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Microsatellite Analysis of Genetic Variation in Sturgeon: New Primer Sequences for *Scaphirhynchus* and *Acipenser*

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Abstract.—Low levels of genetic variation at traditional molecular markers have hampered genetic research within the family Acipenseridae. In an effort to develop a large set of polymorphic genetic markers, 172 clones were sequenced from three subgenomic libraries of shovelnose sturgeon *Scaphirhynchus platorynchus*; the libraries were enriched for two dinucleotide and one tetranucleotide microsatellite motifs (CA, GA, and TAGA). Primers were designed for 113 of the sequences and tested against shovelnose sturgeon, pallid sturgeon *S. albus,* white sturgeon *Acipenser transmontanus,* lake sturgeon *A. fulvescens,* and green sturgeon *A. medirostris.* Of the 113 primer sets tested, 96% amplified in one or more species (58 dimeric and 50 tetrameric). In *Scaphirhynchus* species, 93% of all loci amplified, and 76% were polymorphic. Within the individual *Acipense*r species, 65–80% of loci amplified, with 42–58% being polymorphic. Polymorphic systems for *Scaphirhynchus* species predominately displayed simple, disomic banding patterns, while those for *Acipenser* species typically displayed banding patterns characteristic of tetraploid or higher polyploid levels. These new microsatellite loci provide a group of genetic markers that are detectable with noninvasive sampling and that should prove useful in the preservation of threatened and endangered sturgeon species worldwide.

The family Acipenseridae (sturgeons) contains approximately 20 species from four genera: *Acipenser, Huso, Pseudoscaphirhynchus,* and *Scaphirhynchus.* Worldwide, sturgeon species are threatened or endangered (Birstein et al. 1997a). Sturgeons are vulnerable to extinction owing to a combination of biological characteristics (sexual maturation at a late age and long time periods between spawning) and anthropogenic effects (overharvesting for caviar and meat, construction of dams that impede migration, and habitat destruction). Genetic research on sturgeon has been limited owing to the occurrence of polyploid events (tetraploid to 16n-ploid) (Birstein et al. 1997b) and slow rates of DNA and protein evolution (Birstein 1993).

The genus *Scaphirhynchus* consists of three morphologically similar species: the commercially exploited shovelnose sturgeon *S. platorynchus,* the federally endangered pallid sturgeon *S. albus,* and the rare Alabama sturgeon *S. suttkusi.* Morphological similarity makes positive field identification of each organism difficult. This issue is further complicated by the lack of significant differences between the species at detectable allozyme loci (Phelps and Allendorf 1983), anonymous single-copy nuclear loci (Morizot 1994), and mitochondrial DNA (mtDNA) D-loop sequence (Campton et al. 1995). In situations where genetic differences between closely related taxa (e.g., populations and species) are based on frequency estimates, the number of loci examined is a major determinant of the accuracy of the findings (Baverstock and Moritz 1996). The specific identification of the three putative *Scaphirhynchus* species is an ex-

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ample of this scenario. A lack of molecular variation at nuclear genetic markers and mtDNA markers has left researchers with little statistical power to investigate species boundaries in *Scaphirhynchus.* Hybridization between the species (Carlson et al. 1985) may further complicate the species boundary question and necessitate an increased number of polymorphic loci to address this issue. The polymorphic genetic markers described herein should enable the first definitive testing of reproductive isolation between the three putative *Scaphirhynchus* species.

Recent studies on chinook salmon *Oncorhynchus tshawytscha* (Banks et al. 2000), pink salmon *O. gorbuscha* (Olsen et al. 2000), and Atlantic herring *Clupea harengus* (Shaw et al. 1999) have incorporated microsatellite loci in examining genetic variability within and among species. Microsatellites are short (2–5 base pair) tandem repeats of nucleotides that are codominant, exhibit Mendelian inheritance, reveal extensive polymorphism, and generally render higher discriminatory power than traditional genetic markers, such as allozymes (Estoup et al. 1998). Microsatellite loci are assayed withthe polymerase chain reaction (PCR; Saiki et al. 1988), which allows the use of nonlethal tissue sampling (fin, blood, feces, feather, etc.), whereas allozyme studies usually require lethal sampling of internal tissues (liver, heart, and eye). Use of nonlethal tissue samples is of extreme importance in the genetic analysis of endangered and threatened species.

May et al. (1997) demonstrated that primers designed from a lake sturgeon subgenomic library amplified microsatellites at 8 of 11 loci examined in other *Acipenser* species, as well as in *Scaphirhynchus* sturgeon. Furthermore, six of the nine loci (67%) that amplified in *Scaphirhynchus* species were polymorphic, and *Acipenser* polymorphism ranged from 33% to 80%. This high degree of polymorphism is a vast improvement over traditional genetic markers such as allozymes and mtDNA. The dynamics of the sturgeon genome appear to mandate using a rapidly evolving marker in lieu of the ones used in traditional molecular techniques. Furthermore, the conservation of microsatellite flanking regions among related taxa suggests that the development of microsatellite loci for one species would prove useful on a familywide scale (May et al. 1997).

