soc*201	9	HEX	224–243	49°
	*Soc*243	2	6-FAM	94–106	
	*Soc*419	1.4	6-FAM	238–260	
3	*Soc*19	5	6-FAM	195–267	Touchdown I
	*Soc*85	2.3	6-FAM	80–122	60°–54.5°
	*Soc*138	4.5	HEX	77–123	54°
	*Soc*156	0.4	6-FAM	168–184	
	*Soc*206	0.8	NED	246–265	
	*Soc*410	2.7	6-FAM	306–344	
4	*Soc*11	1.6	6-FAM	217–240	Touchdown II
	*Soc*83	9	HEX	114–142	62°
	*Soc*99	2.2	NED	131–209	56°
	*Soc*407	9	6-FAM	139–157	54°
	*Soc*424	10	HEX	204–230	
5	*Soc*400	3	6-FAM	245–266	Touchdown I
	*Soc*402	6	HEX	134–164	55°–49.5°
	*Soc*404	4	6-FAM	150–212	49°
	*Soc*415	5	HEX	187–235	
	*Soc*432	0.8	HEX	98–118	
6	*Soc*44	7	HEX	211–271	Touchdown II
	*Soc*401	3.7	HEX	174–206	56°
	*Soc*433	0.7	6-FAM	84–102	52°
	*Soc*444	1	HEX	161–165	50°

Primer quantities (in pmol) and fluorescent labels (ABI dye) are given for finalized PCR cocktails. Allele size ranges are given for each microsatellite as previously described in Saillant et al. (2004). The multiplex PCR protocol and annealing temperatures (°C) for each panel are shown in the far right column; further description of the PCR protocols is given in the text. PCR primer sequences for each microsatellite may be found in Saillant et al. (2004).

given microsatellite included in each multiplex panel. The Touchdown II protocol was developed in our laboratory and involved a three-step reduction in annealing temperature. The initial step employed the highest optimal annealing temperature for a given microsatellite included in the panel, while the third step employed an annealing temperature slightly (1–2 °C) below the 'bottom' temperature; the second step employed an annealing temperature intermediate between the other two. The Touchdown I protocol was composed of: (i) initial denaturation at 95 °C for 3 min; (ii) 12 cycles of denaturation at 95 °C for 30 s, annealing (minus 0.5 °C per cycle) for 1 min, and extension at 72 °C for 4 min; (iii) 30 cycles of denaturation at 95 °C for 30 s, 'bottom' annealing temperature for 1 min, and extension at 72 °C for 4 min; and (iv) final extension at 72 °C for 10 min. The Touchdown II protocol was composed of three steps that followed an initial denaturation at 95 °C for 3 min: Step 1—7 cycles of denaturation at 95 °C for 30 s, annealing for 1 min, and extension at 72 °C for 4 min; Step 2—7 cycles of denaturation at 95 °C for 30 s, annealing for 1 min, and extension at 72 °C for 4 min; and Step 3—28 cycles of denaturation at 95 °C for 30 s, annealing for 1 min, and extension at 72 °C for 4 min. Final extension was at 72 °C for 10 min. Annealing temperatures for both Touchdown I and II protocols are given in Tables 1–3.

The next step involved identifying the optimal touchdown protocol and corresponding annealing temperature range for each of the 14 multiplex panels. Choice of touchdown protocol was based primarily on reliability of scoring PCR products at all microsatellites in a given panel. We also evaluated different concentrations of PCR reagents (including primers) to determine optimal conditions for each panel. Optimal PCR reactions across all panels were comprised of 1.5 µl of DNA (approximately 50 ng), 1 µl of 10× reaction buffer

Table 2
Multiplex panels for red snapper (*Lutjanus campechanus*) developed from 20 microsatellite primers

Panel	Microsatellite	Quantity	ABI dye	Range in allele size	Touchdown protocol
1	*Lca*43	5	6-FAM	162–192	Touchdown II
	*Prs*260	0.9	6-FAM	111–129	56°
	*Prs*55	2.7	HEX	180–208	53°
	*Prs*229	0.9	HEX	123–137	50°
	*Prs*248	1.3	NED	212–260	
	*Prs*303	0.6	NED	124–150	
	*Lca*20	5	6-FAM	203–223	
2	*Prs*275	4	6-FAM	133–150	Touchdown II
	*Prs*137	7	6-FAM	155–185	54°
	*Prs*328	0.9	6-FAM	196–214	52°
	*Prs*282	5	HEX	113–143	50°
	*Prs*221	4	HEX	220–266	
	*Ra*6	2	NED	112–130	
	*Lca*64	5.2	HEX	151–181	
3	*Lca*91	5	6-FAM	130–144	Touchdown I
	*Lca*22	4	6-FAM	228–258	56°–50.5°
	*Prs*333	0.7	HEX	156–157	50°
	*Prs*240	1.4	HEX	193–223	
	*Lca*107	4	HEX	96–120	
	*Ra*7	1.6	NED	145–167	

Primer quantities (in pmol) and fluorescent labels (ABI dyes) are given for finalized PCR cocktails. Range in allele size is based on work in our laboratory. The multiplex PCR protocol and annealing temperatures (°C) for each panel are shown in the far right column; further description of the PCR protocols is given in the text. PCR primer sequences for microsatellites with prefixes *Lca* and *Prs* may be found in Gold et al. (2001). PCR primer sequences for microsatellites *Ra*6 and *Ra*7 may be found in Bagley and Geller (1998).

