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Brief Communication

Development of Nuclear Microsatellite Loci and Mitochondrial Single Nucleotide Polymorphisms for the Natterjack Toad, *Bufo* (*Epidalea*) *calamita* (Bufonidae), Using Next Generation Sequencing and Competitive Allele Specific PCR (KASPar)

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Abstract

Amphibians are undergoing a major decline worldwide and the steady increase in the number of threatened species in this particular taxa highlights the need for conservation genetics studies using high-quality molecular markers. The natterjack toad, *Bufo* (*Epidalea*) *calamita*, is a vulnerable pioneering species confined to specialized habitats in Western Europe. To provide efficient and cost-effective genetic resources for conservation biologists, we developed and characterized 22 new nuclear microsatellite markers using next-generation sequencing. We also used sequence data acquired from Sanger sequencing to develop the first mitochondrial markers for KASPar assay genotyping. Genetic polymorphism was then analyzed for 95 toads sampled from 5 populations in France. For polymorphic microsatellite loci, number of alleles and expected heterozygosity ranged from 2 to 14 and from 0.035 to 0.720, respectively. No significant departures from panmixia were observed (mean multilocus F_{IS} = –0.015) and population differentiation was substantial (mean multilocus F_{ST} = 0.222, $P < 0.001$). From a set of 18 mitochondrial SNPs located in the 16S and D-loop region, we further developed a fast and cost-effective SNP genotyping method based on competitive allele-specific PCR amplification (KASPar). The combination of allelic states for these mitochondrial DNA SNP markers yielded 10 different haplotypes, ranging from 2 to 5 within populations. Populations were highly differentiated (G_{ST} = 0.407, $P < 0.001$). These new genetic resources will facilitate future parentage, population genetics and phylogeographical studies and will be useful for both evolutionary and conservation concerns, especially for the set-up of management strategies and the definition of distinct evolutionary significant units.

Subject areas: Population structure and phylogeography; Conservation genetics and biodiversity
Key words: amphibians, *Bufo calamita*, conservation genetics, genetic diversity, Kompetitive allele-specific PCR, mtDNA SNPs, nuclear microsatellites, parentage

Amphibians have dramatically declined worldwide over the past 30 years (Beebee and Griffiths 2005). One of the major processes currently acknowledged to be involved in the increase of the number of threatened amphibian species is landscape fragmentation and associated habitat loss. The ensuing genetic erosion due to decreasing gene flow among populations and reduced effective population sizes lowers the evolutionary potential of populations (Allentoft and O'Brien 2010). Hence, conservation genetics studies of amphibians are essential for establishing conservation priorities and for the delineation of management units with adequate evolutionary capabilities (Beebee 2005; Igawa et al. 2015; McCartney-Melstad and Shaffer 2015).

The natterjack toad, *Bufo (Epidalea) calamita* (Laurenti, 1768) (see Dubois and Bour 2010 for recent changes in taxonomical status), has a wide geographical distribution range, extending from continental southern Europe (Spain and Portugal) to north-western Europe (Denmark and southern Sweden) and including Ireland and United Kingdom (Rowe et al. 2006). This emblematic pioneering species is confined to temporary ponds in highly specialized habitats as marshes, coastal sand dunes, or heathland habitats (Beebee and Denton 1996). In spite of its large geographical distribution, *B. calamita* is a highly protected species classified as rare and vulnerable, and suffering from the loss of suitable breeding ponds and favorable connected habitats, especially in the northern parts of its range (Allentoft et al. 2009). Consequently, this species now often inhabits human-made areas as substitution habitats, such as quarries, and is subject to numerous local conservation actions devoted to protect and reinforce isolated and declining populations (Beebee and Denton 1996; Godin 2002; Flavenot et al. 2015).

