Letter to the Editor

Patterns of Size Homoplasy at 10 Microsatellite Loci in Pumas (*Puma concolor*)

Melanie Culver, Marilyn A. Menotti-Raymond, and Stephen J. O'Brien

Laboratory of Genomic Diversity, National Cancer Institute–Frederick, Frederick, Maryland

Microsatellites, repetitive simple sequences of 1–6 nt in length, are abundant in eukaryotic genomes (Weber 1990*a*). Because of extensive variability in the number of repeat units for any one locus among members of a population, microsatellite loci exhibit high polymorphism. Microsatellite variation has become a useful class of genetic markers in population assessment for numerous species for questions of genetic identification, parentage, kinship, and population variability assessment (Jarne and Lagoda 1996; Goldstein and Pollock 1997).

The high level of polymorphism at microsatellite loci is believed to result from slipped-strand mispairing during DNA replication (Levinson and Gutman 1987; Weber 1990*b*; Weber and Wong 1993; Krugylak et al. 1998), most commonly causing the gain or loss of one or more repeat units. This mutation mechanism would be expected to generate allelic homoplasy, i.e., comigrating allele size fragments which are not identical by descent or in DNA sequence among different individuals.

Because mutational slippage does not conform to the infinite-alleles model (Kimura and Crow 1964), a stepwise mutation model (SMM) (Ohta and Kimura 1973; Chakraborty and Nei 1982) has been invoked to explain allele frequency distributions and population variability at microsatellite loci (Valdez, Slatkin, and Freimer 1993; Kimmel et al. 1996). A distinguishing feature of the two models is that homoplasy is not considered under the infinite-alleles model, while it is an expectation of the stepwise mutation model (Viard et al. 1998). The SMM (but not the infinite-alleles model) makes the assumption that differences in allelic length are due to variation in the number of repeat units and not to insertions and deletions in the flanking sequences.

Allelic size homoplasy, a condition under which comigrating alleles represent different sequence motifs, has been demonstrated through sequence analysis of electromorphs of compound or imperfect repeats of primates (Blanquer-Maumont and Crouau-Roy 1995), several fish species (Angers and Bernatchez 1997), invertebrates (Viard et al. 1998), and the fungus *Candida albicans* (Metzgar et al. 1998). Insertion and deletion events in genomic regions flanking microsatellites confer allelic size homoplasy in several marine turtle species (FitzSimmons et al. 1995) and humans (*Homo sa-*

piens) (Grimaldi and Crouau-Roy 1997). The occurrence of allelic size homoplasy is informative, since ignorance of its extent in a population may confound forensic, phylogenetic, or population diversity assessment. A few studies of microsatellite size homoplasy in natural populations have been reported (Estoup et al. 1995; Angers and Bernatchez 1997; Ortí, Pearse, and Avise 1997; Viard et al. 1998), but none of these studies assess mammalian populations.

A sampling of 277 free-ranging puma (*Puma concolor*) individuals from throughout the geographic distribution of this species (from the Canadian Yukon to the Patagonia region of Chile and Argentina) was analyzed for genetic diversity (Culver et al. 2000). In that phylogeographic study, relationships of 31 recognized puma subspecies were evaluated based on mitochondrial DNA (mtDNA) haplotypes and allele distributions of 10 feline microsatellite loci isolated from a genomic library of the domestic cat *Felis catus* (Menotti-Raymond and O'Brien 1995). Here, nucleotide sequences from 64 pumas homozygous for various length alleles at each of the 10 dinucleotide loci were determined. Microsatellite allele sequences were compared with each other and with the homologous domestic cat microsatellite locus in order to assess locus sequence structure between Felidae species and to estimate the incidence of allelic size homoplasy in pumas. Figure 1*A* and *B* presents sequence alignments for 76 different homozygous puma alleles. (Ten pumas were homozygous at more than one locus.) These results have bearing on the application of microsatellite locus genotypes in diversity and phylogeographic assessment of wide-ranging species of mammals.