In this study, primers were developed for 108 new microsatellite loci from a shovelnose sturgeon microsatellite-enriched subgenomic library. These primers were tested for amplification efficiency with shovelnose sturgeon, pallid sturgeon, white sturgeon *A. transmontanus,* lake sturgeon *A. fulvescens,* and green sturgeon *A. medirostris.* The results of this study will prove useful in a variety of future sturgeon genetic studies, ranging from stock assessment to mapping of quantitative trait loci.

Methods

DNA was extracted from gill tissue of a shovelnose sturgeon specimen caught at Mississippi River km 177 (measuring from the river's mouth) with the QIAamp tissue kit (QIAGEN, Valencia, California). A subgenomic library was created by Genetic Identification Services (Chatsworth, California) by partially digesting whole genomic DNA with a mixture of the following restriction enzymes: *Bsr*BR1, *Eco*R V, *Hae* III, *Pvu* II, *Sca* I, and *Stu* I. An oligonucleotide linker containing a *Hind* III site was ligated to fragments in the range of 300–700 base pairs. These fragments were enriched by magnetic bead capture to create four separate libraries for the repeat motifs: $(CA)_n$, $(GA)_n$, $(CATC)_n$, and $(TAGA)_n$. The captured fragments were ligated into the *Hind* III site of the plasmid pUC19 and the ligation products electroporated into *E. coli* DH5α. From each of the four microsatellite-enriched libraries, 52 $(CA)_n$ and $(GA)_n$ and 91 $(TAGA)_n$ recombinant clones (white colonies) were sequenced to determine the efficiency of enrichment. In all, 45 $(CA)_n$, 46 $(GA)_n$, and 81 $(TAGA)_n$ clones contained a microsatellite, corresponding to 87, 88, and 89% efficiency of enrichment, respectively. The initial sequences for the $(CATC)_n$ library yielded no microsatellite loci, and work on this library was discontinued. Ultimately, 172 clone sequences were obtained on an ABI 373A sequencer (PE Biosystems; now Applied Biosystems, Foster City, California) by means of dye terminator chemistry. Primers were designed for 113 of the 172 sequences with Primer3 (Rozen and Skaletsky 1999) and MacVector 6 (Oxford Molecular, Beaverton, Oregon).

Oligonucleotide primers were tested on DNA from three pallid (Gavin Point National Fish Hatchery), three shovelnose (Missouri River), five lake (Mattagami River), six white (Sacramento River), and four green (Columbia River) sturgeons. DNA was extracted from 1-cm^2 fin samples using the TNES-Urea method (White and Densmore 1992).

Reaction mixes were prepared in a total volume of 20 μL with 50 ng DNA template, 0.4 μM of each primer, 3.125 mM $MgCl_2$, 0.22 mM of each

deoxynucleotide triphosphate (dNTP), 0.5 unit *Taq* DNA polymerase, and 1× Gibco PCR buffer (200 mM tris-HCl, pH 8.4; 500 mM KCl). Double-distilled water was used to bring the reaction mixture to the desired final volume. Amplification was conducted with a PTC-100 thermocycler (MJ Research, Watertown, Massachusetts), with an initial denaturation at 94°C for 3 min, followed by 35 amplification cycles (94°C, 1 min; 57°C, 30 s; 72°C, 30 s), and a final elongation at 72°C for 5 min. Following amplification, 20 μL of formamide loading dye (98% formamide, 0.09% bromophenol blue, 0.09% xylene cyanol FF, and 0.01mM pH 8.0 stock EDTA at a pH of 8.0) was added to the amplified product. This mixture was denatured at 95°C for 4 min and then cooled on ice for 5 min before gel loading. Then 5 μL of each sample was loaded on a 5% denaturing acrylamide:bisacrylamide (19:1; 7.5 M urea, 0.5X tris−boric acid (borate)−EDTA [TBE]) gel and electrophoresed for 1 h at 35 W. The gel was stained using the agarose overlay method of Rodzen et al. (1998) and visualized on the Molecular Dynamics (Sunnyvale, California) FluorImager 595. Fragments were sized by comparison with a BIO-RAD Fluorescent Low Range DNA standard.

Since this study, we have used these primers to develop species-specific primers in other sturgeon species. As an example, white sturgeon genomic DNA was first amplified using the *Spl-113* primers and the amplification products excised from a denaturing 5% polyacrylamide gel electrophoresis (PAGE) gel. DNA from the gel-isolated bands was extracted overnight at 50°C in 150 μL of TE (tris, 10 mM; EDTA, 0.1 mM) buffer. The supernatant was used as a template in a second PCR reaction with the original *Spl-113* primers. This PCR product was purified using the QIAQuick PCR Purification Kit (QIAGEN). Fragments were sequenced by Davis Sequencing (Davis, California) on an ABI PRISM 377 DNA sequencer using the *Spl-113* forward and reverse primers. The resulting sequence information was used to design white sturgeon-specific primers for this locus, since renamed *Atr-113*.