Nuclear microsatellite loci and mitochondrial DNA (mtDNA) markers are powerful tools to get insights into patterns of dispersal events among populations, to delineate maternal lineages and to identify evolutionary significant units for the set-up of subsequent conservation management strategies (Slatkin 1995; Frankham et al. 2010; Allendorf et al. 2013; Ritchie et al. 2016; Wirtz et al. 2016). The natterjack toad was among the first European amphibian for which microsatellite markers were isolated for population genetics and phylogeographical inferences (Rowe et al. 1997, 1998, 2000, 2006; Rogell et al. 2005). To gain further insights into fine-scaled population structure and individual siring success in the natterjack toad, this study aims at 1) isolating and screening an additional set of nuclear microsatellite markers of high resolution using high-throughput sequencing techniques and 2) developing fast and cost-effective mtDNA markers using the KBiosciences competitive allele-specific PCR amplification (KASPar™) of target SNPs. The KASPar method is simple and cost-effective as compared to other SNP genotyping assays: it allows bi-allelic scoring of SNPs through competitive binding of 2 allele-specific forward primers (Cuenca et al. 2013; Semagn et al. 2014). We successfully isolated and characterized 22 nuclear microsatellite markers and 18 mitochondrial SNP markers suitable to shed light on the intensity of gene flow among subdivided populations and to delineate maternal lineages of conservation relevance for remnant populations.

Materials and Methods

DNA Extraction

A nondestructive sampling method was used. Plain sterile 15SC Copan (Brescia Italia) swabs were used to collect buccal cells for *B. calamita* individuals which were released after sampling. Swabs were dried prior DNA extraction. Total genomic DNA was isolated from these buccal swabs using the Macherey Nagel (Düren, Germany) NucleoSpin® 96 trace kits following the manufacturer instructions.

Isolation of Nuclear Microsatellite Loci

Total genomic DNA was sent to the GenoScreen genomic platform, Lille, France (www.genoscreen.fr). A random pool of 8 toads was chosen for Shotgun sequencing of a 55.2 ng genomic library. A high-throughput method for isolating high-quality microsatellite markers for nonmodel organisms was then used as described in Malausa et al. (2011). By coupling next-generation sequencing and multiplex microsatellite enrichment, 1 µg of genomic DNA was used for the development of microsatellite libraries through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries, as in Favre-Bac et al. (2014) and Poux et al. (2015). GS-FLX libraries were realized according to manufacturer's protocols and sequenced on a GS-FLX Titanium PicoTiterPlate (Roche Diagnostics, Mannheim, Germany). Of 26 136 randomly fragmented sequences, 47 loci with the longest repeat sequences were initially tested for further development and routine genotyping on 8 individuals of *B. calamita*.

Nuclear Microsatellite Loci Genotyping

PCR reactions were performed in optimized cost-effective multiplexes that successfully amplified different sets of microsatellite loci in a single PCR reaction (Table 1). Forward primers for the selected loci were labeled with PET™, NED™, 6-FAM™, or HEX™ fluorescent dye (Applied Biosystem, Foster City, CA). We used 10 µL volume containing 1 µL (5–20 ng) of genomic DNA, 1X multiplex PCR master mix (QIAGEN Hilden, Germany), and 0.1–0.3 µM of labeled forward and reverse primer. The PCR cycling program had an initial denaturation of 95 °C for 15 min; 35 cycles of 94 °C for 30 s, annealing temperature of 55 °C for 1 min 30 s, and 72 °C for 1 min; and a final extension at 60 °C for 30 min. PCR was conducted on a Eppendorf Mastercycler pro 384 (Applied Biosystem). 1.5 µL of PCR product were pooled with 0.25 µL of GeneScan 500 LIZ size standard (Applied Biosystems) and 9.75 µL of deionized formamide (Applied Biosystems). The PCR amplicons were subsequently electrophoresed and sized using an ABI PRISM 3130 Sequencer (Applied Biosystems) and the software GeneMapper version 5 (Applied Biosystems), respectively. Individuals with failed amplifications or dubious genotypes were checked with a second round of genotyping.