Six puma loci exhibited simple uninterrupted repeats (fig. 1*A*), and four loci exhibited compound uninterrupted or interrupted repeat structures (fig. 1*B*). Domestic cats also displayed six simple uninterrupted repeat loci (Menotti-Raymond et al. 1999). Four of these loci (FCA043, FCA090, FCA117, and FCA249) were homologous to puma simple uninterrupted repeat loci, and two (FCA008 and FCA262) were homologous to puma compound interrupted repeat loci. Two loci (FCA096 and FCA166) were compound in both species. Repeat numbers among puma microsatellite alleles ranged from 8 to 49 (table 1), and two loci (FCA035 and FCA166) had nonoverlapping allele repeat and size ranges between the puma and the domestic cat.

Marked differences in flanking regions immediately 5′ and 3′ of the repeat were detected between the domestic cat and the puma and contributed to allele size homoplasy between the species (fig. 1*A* and *B*). For 874 nt (the total alignment length of flanking sequence for 10 loci), the percentage of sequence difference between the cat and the puma was 5.0% (44 nt) ranging from a low of 2.0% (FCA262) to a high of 14.6% (FCA008).

A

FIG. 1.—*A*, Alignments of flanking sequences and the repeat region of the domestic cat (Fca-MR) and individual pumas (Pco) for six microsatellite loci which exhibit simple repeat structure in pumas (FCA035, FCA043, FCA082, FCA090, FCA117, and FCA249). A dot indicates identity to the reference Fca MR sequence; a dash indicates deletion of a nucleotide. Subspecies abbreviations (Culver et al. 2000) are in parentheses, with South American subspecies indicated in bold. [a] Total number of dimers for each allele; alleles with the same dimer number have the same allele product size. [b] Size in base pairs. [c] Repeat structure; SU (simple uninterrupted) consists of one uninterrupted tandemly repeated dimer; CU (compound uninterrupted) consists of two different and adjacent tandemly repeated dimers, and CI (compound interrupted) has one or more interruptions of the tandemly repeated dimers. [d] Includes dimers and tetramers. (GenBank accession numbers AF130487, AF130516, AF130527, AF130584, AF130510, AF130486, AF130589, AF130519, and AF130477; puma sequence GenBank accession numbers AF339931–AF340006). Domestic cat sequences, primer pairs for locus amplification, and genomic map position in the domestic cat are reported in Menotti-Raymond et al. (1999) for all markers but locus FCA166: 5'-AGGTATTCTTCATCCTAGGCA, 5'-TGTGCTGACAGCACCGAG. *B*, Alignments of flanking regions and repeat region of domestic cat and puma sequence for microsatellite loci which exhibit compound repeat structure in pumas (FCA008, FCA096, FCA166, and FCA262). ^ indicates a tandem array that varies in repeat number among pumas; *, ~, and † indicate homoplastic allele pairs; A, B, C, D, and E (locus FCA008) refer to regions of the repeat structure. [a-c] SU, CU, and CI as in A. PCR products from homozygous individuals were sequenced. Sequencing technology was such that it would have been possible to identify sequence variants in the two electromorphs for each individual. Each sequence represented in the table is therefore assumed to be represented two times in the data set. Eleven hundred sixty-eight is the sum of all pairwise comparisons of 12, 12, 12, 12, 24, 12, 18, 20, 18 sequences for Fca043, Fca090, Fca082, Fca117, Fca249, Fca035, Fca166, Fca262, Fca096, and Fca008, respectively. Five hundred sixty-two is the sum of all pairwise comparisons of 12, 18, 20, and 18 sequences for Fca166, Fca262, Fca096, and Fca008, respectively.