Results

Amplification (the presence of PCR product resolved by electrophoresis) in one or more species occurred in 96% (108 of 113) of the primer pairs tested. Fifty-five percent (62 of 113) of the microsatellite loci amplified in all tested species. Of the 108 amplified microsatellite loci, 58 had dimeric repeats and 50 had tetrameric repeats (Appendix 1). The resolution of monomorphic and polymorphic loci was classified as good (G), fair (F), and poor (P) (Appendix 1). Amplification was considered good if alleles were clear and sharp, with no perceptible stutter bands; fair if allele amplification was clear, but stutter bands were evident; or poor if multiple loci amplified or if product amplification was apparent, but it was not possible to distinguish individual alleles. Because of the close taxonomic relationship between pallid and shovelnose sturgeon (Phelps and Allendorf 1983; Wirgin et al. 1997), these two species were treated as a single group (*Scaphirhynchus* spp.) when scoring the gels.

Among the *Scaphirhynchus* spp., 93% of all loci amplified, and 76% of those were found to be polymorphic. Within the white sturgeon, lake sturgeon, and green sturgeon groups, 65, 80, and 68% of loci amplified, and 42, 58, and 47% were polymorphic. Nearly all the polymorphic systems for *Scaphirhynchus* species displayed the simple banding patterns characteristic of disomic loci (i.e., single-banded or symmetrical two-banded patterns), while those for *Acipenser* species usually displayed banding patterns characteristic of tetraploid or higher polyploid levels (more than two bands per individual, with asymmetry in band intensity within individual patterns).

Discussion

The goal of this study was to increase the number of microsatellite loci that could be used for genetic studies of various sturgeon species. The study was based on three shovelnose sturgeon microsatellite-enriched subgenomic libraries. Primer pairs developed from the libraries were tested in *Scaphirhynchus* and *Acipenser* species for polymorphisms. The cross-amplification across genera that we found concurs with similar findings that primers developed in one species often cross-amplify in other species (Lyons et al. 1997; May et al. 1997; Davis and Strobeck 1998; Smith et al. 1998). We anticipate these new primers to be applicable in other sturgeon species with minimal development and optimization.

Despite testing the primers with only three to six individuals from each species, a high level of polymorphism within each species was resolved. Fifty-six percent of all species−locus combinations were polymorphic. The use of more individuals, as well as further optimization of the reactions, would probably reveal new alleles at polymorphic loci and possibly detect polymorphism in loci that are reported here as monomorphic (May

et al. 1997). In addition, the use of ^{32}P end-labeled primers and visualization of amplicons via autoradiography appeared to result in increased quality of amplification at the dimeric microsatellite loci (results not shown). Reaction optimization or redesign of species-specific primers for one sturgeon species may reveal amplification in other sturgeon species that either failed to amplify originally or yielded a nonspecific product. The redesign of *Spl-113* for white sturgeon amplification illustrates the relative ease of this approach. The new *Atr-113* primers produced amplicons that amplified more effectively and added an allele not seen with the *Spl-113* primers from Appendix 1. The availability of the entire clone sequences in GenBank will permit researchers to create new species-specific primers.

Management agencies have begun conservation and restoration programs for some endangered sturgeon populations (Beamesderfer and Farr, 1997; Smith and Clugston, 1997). However, little is known about the sturgeon polyploid genome or the genetic structure within and among sturgeon populations. Microsatellites have high levels of variation and have become important genetic markers for studies in parentage, linkage, and intraspecific population structure in many organisms (Schug et al. 1998). The primers described herein will facilitate the use of microsatellite loci for the identification and evaluation of genetic variation between individuals and populations within many, if not all, sturgeon species.

The inheritance of allelic variation at microsatellite loci revealed by new primers should be tested. Initial work on inheritance of microsatellite loci in lake sturgeon (Pyatskowit et al., in press) suggests that many of these loci segregate disomically; green sturgeon and white sturgeon exhibit much more complex systems and await detailed inheritance studies to ascertain their genomic structure. The primers designed in this study detected variation in both *Scaphirhynchus* and *Acipenser* species, indicating that they should provide a useful set of DNA markers for studying the genetic structure of all sturgeon species. Given the current threatened or endangered status of most Acipenseridae (all members in the family are listed in CITES 1998), identification and implementation of this suite of molecular markers should prove timely in furthering the cause of sturgeon conservation efforts worldwide.