mtDNA Acquisition from Sanger Sequencing

In addition to these nuclear microsatellite loci, we further identified mtDNA single nucleotide polymorphisms (SNPs) from mitochondrial sequence data acquired from Sanger sequencing. These mtDNA SNPs were located in the 16S and D-loop region (see GenBank accessions in Supplementary Table S1). Based on the entire mitochondrial genome of a related species (*Bufo japonica*, GenBank accession number: AB303363.1) described in Igawa et al. (2008), 2 mtDNA regions were selected for Sanger sequencing by adapting universal primers. The D-loop, situated in the control region, is known to be the most variable region along the circular mitochondrial genome (Vanbrabant et al., 2009). The 16S region covers conserved coding and hypervariable noncoding regions. The set of slightly modified universal primers were as follows: 16sar_F (CGCCTGTTTACCAAAAACAT) and 16sbr_R (CCGGTCTGAACTCAGATCACGT), LX12SN1_F (TACACACCGCCCGTCA) and LX16S1R_R (GACCTGGATTTCT CCGGTCTGAACTC), LX16S1_F (GGTTTACGACCTCGATGTTG

Table 1. Locus name, primer sequence (5′–3′), repeat motif from the original sequence, allelic size range (bp), multiplex number, fluorescent dye used, and the GenBank accession number for 22 newly isolated microsatellite loci in the natterjack toad *Bufo* (*Epidalea*) *calamita*

Locus name	Primer sequences (5′–3′)	Repeat motif	Allelic size range	Multiplex number	Dye	GenBank accession no.	A_n	H_o	H_e	F_{IS}	F_{ST}	R_{ST}
BC05	F: CATTGATATGGCTGCCAACTT R: CATGGGGATCAATGGCTACT	$(GT)_{13}$	106–116	1	NED	KX237573	4	0.548	0.519	−0.049^{NS}	0.275***	0.391***
BC08	F: CTCTTGTGCAAGATCTCTGGG R: TACTGACTGCTGCCCTCTCC	$(TAGA)_{11}$	241–279	1	HEX	KX237574	14	0.642	0.654	0.005^{NS}	0.137***	0.193***
BC11	F: AGCCTTCTTTGCATCACTGC R: TAGCGGGAAGAGATGTACGC	$(GATA)_{11}$	128–158	1	HEX	KX237575	8	0.581	0.594	0.035^{NS}	0.319***	0.320***
BC19	F: CCAAGGAAGAAACTGTGGCA R: AACATACATACACTCACACCCACA	$(TG)_{10}$	170–174	1	FAM	KX237576	3	0.502	0.451	−0.112^{NS}	0.365***	0.457***
BC22	F: TGCAGATTGCCAGCAGTTTA R: CACTTCCTCAAGGTGGTGCT	$(GATA)_9$	314–339	1	FAM	KX237577	7	0.696	0.720	0.074^{NS}	0.137***	0.243***
BC29	F: GTTGCGACTGGGGAAATAAC R: GCTTCACAAGACATGCAGGA	$(ATCT)_9$	172–188	1	NED	KX237578	5	0.550	0.602	0.032^{NS}	0.198***	0.177***
BC38	F: TACAGTTAAGGACCCCGTCG R: GGCCACTGTCCGTGGTTAC	$(CA)_8$	251	1	PET	KX237579	1	—	—	—	—	—
BC46	F: TGAATAGACAGACATTTGTCCAAGA R: TTCTACCGGTCAACCTATCCA	$(AGAT)_8$	111–131	1	FAM	KX237580	6	0.667	0.667	0.016^{NS}	0.155***	0.265***
BC01	F: TCCATAATCAGGCGCTCATA R: TCTATTCTCTTAAACCGGAGAGG	$(TAC)_{16}$	85–127	2	FAM	KX237581	9	0.564	0.519	−0.082^{NS}	0.177***	0.210***
BC02	F: TTGCTTGAGAAAAGTCCAACA R: ACTTGCCAACTCTCCCAGAA	$(GATA)_{14}$	191–218	2	FAM	KX237585	8	0.603	0.680	0.081^{NS}	0.166***	0.496***
BC04	F: TGCTCCTGACAATTAACTTTGG R: ATCTGTGTCAGGGCATCTCC	$(CA)_{13}$	132–143	2	NED	KX237584	6	0.630	0.597	−0.082^{NS}	0.178***	0.068***
BC24	F: ACGGTTTTCTGAAGCAATGG R: GCATGTGCAGAAGACTTCAAA	$(AC)_9$	206–213	2	NED	KX237586	4	0.610	0.623	0.034^{NS}	0.193***	0.271***
BC25	F: CAGTTGTTTTCCGAGGTGGT R: AAGGAAGCTGAATTTTGGTTGA	$(CT)_9$	201–207	2	PET	KX237587	3	0.187	0.178	−0.058^{NS}	0.450***	0.368***
BC28	F: ACTTTGGCAAGGGAACCAG R: TTGTCAGTTAACCAGCGTGC	$(GA)_9$	175–183	2	HEX	KX237588	3	0.487	0.432	−0.172^{NS}	0.242***	0.155***
BC34	F: TGCATAGCCTTGTGAAGCTG R: GTGACACCATGTCCCAGATG	$(AC)_9$	108–112	2	HEX	KX237582	3	0.381	0.361	−0.033^{NS}	0.424***	0.593***
BC35	F: GGGTATGGTGGCTGGTAATG R: TCACAAGTAGCCACAGTAAAGGA	$(TC)_9$	108–110	2	PET	KX237583	2	0.035	0.035	−0.021^{NS}	0.013^{NS}	0.013^{NS}
BC15	F: TGCTCCTCAAGTGTTGTTGG R: TGGGACGACAGGAACGTACT	$(AG)_{11}$	89–99	3	FAM	KX237589	4	0.222	0.180	−0.258^{NS}	0.204***	0.151***
BC18	F: CCTTAATGGCCCAAGCCTAT R: AGACAGGGATGGATAGATGGA	$(ATCT)_{10}$	175–191	3	FAM	KX237590	5	0.682	0.659	0.003^{NS}	0.135***	0.094***
BC37	F: TCACCTGTACCCCTCTGGG R: CCATCCATGACACAGACCAG	$(ATCT)_9$	87–116	3	HEX	KX237591	8	0.693	0.711	−0.015^{NS}	0.130***	0.243***