B

FCA166

Fca-MR clone																			dimersa	sizeb	structc
Fca-MR clone	5' ACACTAATTN	(AT)₁₉	(GT)₁₉	AT	GT	(AT)₂	GGTAAG	(ATTT)₃	(AG)₆	(ACAC)₆	AG	AC	AATGCAACCGCGGGANGGGCAGAGAGAGAGGANGNANAGAGNGNGTCCATGCAGG 3'					59ᵈ	206	CI
Pco-094 (ORE)	.........T	(AT)₃	(GT)₁₉	AT	GT	(AT)₂	....	(ATTT)₄	(AG)₉	(AGGG)₃	(AG)₁₃	AC	.............G............A.A.G....A.A........GC.					46ᵈ	174	CI
Pco-070 (KAI)	.........T	(AT)₃	(GT)₂₁	AT	GT	(AT)₂	....	(ATTT)₄	(AG)₁₀	(AGGG)₃	(AG)₁₃	AC	--............G............A.A.G....A.A........GC.					49ᵈ	180	CI
Pco-556 (MAY)	.........T	(AT)₃	(GT)₂₁	AT	GT	(AT)₂	....	(ATTT)₄	(AG)₉	(AGGG)₃	(AG)₁₃	AC	--............G............A.A.G....A.A........GC.					48ᵈ	178	CI
Pco-594 (MAY)	.........T	(AT)₃	(GT)₂₁	AT	GT	(AT)₂	....	(ATTT)₄	(AG)₉	(AGGG)₃	(AG)₁₃	AC	--............G............A.A.G....A.A........GC.					48ᵈ	178	CI
Pco-600 (CAP)	.........T	(AT)₃	(GT)₂₁	AT	GT	(AT)₂	....	(ATTT)₄	(AG)₁₀	(AGGG)₃	(AG)₁₃	AC	--............G............A.A.G....A.A........GC.					49ᵈ	180	CI
Pco-583 (ARA)	.........T	(AT)₃	(GT)₁₄	AT	GT	(AT)₂	....	(ATTT)₄	(AG)₉	(AGGG)₃	(AG)₁₃	AC	--............G............A.A.G....A.A........GC.					41ᵈ	164	CI
			^						^											

FCA262

Fca-MR clone	5'												TTTATGTATAGAAACATGCAGAAAAATCTTTGTTGAACTGAAGAGAAATTATCTTAAGGTTGCCATCGATGTGTGGTGTCCATCCAATTAAGATTATTCCG 3'	dimersa	sizeb	structc
Pco-109 (MIS)		(CA)₂	TA	(CA)₄	GA	CA	TA	(CA)₁₁	TA	(CA)₂₁			....T.	21	140	CI
Pco-249 (MIS)		(CA)₂	TA					(CA)₁₅	TA				....T.	22	142	CI
Pco-339 (MIS)		(CA)₂	TA					(CA)₁₄	TA				....T.	19*	136	CI
Pco-080 (HIP)		(CA)₂	TA					(CA)₁₄	TA				....T.	18	134	CI
Pco-691 (BRO)		(CA)₂	TA	(CA)₄	GA	CA	TA	(CA)₁₁	TA				....T.	22	142	CI
Pco-356 (STA)		(CA)₂	TA					(CA)₁₅	TA				....T.	19*	136	CI
Pco-586 (STA)		(CA)₂	TA	(CA)₆		TA	(CA)₈	TA					....T.	19*	136	CI
Pco-701 (ACR)		(CA)₂	TA	(CA)₆		TA	(CA)₉	TA					....T.	19*	136	CI
Pco-215 (PDM)		(CA)₃	TA	(CA)₆		TA	(CA)₁₀	TA					....T.	21	140	CI
				^			^									

FCA096

Fca-MR clone	5' CAGTGCAGAGCCTCCTTCGGGGATTCTGTCCCCTCTCTCTCGGCCATCCCTT				CAAATAAACATTTAAACAAAATAAATAAAAGTATGTATTTAATATGT 3'	dimersa	sizeb	structc
Pco-246 (ORE)	....GC.	(AC)₁₉	(TC)₁₈	AC	(TC)₁₂	....	38	173	CI
Pco-029 (STA)	....GC.	(AC)₁₀	(TC)₅	AC	(TC)₁₀	.................T.....................	28	153	CI
Pco-585 (STA)	.......G....................................GC.	(AC)₁₃	(TC)₁₂	AC	(TC)₁₁	.................T.....................	37*	171	CI
Pco-553 (COS)	.......G....................................GC.	(AC)₁₃	(TC)₁₃	AC	(TC)₁₂	.................T.....................	37*	171	CI
Pco-409 (INC)	.......G....................................GC.	(AC)₁₀	(TC)₅	AC	(TC)₁₂	.................T.....................	28	153	CI
Pco-582 (ACR)	.......G.............A......................GC.	(AC)₁₄	(TC)₁₀	AC	(TC)₁₁	.................T.....................	36	169	CI
Pco-580 (CAB)	.......G.............A......................GC.	(AC)₁₀	(TC)₂₃			.................T.....................	33	163	CU
Pco-218 (ARA)	.......G.............A......................GC.	(AC)₁₀	(TC)₂₁			.................T.....................	33	163	CU
Pco-583 (ARA)	.......G.............A......................GC.	(AC)₁₁	(TC)₁₈	AC		.................T.....................	30	157	CI
Pco-205 (PAT)	.......G.............A......................GC.	(AC)₁₂	(TC)₁₀	AC	(TC)₁₁	.................T.....................	34	165	CI
		(AC)₁₂	(TC)₁₀	AC	(TC)₁₁		34	165	CI
		^			^				