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Appendix 1: Results of Primer Tests in Sturgeon

TABLE A.1.—Primer, repeat motifs, primer sequences, GenBank accession number, product size in clone, polymorphism, and quality of amplification for microsatellite loci derived from a shovelnose sturgeon genomic library. Primers were tested on six white, six pallid–shovelnose, five lake, and five green sturgeon individuals. Locus polymorphism in each species is designated as monomorphic (M), polymorphic (P), no activity (N), or nonspecific (i.e., did not amplify in all individuals) (Y). Product amplification was considered good (G) if alleles were clear and sharp with no stutter bands, fair (F) if alleles were clear but stutter bands existed, or poor (P) if multiple loci amplified or individual alleles could not be distinguished even though product amplification was evident. Individual species columns consist of level of polymorphism followed by quality of amplification.

Primer	Repeat motif	Forward (above) and reverse (below) primer sequences (5′ to 3′)	GenBank accession number	Product size (bp)	White ($N = 6$)	Pallid–shovelnose ($N = 6$)	Lake ($N = 5$)	Green ($N = 5$)
Spl-1	*(CA)*$_{24}$	CGAACAGGAAATACAGACGGTC TTGAGTCCAGACATAAAGCACAGTC	AF276109	301	P/F	P/F	P/F	P/F
Spl-2	$(TG)_{13}$	GGCAAAGGAGTTGCGGAGTTTAGAG GAATACAGGGGTGACCTCAAGATGG	AF276110	342	M/P	M/G	P/F	N
Spl-3	$(AC)_{18}$	CGCAACAGTATGCTTTTGCAGCAC GCAGCAACCTTTAATTCAAGGGG	AF276111	245	M/F	M/P	P/P	P/P
Spl-4	$(TG)_{12}$	AGCAAGGCAGGCTGTAAGCAAAG TGTTTTTCGGCTTCACCAGGG	AF276112	187	M/P	M/P	M/P	M/P
Spl-5	$(TC)_{12}(AC)_{11}$	GCGGGGAGTTAAGTTCTGGGATAG GGCGACGTATAAATCATAGTGCTG	AF276113	325	N	P/G	P/P	N
Spl-6	$(GA)_{28}$	GCAGACTGCAATCAGAAAGCGATG TAGCAACCCTCACCCTGTAGATGG	AF276114	318	P/F	P/F	M/F	M/F
Spl-7	$(GA)_{25}$	CCAGGAGGGGTAGCACTTTATC GGCAAACAGGGAAGTTTTAGG	AF276115	193	P/F	P/F	M/F	M/F
Spl-8	$(TC)_{29}$	AGCCTTCCACACCGCAG TAGCTGTTGGGGGAGCCAG	AF276116	164	P/P	P/P	M/P	M/P
Spl-9	$(CA)_{20}$	TCACTGGAACGCACACACTC CGATTTCAGCAGCTTCAATC	AF276117	237	Y	M/P	M/P	M/P
Spl-10	$(CT)_{27}(AC)_{19}(GACA)_{7}(CACT)_{14}$	GCTTCCAGCACTCGTAACAG TGTGAGCGTGATCGAGCAAG	AF276118	289	P/G	P/F	N	P/G
Spl-11	$(TG)_{15}$	TGGGATTGGAGCAACGTAAC TGAGCAGTGACAAGAGAGCC	AF276119	185	P/P	P/F	P/F	P/F
Spl-12	$(TG)_{14}$	AGCTGGACCTGCAACATCAG TCGCCTGAGCAGAGATGAAC	AF276120	175	P/P	M/F	P/P	N
Spl-13	$(TG)_{19}(CGTGTG)_{8}$	TGAGCTGATCGTGTCTGTCC AAAGGCATCGGCGATAAC	AF276121	339	M/G	P/F	P/F	P/F
Spl-14	$(TG)_{12}$	CCCGCTGAAACCAATACCTG TGCAGTGATTGACAGCTTGG	AF276122	192	P/P	M/P	P/P	M/F
Spl-15	$(CA)_{19}$	TGCACCTGAACACAACTGGG TCGTGTGGTTAGGCTGTCTG	AF276123	202	M/F	P/F	M/F	P/F
Spl-16	$(TG)_{16}$	GTCCAAGCACTCCTGATGTG CAACCAGGGCATTTATCCAC	AF276124	239	P/F	P/F	P/F	P/G
Spl-17	$(AC)_{13}$	TCTACCTCTCGACTCTTGTTCCACC AGCAAACAGACACACACTGGGATAG	AF276125	442	P/F	M/G	M/F	P/P
Spl-18	$(CA)_{16}$	CCACAATGAGATCCTAGCCC CAACGCAATGGATACAGCC	AF276126	245	N	M/P	P/G	N
Spl-19	$(TG)_{21}$	GCCCATTTACAAACCTAGTTTTCG ATGCTGTTTTGGAAGGTAGAAGAAC	AF276127	241	P/G	P/P	P/F	P/P
Spl-20	$(TG)_{20}$	ACCCTCCCATGATGATTCTG TTCAGCAACAACACTGCATG	AF276128	214	M/P	P/P	P/F	Y
Spl-21	$(CA)_{20}\ldots(AC)_{26}$	CCTTTGAATTTGGTGTGCAG ATTGCGTGTGTGTGCGAGAG	AF276129	209	M/P	M/P	P/P	M/F
Spl-22	$(CA)_{15}$	GCGGACCACTACTGTCATTG TGTATTGTTCTGGGGTGGG	AF276130	146	M/P	M/P	M/P	M/P
Spl-23	$(CA)_{n}$	AATCTTCCACTAACCGCCC TTCAGCTCATGCTGCTGCAC	AF276131	269	M/P	P/F	P/G	N
Spl-24	*(TG)*$_{20}$	CGCCGCCAGCTATCTTAAAC AACCCAGCAGCTTTCTTAGG	AF27613	207	M/F	P/P	M/P	N
Spl-25	$(CA)_{14}$	CTCAGCCTGCTTCCTTAACTC TAGGGAAAACGACAGCGGTG	AF276133	382	N	P/G	P/P	M/G