Table 3
Multiplex panels for cobia (*Rachycentron canadum*) developed from 35 microsatellite primers

Panel	Microsatellite	Quantity	ABI dye	Range in allele size	Touchdown protocol
1	*Rca*1B-E08A	1.1	6-FAM	181–229	Touchdown II
	*Rca*1-E05	8	NED	221–261	55°
	*Rca*1-E04	0.7	HEX	215–237	53°
	*Rca*1-A11	2.5	NED	165–201	51°
	*Rca*1-E11	4.5	HEX	167–183	
	*Rca*1B-G10	1.1	6-FAM	153–155	
2	*Rca*1-D11	0.9	6-FAM	204–212	Touchdown II
	*Rca*1B-F06	2.2	HEX	260–308	55°
	*Rca*1-B12	1.2	NED	176–196	53°
	*Rca*1-D08	1.1	6-FAM	172–178	51°
	*Rca*1-F11	0.5	6-FAM	119–123	
	*Rca*1B-F07	0.8	HEX	131–143	
	*Rca*1-F10	1.6	NED	287–351	
3	*Rca*1B-A10	0.4	6-FAM	169–187	Touchdown II
	*Rca*1B-E02	4	6-FAM	297–315	60°
	*Rca*1-H10	1.2	HEX	119–139	57°
	*Rca*1-A04	1.8	HEX	196–206	54°
	*Rca*1-G05	1.9	HEX	269–285	
	*Rca*1-D07	1.5	NED	154–162	
	*Rca*1-F07	1.9	NED	235	
4	*Rca*1B-E08B	0.8	6-FAM	117–123	Touchdown II
	*Rca*1-F01	1.2	6-FAM	199–205	60°
	*Rca*1-D04	1.2	HEX	125–131	57°
	*Rca*1-H04A	2.5	HEX	156–162	54°
	*Rca*1-G02	2	HEX	240–244	
	*Rca*1B-D09	5	NED	168	
	*Rca*1-H08	10	NED	273–299	
5	*Rca*1B-C06	4	6-FAM	336–404	Touchdown I
	*Rca*1B-D10	1.4	6-FAM	143–223	50°–44.5°
	*Rca*1B-E06	1	HEX	305–327	44°
	*Rca*1B-H09	10	HEX	168–224	
	*Rca*1-A08	4	6-FAM	287–321	
	*Rca*1-C04	3	NED	217–253	
	*Rca*1-E06	3.2	NED	144–186	
	*Rca*1-H01	3	NED	275–311	

Primer quantities (in pmol) and fluorescent labels (ABI dye) are given for finalized PCR cocktails. Allele size ranges are given for each microsatellite as previously described in Renshaw et al. (in press). The multiplex PCR protocol and annealing temperatures (°C) for each panel are shown in the far right column; further description of the PCR protocols is given in the text. PCR primer sequences for each microsatellite may be found in Renshaw et al. (in press).

[500 mM KCl, 200 mM Tris–HCl (pH 8.4)], 2 mM $MgCl_2$, 2.5 mM of each dNTP, 0.75 units *Taq* DNA polymerase (Gibco BRL), and various quantities of primers. Concentrations of different primers were adjusted relative to obtaining homogeneous amplification product intensity at each microsatellite within a given panel, achieved by raising and lowering primer concentrations in response to PCR outcomes. Final volumes in all PCR 'cocktail' reactions were adjusted with double-distilled water to bring total cocktail volume to 11.5 μl. Optimized PCR protocols for each of the 14 multiplex panels are given in Table 1 (red drum), Table 2 (red snapper), and Table 3 (cobia).

Development of the panels required evaluating an initial 'mega-cocktail' of PCR primer compatibility, reagents, and protocols, followed by the testing of primer concentrations to generate similar quantities of amplified products across all microsatellites. The latter allowed straightforward scoring of all the microsatellites included in the panels for all three species. The impetus for the work was the potential reduction in both personnel time (labor) and consumable supplies generally required for large genotyping projects (Neff et al., 2000). We assessed cost effectiveness by estimating expenses (in U.S. dollars) of supplies and labor required for running single microsatellite gels versus four- and eight-microsatellite panels. PCR supplies included *Taq* polymerase kits, primers and dyes, dNTPs, 96-well plates, tubes, pipet tips, and mineral oil; gel supplies included 44 HD Rox size standards, 96-well plates, long ranger singel packs, sequencing combs, pipet tips, and formamide. The total costs per microsatellite for 96-well reactions (=96 samples) was ~\$64.00 (single microsatellite gels) versus ~\$18.25 (four-microsatellite panel) versus ~\$9.50 (eight-microsatellite panels). Personnel time per microsatellite (also estimated for 96 samples) was reduced as well; single microsatellite gels involved ~2.5 h, whereas four- and eight-microsatellite panels involved ~45 min and ~30 min, respectively. The estimates of personnel times were based on an experienced research assistant.

3. Synopsis

Multiplex panels of nuclear-encoded microsatellites were developed for three species of marine fishes of interest to aquaculture: red drum (*Sciaenops ocellatus*), red snapper (*Lutjanus campechanus*), and cobia (*Rachycentron canadum*). Optimization of each panel resulted in efficient assay and unambiguous scoring of microsatellites in all three species. The multiplex panels can be utilized in a variety of applications, including strain and hybrid identification, parentage assignment, pedigree reconstruction, estimating genetic diversity and/or inbreeding, mapping of quantitative trait loci, and marker-assisted selection. We are presently using the red drum panels drum to identify parentage in 'common-garden' experiments to estimate heritability of juvenile growth rates and thermal tolerance in red drum; the red snapper panels are being used to study stock structure and genetic-effective size of 'wild' red snapper in the northern Gulf of Mexico.

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