FCA008

Fca-MR clone	5' ACAGTTTAA											GCTAGTTACTTCAGGCATACAGGCTATCTCTTTTCTGCATA 3'	dimersa	sizeb	structc
Pco-324 (MIS)	.........-							(CA)₂₆	C............................C.........-T.G					26	100	SU
Pco-275 (ORE)	.........-			(CG)₄	(CA)₁₉		TA	(CA)₂	TA	C............-T.....G....C.........-T.G					36	118	CI
Pco-405 (CAL)	.........-				(CA)₂₆		TA	(CA)₂	TA	C............-T.....G....C.........-T.G					30*	106	CI
Pco-590 (AZT)	.........-			(CG)₄	(CA)₁₄		TA	(CA)₂	TA	C............-T.....G....C.........-T.G					36	118	CI
Pco-550 (COS)	.........-			CG	(CA)₁₄		TA	(CA)₂	TA	C............-T.....G....C.........-T.G					30*	106	CI
Pco-549 (COS)	.........-		TA	(CG)₅	(CA)₁₉	TA	TA	(CA)₂	TA	C............-T.....G....C.........-T.G					39-	124	CI
Pco-199 (CAP)	.........-				(CA)₁₆		TA	(CA)₂	TA	C............-T.....G....C.........-T.G					20+	86	CI
Pco-409 (INC)	.........-		TA		(CA)₁₅		TA	(CA)₂	TA	C............-T.....G....C.........-T.G					20+	86	CI
Pco-205 (PAT)	.........-	(CA)₄	TA	TG	CG	(CA)₃	(CG)₃	(CA)₁₀	TA	(CA)₇	TA	(CA)₃	TA	C............-T.....G....C.........-T.G		35	116	CI
		(CA)₁₅	GA			(CA)₂	TA	(CA)₂	TA					C............-T.....G....C.........-T.G		39-	124	CI
	^				^		^		^					^				

Repeat Regions	A	B	C	D	E

FIG. 1 (*Continued*)

Table 1
Numbers of Repeats, Lengths, and Complexity of Microsatellite Allelles Found in Domestic Cats and Pumas among 10 Microsatellite Loci

	DOMESTIC CAT[a]				PUMA			
LOCUS	N[b]	No. of Repeats	Length[c] (bp)	Rep. Struct.[d]	N[b]	Range in No. of Repeats	Length[c] (bp)	Rep. Struct.[d]
FCA043	1	17	88	SU	6	14–22	79–95	SU
FCA090	1	17	67	SU	6	8–20	49–73	SU
FCA117	1	24	121	SU	6	15–23	105–121	SU
FCA249	1	14	210	SU	6	11–16	202–212	SU
FCA082	1	19	198	CI	6	19–25	198–210	SU
FCA035	2	16–21	93–103	CI	12	8–14	77–89	SU
FCA166[e]	1	59	206	CI	6	41–49	164–180	CI
FCA262[f]	1	21	140	SU	9	18–22	136–144	CI
FCA096[f]	1	38	173	CI	10	28–37	153–171	CU, CI
FCA008[f]	1	26	100	SU	9	20–39	86–124	CI

[a] One domestic cat for each locus was sequenced from a recombinant clone selected in a library screen (Menotti-Raymond et al. 1999).

[b] Number of sequenced individuals.

[c] Sequence length includes the repeat and the flanking regions but not the primer.

[d] Repeat structure: SU (simple uninterrupted) consists of one uninterrupted tandemly repeated dimer, trimer, or tetramer; CU (compound uninterrupted) consists of two different uninterrupted tandemly repeated dimers, trimers, or tetramers; and CI (compound interrupted) occurs when the repeat has one or more interruptions from repeated or nonrepetitive DNA.

[e] At this locus, nine of the domestic cat and seven of the puma repeats are tetramers.

[f] This locus contained homoplastic alleles among pumas.