Appendix 1: Continued.

Primer	Repeat motif	Forward (above) and reverse (below) primer sequences (5′ to 3′)	GenBank accession number	Product size (bp)	White (N = 6)	Pallid–shovel-nose (N = 6)	Lake (N = 5)	Green (N = 5)
Spl-26	$(CA)_{28}$	TGCCCTCCGTATTTTTCAAG TTGCCTTTAACCACTGGACC	AF276134	311	N	P/P	N	N
Spl-27	$(CA)_{21}$	TCGATGGAGGAACAGTCCAC AGATGGGGATTACTGCGAGG	AF276135	458	M/G	P/F	P/G	P/G
Spl-28	$(AC)_{21}$	TGACTGTCGCACAGCAGTTG TGCCATGCAGCAAATTACTG	AF276136	183	P/P	P/F	P/F	M/F
Spl-29	$(AC)_{32}$	TCTTCAGGTGGGTGCAATC GCAGAAGCATGACTTGTGTGG	AF276137	289	N	N	N	N
Spl-30	$(AC)_{18}$	AGCGATCAGGCTGTTCCTAC ACAGGCGATTTCACTGGAGG	AF276138	259	P/P	P/G	M/F	M/P
Spl-31	$(CA)_n$	GTTGCAGAGAAGAACTGAACCAATG ACACAGGCAGGGCAATACATAGAG	AF276139	396	N	P/G	P/G	P/F
Spl-32	$(TG)_{26}$	GCGGTCAATTAGCACCTACTG TCTCACTTGCTTTATCACTAGCC	AF276140	188	Y	Y	P/F	Y
Spl-33	$(T)_{18}\ldots(TG)_{13}$	ACGTGATTTATCAGCCACAGTCTG GCAGGTTGTTGTACCAGAATGTTAG	AF276141	300	P/P	M/F	M/F	M/P
Spl-34	$(CA)_{17}$	TTTGATTTGTTGGAGAGGGG TCAGTTGCGTCTCATGGATTG	AF276142	321	N	P/F	N	N
Spl-35	$(TG)_{13}$	TCATGGCTCCCTTTTGCTC ATAGCTGGCGGTATGTGAGG	AF276143	237	M/F	P/F	P/F	M/F
Spl-36	$(TC)_{32}$	TGCTTTAATCCAGAGTCTTAGCC AATAGGAGCCCATTGGTAACC	AF276144	367	Y	P/G	P/F	P/P
Spl-37	$(TC)_{23}$	TTGCTTGCTCTCTCCGTCAC AGTCAGGCGATTCACAGCAG	AF276145	225	M/F	P/P	M/F	M/F
Spl-38	$(TC)_{21}$	TGAACAGAGCGATGCAGTG TCAGAGATGGAAGGAGGGAG	AF276146	230	P/F	P/P	P/F	P/F
Spl-39	$(TC)_{29}$	ACATGCCGACTTTGACGTG TCAGCCGAACCCATTTCCTC	AF276147	307	M/F	M/P	M/P	M/F
Spl-40	$(GA)_{24}$	ATGCTCACAACTCCCACATATC TCAGACATCCCAAAGGATTTTG	AF276148	235	M/G	P/P	P/G	M/G
Spl-41	$(CT)_9$	ATCCCAGGCTCACCCATTTG TGTTCTGACGGACAACCCTG	AF276149	348	N	M/P	N	N
Spl-42	$(TC)_{27}$	CCAGAATCAGCACTGGCAAC GGAAGCAGAGGGGAAACAAG	AF276150	248	P/G	P/F	M/G	P/G
Spl-43	$(TC)_{23}$	GCAAATGAGGAGTGTCGATG AGGTGAACTGCCAGGTATGG	AF276151	140	M/F	P/F	P/F	P/P
Spl-44	$(GA)_{15}$	CATCTCCCAGAGCGTCACTC CTGGTGGCTTTAGTTCCCTC	AF276152	151	P/F	P/P	P/F	P/F
Spl-45	$(TC)_{28}\ldots(T)_{15}$	ACAGGAACATTTAACAAGCTGG AACCGATCAAAGGCTTTTTC	AF276153	326	P/F	P/F	P/F	M/P
Spl-46	$(TC)_{10}\ldots(TC)_9$	TGCATTCTCTTCATTGGCTG AACAGGGAGAGGGAGAGGG	AF276154	211	M/F	N	P/P	M/P
Spl-47	$(TC)_{31}$	ATATTTGCTGTCGATGCAGGG TCGCACACAAACAAGCACTC	AF276155	236	P/F	M/F	P/F	M/F
Spl-48	$(GA)_{27}(G)_{10}$	TGGCAACCGTGACAGCTTAC GGTCATCACATTCCAATGCTG	AF276156	254	P/F	P/F	P/F	P/F
Spl-49	$(TC)_{15}$	GACTTCGTGGACTGCCAAAG AACTTGCCAGCACCAGGGTC	AF276157	167	M/G	P/F	P/F	P/F
Spl-50	$(GA)_{33}$	CGGAAAGTCATTTCTCATCCC CCTTGTTAAAGCTGCTTCCC	AF276158	231	M/G	P/P	P/P	P/G
Spl-51	$(TC)_{16}$	ACGGAGACAAACAAACACGATG AGCACTCACTCGGGGTTGGG	AF276159	137	N	N	N	N
Spl-52	$(TC)_{30}$	TTGCACGCTAGACAATGGG GACCGTGGCTGGGATTG	AF276160	249	P/P	P/P	P/P	M/F
Spl-53	$(GA)_{16}$	CGAGGGAGGTGTCTCCTATC GCAGTTGCATGGGGTTTAG	AF276161	231	P/P	P/G	P/P	P/F
Spl-54	$(GA)_{50}$	TGGACCAGGACCAGGCATG AGGAATACCAGTCATTTAGAAGGTC	AF276162	251	M/G	M/G	P/G	P/G