A total of 18 transitions and 16 transversions were observed. Ten flanking region insertion/deletion events were observed between the domestic cat and the puma at six loci, but these events did not cause large size shifts. The insertion/deletion events resulted in a 1-bp shift in allele size at 1 locus, a 2-bp shift in allele size between the domestic cat and the puma at 3 of the 10 loci, and a 3-bp shift at 1 locus. These differences would suggest that, due to flanking-region differences at 50% of the loci in this data set, interspecific comparisons of electromorphs would misclassify allele size relative to the number of repeat units by one repeat unit. Two loci (FCA035 and FCA166) displayed large shifts in allele size between the domestic cat and the puma; both were the result of repeat structure differences and not flanking-sequence changes.

Mutational differences in the structures of repeat units between cat and puma microsatellite homologs resulted in interrupted repeats of the long repeat arrays (fig. 1*B*). Such interruptions (particularly as observed in FCA008 and FCA262) can reduce the length of the repeat, which precedes the "decay" of the repeat region, a process suggested to lead to "death" of the microsatellite (Kruglyak et al. 1998; Taylor, Sanny, and Breden 1999). The sum of these differences between homologous puma and domestic cat microsatellite locus structures would raise questions around their efficacy in phylogenetic comparisons among species or genus-level comparisons of distantly related mammalian genera such as Puma versus Felis, estimated at 10 Myr divergence (Johnson and O'Brien 1997). In addition, compound repeat structures among four loci in the cat and four loci in the puma also contribute substantially to size homoplasy. In sum, approximately 80% of comigrating alleles between these two species reflect size homoplasy of sequentially distinctive alleles.

Within pumas, no differences in 874 bp of flanking DNA were observed across 10 loci, including 76 individuals homozygous for microsatellite alleles (fig. 1*A* and *B*). Thus, microsatellite estimates of population diversity and diversification were derived exclusively from the differences in repeat structural motifs. Similar invariance in microsatellite flanking regions have been observed in bees (Angers and Bernatchez 1997).

Three of the four interrupted repeat loci in pumas (FCA262, FCA096, and FCA008; fig. 1*B*) exhibited allele size homoplasy whereby identical size alleles displayed distinctive repeat structure compositions in different parts of the pumas' geographical range. Locus FCA262, a simple interrupted repeat in the puma, demonstrated size homoplasy among puma electromorphs containing 19 dinucleotide repeats (fig. 1*B*). Electromorphs at this locus had two distinctly different repeat structures: one electromorph with a pattern of $(CA)_6TA(CA)_8$, and a second electromorph of $(CA)_{15}$ in the variable regions of the repeat. A North American subspecies (MIS [Mississippi]) contained the $(CA)_{15}$ form, a South American subspecies (ACR [Brazil]) contained the $(CA)_6TA(CA)_8$ form, and a more centrally located subspecies (STA [Texas/Mexico]) contained both forms. Within the STA region, the individual from farther north (Texas) contained the northern form of the electromorph, and a puma from farther south (Mexico) contained the southern form.

Locus FCA096 consisted of a compound repeat that contained both uninterrupted and interrupted repeat structures within the puma (fig. 1*B*). This locus exhibited size homoplasy for the allele containing 37 dinucleotide repeats, and both electromorphs occurred in the STA subspecies. A compound uninterrupted allele at this locus (size 33) which does not exhibit size homoplasy appears to be regional, occurring only in two adjoining central South American subspecies (ACR and CAB).

The FCA008 locus, a simple uninterrupted repeat in the domestic cat, exhibited a complex compound interrupted repeat in the puma (fig. 1B). Three pairs of compound repeat alleles exhibited size homoplasy, those containing 20, 36, and 39 dinucleotide repeats. Different repeat regions of FCA008 (labeled A, C, and E in fig. 1B) occurred in alleles found throughout all geographical areas. However, alleles from south of Honduras contained dimers only in repeated regions B and D, resulting in a cline of repeat complexity at this locus. The other compound imperfect repeat locus (FCA166) contained a combination of dinucleotide and tetranucleotide repeats but exhibited no geographical component. Simple uninterrupted repeat loci (SU, fig. 1A) showed no apparent size homoplasy, although back mutation of repeat motif number represents a type of homoplasy not detectable by sequence analysis.

Of 1,168 possible pairwise allele comparisons (the sum of allele size pairwise combinations for each of 10 loci; fig. 1A and B), homoplasy was observed in 32 pairwise comparisons made between individuals (2.7%). Since alleles were sequenced using technology diagnostic for sequence polymorphism, each sequence was considered to represent two identical alleles. Thus, 97.3% of allele size comigrations within the puma species would represent authentic sequence identities.

The incidence of allele size homoplasy for a puma specieswide comparison is estimated as the product of included homoplastic allele sequence frequencies. Thus, for a microsatellite size allele of frequency p which includes two homoplastic sequences of equal frequency in our sequenced sample (as is the case for FCA262, FCA096, and FCA008; Fig. 1B), the estimated homoplasy frequency equals $(p/2)^2$. The observed allele frequencies across 277 sampled pumas (Culver et al. 2000) for size alleles which display homoplasy are as follows: FCA262 electromorph size 136 = 0.63; FCA096 electromorph size 171 = 0.16; FCA008 electromorph sizes 86, 106, and 124 = 0.04, 0.63, and 0.03, respectively. The estimated homoplasy incidences per locus are 9.92% for FCA262, 0.64% for FCA096, and 9.98% for FCA008. For the 10 sampled microsatellite loci, the average size homoplasy incidence is (9.92% + 0.64% + 9.98%)/10 = 2.05%. In a subset of the four compound loci (FCA008, FCA096, FCA166, and FCA262) where it was possible to observe size homoplasy, 32 of 562 pairwise comparisons (5.7%) exhibited size homoplasy. Thus, for comparisons within the puma, a species which exhibits considerable genomic diversity, the incidence of allele size homoplasy was rather low (2.1%–5.7%).

Alternatively, Taylor, Sanny, and Breden (1999) suggested that simple uninterrupted microsatellite homoplasy could be estimated by assuming that compound microsatellites have the same mutation rate and pattern as simple uninterrupted microsatellites. From our data (fig. 1B), dividing the number of allele sequences by the number of allele sizes (18/13 = 1.4), we estimate that for every microsatellite allele size, there are approximately 1.4 sequences, a somewhat smaller estimate than the 1.66 alleles estimated by Taylor, Sanny, and Breden (1999), but larger than what was actually observed.

In our sample, we observed five pairs of comigrating homoplastic alleles. Two pairs originated from North America, one pair originated from North America/South America, and two pairs originated from Central America/South America (fig. 1B). One pair (FCA096-171 nt) of alleles originated from the same subspecies, and four pairs (FCA262-136 nt, FCA008-106 nt, FCA008-124 nt, and FCA008-86 nt) were from widely different geographical areas. The overall incidence of allele size identity across all pumas was approximately 29%, but the estimates were even higher among geographically adjacent populations (~61%). Although comigrating alleles were more common within adjoining or closely adjoining subspecies, the comigrating alleles from distant geographic regions were more likely to exhibit size homoplasy, perhaps because spacial isolation over time increases the incidence of compound interrupted repeat allele formation. The pattern of compound repeat distribution over space was clinal in at least two cases (FCA008 and FCA262), providing additional information relevant to population assessment in phylogeographic analysis (see also Angers and Bernatchez 1997; Taylor, Sanny, and Breden 1999).

Models used to explain the mutational process at microsatellites rest on the assumption that differences between alleles are due entirely to changes in the number of repeat units (Tautz 1989; Weber 1990a). A number of genetic distance measures describing evolution at microsatellite loci also rely on the same assumption (Goldstein et al. 1995; Slatkin 1995). This data set examines this assumption on inter- and intraspecies levels. Among examined compound repeats, 74% of inferred mutational events were reflected by allele length differences within puma species. In contrast, the vast majority of mutational events between puma and cat alleles were not reflected in differences in allele length. The exact magnitude of interspecies changes could not be determined with any level of confidence due to extensive molecular signature changes within some of the repeat structures between the two species. These observations affirm the utility and power of microsatellite analyses in population and phylogenetic analyses within adequately sampled species (Culver et al. 2000). In contrast, the high incidence of size homoplasy between species identifies a potential bias around the efficacy of microsatellites in comparisons of distantly related species. Illustrated here and elsewhere, allele length comigrations of homologous loci from evolutionarily divergent species exhibit size homoplasy so frequently as to invalidate available phylogenetic models (Estoup et al. 1995; Garza, Slatkin, and Freimer 1995; Angers and Bernatchez 1997; Primmer and Ellegren 1998; Viard et al. 1998; Colson and Goldstein 1999).

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