Appendix 1: Continued.

Primer	Repeat motif	Forward (above) and reverse (below) primer sequences (5′ to 3′)	GenBank accession number	Product size (bp)	White ($N = 6$)	Pallid–shovelnose ($N = 6$)	Lake ($N = 5$)	Green ($N = 5$)
Spl-55	$(T)_{20}...(GA)_{21}$	AGGCGAAGAAATGAAGACACTC TCCGAAAGTTGGCAGGTATG	AF276163	329	Y	P/F	P/G	P/G
Spl-56	$(TC)_{22}$	AGCTCTGGGAGATAAGCCTG CGTTCTGTGTGCAGTCAACC	AF276164	214	P/G	P/F	M/G	P/F
Spl-57	$(GA)_{35}$	AGAGCGAACACTGATGGCTC ACTTGGATGCACAGGGATG	AF277165	275	P/P	P/P	P/P	N
Spl-58	$(TC)_{13}$	TACCCGTGCTCTTCCACTCC TTCACCCACCTCCCTGACAG	AF276166	151	Y	P/F	M/P	N
Spl-59	$(GA)_{31}$	CCTGTGCAGAAGTGACCATC ACAAACACAACCCCTATCCC	AF276167	278	M/P	M/G	P/G	N
Spl-60	$(C)_{10}...(TC)_{15}$	TGTTAAGCTGTCGCCCCTCC ATTCCTGCACACTGAGAGTCAC	AF276168	201	P/F	P/F	P/G	M/P
Spl-100	$(TCTR)_{20}$	CCATGCCTGTTCATAATTGTTC TTTTCACCATTGGGTGTTCA	AF276169	226	P/F	P/G	P/G	P/F
Spl-101	$(TCTA)_{7}$	CCCTCCACTGGAAATTTGAC GCAATCAACAAGGTCTCTTTCA	AF276170	272	P/F	P/G	P/G	P/F
Spl-102	$(TCTA)_{18}$	TGATCTCAGACGCTGTGAATG GCCTGTAGTTATAATCAACCAATCAA	AF276171	174	N	N	N	N
Spl-103	$(TCTA)_{18}$	TGAATGATCTCAGACACTGTGAA GCTGTTTTTAAACGAAGCCG	AF276172	290	N	N	N	N
Spl-104	$(TCTR)_{12}$	TTATATGGGTGGGGTGGATG TCCTCTTTGGCATTTGTTCC	AF276173	270	P/G	P/G	P/G	P/G
Spl-105	$(TAGA)_{12}$	GCGATTTGATTGGCTCTTGT GGCACTGAATAAATGGACCG	AF276174	136	P/G	P/G	P/G	P/F
Spl-106	$(CTAT)_{12}$	CACGTGGATGCGAGAAATAC GGGGAGAAAACTGGGGTAAA	AF276175	224	M/F	P/G	P/G	M/G
Spl-107	$(TAGA)_{15}$	GCAAGAAAGTCCAAGGCAGA CAGCCATGGTGTGTGAGAAC	AF276176	270	P/P	P/P	P/P	P/P
Spl-108	$(GATA)_{6}(TATA)_{1}(GATA)_{1}$	GTGGACATGCTCACACCAAA GCACTGGTGTCACTGTTGTG	AF276177	289	N	P/F	P/F	N
Spl-109	$(TCTA)_{9}$	CCGCAGAAGCAATTGAAAAG TATAAATGGTGGGGTGGCAT	AF276178	212	P/P	P/G	P/F	P/G
Spl-110	$(TAGA)_{14}$	ATTTTCACCCACAACTTGGC TCCCAGAGCCCTAACCACTA	AF276179	288	Y	M/P	M/F	N
Spl-111	$(TAGA)_{18}$	CTGTTCGGTTGAGTGGTTGA ATCAGGTTCAATGCATTCCC	AF276180	138	M/P	P/P	M/P	P/P
Spl-112	$(ATAG)_{9}$	CCCATCTGTCCGATCTGATT AACCACAGGAAAGCACAAGG	AF276181	186	Y	P/F	M/F	P/F
Spl-113	$(AGAT)_{14}$	TCCCACATGGCTTGTATTGA ACCACACCATGCGTCATAAG	AF276182	284	P/G	P/G	P/G	P/G
Spl-114	$(TCTA)_{14}$	TCAGGGTTCATTCAGGGCTA TGCAATTCGTGTCATGTTCA	AF276183	298	P/F	P/G	P/G	N
Spl-115	$(TCTA)_{14}$	CAAGCAGGAGGGGTTACTGA GGACAGACAGACAGGAAGGC	AF276184	253	P/F	P/P	P/F	P/P
Spl-116	$(TATC)_{15}(TGTC)_{5}(TATC)_{2}$	CCTGCAGCCTTTATGTACTGC AAGATGTACATACCGACAAACAT	AF276185	283	P/F	P/G	P/F	P/F
Spl-117	$(GATA)_{11}$	CAGCCAAAACCATGCTTACA CCGCCTCTCAATAGCAACAT	AF276186	236	M/P	P/F	P/G	P/G
Spl-118	$(TATC)_{11}$	TAGCCTCCAATCTGGCTT CCAAGCTGGGTGTTCAGAGT	AF276187	194	N	P/F	N	N
Spl-119	$(AGAT)_{15}$	GCATGCATATCCAAAATGTCTT TTTGGAGAGTTGTGAGCAGC	AF276188	264	P/F	Y	Y	M/F
Spl-120	$(TATC)_{15}$	ATTCCATGAGCAACACCACA TGATGGTCTGATGAGATCGG	AF276189	285	P/P	P/G	P/F	P/F
Spl-122	$(TCTA)_{11}$	CAAAATGTCAGGCAGTGCTC CATGAGACAGAGGTGCCAAG	AF276190	253	P/P	P/P	M/F	P/F
Spl-123	$(CTAT)_{11}$	GGACCATTTTGTGTTGGGAC TGCACAGTTCACAGCCTACC	AF276191	218	P/F	P/G	P/G	P/F

Appendix 1: Continued.

Primer	Repeat motif	Forward (above) and reverse (below) primer sequences (5′ to 3′)	GenBank accession number	Product size (bp)	White ($N = 6$)	Pallid–shovelnose ($N = 6$)	Lake ($N = 5$)	Green ($N = 5$)
Spl-124	$(GATA)_{12}$	CGCCACCAGCCATTATACTT TGTGGAGTTTTGGCATGTGT	AF276192	275	M/P	P/G	M/P	P/F
Spl-151	$(TCTA)_{16}$	CACAGTGGCAATAAGCCAAG TTCAAAGTGTTCTGCAAGCCT	AF276193	132	Y	P/F	P/F	P/F
Spl-152	$(TATC)_{27}$	GGTTGAAAACGAGGACAATCA TTGGGATACTGAAATGTTTGACA	AF276194	221	N	P/P	N	N
Spl-153	$(TAGA)_{14}$	GACTTCCCCAGCAAAACG TGTGGAAAAGTCCAAGGGAT	AF276195	149	N	P/P	Y	N
Spl-154	$(TAGA)_{13}$ . . .$(CA)_8$	TGTATGCAGTTATCCAGTATGCAA TGACGCTTTCACAGCTTGTT	AF276196	154	P/P	P/G	M/G	N
Spl-155	$(TCTA)_{11}$	TGCATAAGTATTCAACCCCCACAT TGAGCTTGATTAGCCCCAGT	AF276197	236	N	P/G	N	N
Spl-156	$(TAGA)_{12}$	CCGACTGGTATTTAATTTAACCA AACCATTAATTTCTTGGGGAAAG	AF276198	110	N	P/F	N	N
Spl-157	$(CTAT)_{10}$	AAGGCTTTTCAGCCTGTGAC CCACAAACAAAGGATCACCA	AF276199	212	N	P/F	N	N
Spl-158	$(TAGA)_{15}$	CCACATTCATGGGGTAACTAGAG GTGCTTCAGGAGCTGCTTTT	AF276200	204	N	P/G	N	N
Spl-159	$(TCTA)_{21}$	TTCAGGAGCTGCTTTTCAGTT TCACCTGATGACGACTTACTTACC	AF276201	164	N	P/G	N	N
Spl-160	$(TCTA)_{12}$	GCTGCTGGGGAAATACAGTC TTGCATCACAGCACGATAGAG	AF276202	188	N	P/G	N	N
Spl-161	$(TCTA)_9$	GGAAACCTGGGAGAAGATTCA CATTTTGCATTGGCAACAGA	AF276203	184	N	P/G	N	P/P
Spl-162	$(TAGA)_{21}$	GGCTTAAGCAGCTACAGCACT CATACAGAACTACTGAAACGACACA	AF276204	160	N	P/G	P/F	N
Spl-163	$(GATA)_{17}$	TGCTTGTAAACTGCCCCACT CCACATGCAGTTTGAGCTGC	AF276205	208	Y	P/G	P/G	P/G
Spl-164	$(TATC)_7$	GAATTGGTCAGGGGTCTGAA GCAATAGAAGATTCAGGGGAGA	AF276206	153	N	P/F	N	P/F
Spl-165	$(TCTA)_{31}$	TCACAGTTTTCCCAGGATCTC CCTGTATCTTTCTCTAGCTTTATGACA	AF276207	195	Y	P/F	N	P/F
Spl-166	$(TCTA)_{20}$	TGTTTGAGTGATCTGCAGGG GGTTAAGGTTAGTGTTGAAGTTAGG	AF276208	187	N	M/F	M/F	P/F
Spl-167	$(TCTA)_{10}$	CCAATCTGTATCACCCCTGA GCAGAACCATTTTATTGACATTGA	AF276209	164	N	N	N	N
Spl-168	$(TATC)_{18}$	CACTGATTCGCTACAACCGT AGAAGGACTTGCAGTCCGAA	AF276210	149	P/G	P/G	P/G	N
Spl-169	$(TAGA)_{17}$	CAAATGCGACTCATGATGGTT AGTTTTGGGGGTAAGGTGGT	AF276211	192	N	P/G	P/G	N
Spl-170	$(TAGA)_{15}$	ACTGGAAAAATGGGGGTGTT TGTTCACCCAACCAGTTTGT	AF276212	188	N	P/G	N	N
Spl-170a	$(GAT)_5(ATAG)_{11}$	GGACGCACTAGACAGGCTTT CACCAAACACAGCAGATTTCA	AF276213	229	P/G	P/G	P/G	P/F
Spl-171	$(TCTA)_{15}$	ACCACAAAAGCATTGCGAC GGTGGGCTGGTCTTTTGATA	AF276214	157	M/F	P/F	P/P	P/P
Spl-172	$(TAGA)_6(TC)_7$ . . .$(TAGA)_8$	CTGACGAGATGCTGTAGAACG TGGAAATAAAGCGCTGGTAAA	AF276215	195	P/F	P/F	P/F	P/G
Spl-173	$(TCTA)_{10}$	GGCTTTTGTCTGAAACGTCC TGGTGTGTGATTTTGAAGGC	AF276216	219	P/G	P/G	P/F	P/F
Spl-174	$(TCTA)_{15}$	CCAGTCGGTAGGTGTTTATTGTT GCAAAATTGGTCTTATTTGGTAGC	AF276217	155	P/P	P/P	P/F	P/F
Spl-175	$(TCTA)_8(TC)_{27}$	CACTGAGCTTACCCTATCAAAACA CAGTGAGCTACAGAGTGTGTGAGA	AF276218	223	Y	P/P	M/P	M/P
Spl-176	$(TAGA)_{14}$	AAATGTGCATTGTGCTGCAT GAGTTCAGACTTACTTCGCTTGC	AF276219	169	P/P	P/G	P/G	P/F
Spl-177	$(TAGA)_{12}$	AGCTAACCAGAGTGCCAGGA TGCTCTCAGGGCCATCTTTA	AF276220	164	N	P/F	P/F	P/P
Spl-178	$(TCTA)_{12}$	ATTTATTGTTCATAAACCCAGGTGA CATGTGCTCAGATCAGTTTGG	AF276221	158	P/F	P/G	P/P	P/P