Table 1. *Continued*

Locus name	Primer sequences (5′–3′)	Repeat motif	Allelic size range	Multiplex number	Dye	GenBank accession no.	A_n	H_o	H_e	F_{is}	F_{st}	R_{st}
BC39	F: TCTGTCCTTCTGTCCAATCTG R: GCACCTTTGTTCAGGATGGT	$(TCTA)_8$	167–195	3	HEX	KX237592	8	0.690	0.640	-0.049^{NS}	0.142^{***}	0.310^{***}
BC09	F: GGTGGTGGCACATTTCTTTT R: GTAGTTTGCCAGCAATGCCT	$(TAGA)_{11}$	237–273	3	NED	KX237593	7	0.714	0.670	-0.058^{NS}	0.222^{***}	0.314^{***}
BC45	F: CCCTTGCAGCCAAAATAAAA R: TAACAGGAAACGGATTTGGG	$(TAGA)_8$	118–156	3	PET	KX237594	9	0.583	0.592	0.047^{NS}	0.338^{***}	0.300^{***}
Mean over all loci							5.77	0.509	0.504	-0.015^{NS}	0.222^{***}	0.273^{***}

Also presented are the mean measures of genetic diversity and genetic differentiation estimated over 5 populations (totalizing $N = 95$ toads): the total number of allele (A_n), the mean observed (H_o) and expected (H_e) heterozygosity, the mean intrapopulation fixation index (F_{is}), and the mean population genetic differentiation (F_{st} and R_{st}). NS, nonsignificant; —, not calculable.

$^{***}P < 0.001$.

GATCA) and Met3850H_R (GGTATGGGCCCAAAAGCTT), adding up to 3592 bp for the 16S region; Control BH_R (GTCCATTGGAGATTAAGATCTACCA) and ControlWrev-L_F (GACATACTATGTATAATCGAGCAT), CytA-L_F (GAATCGGGG GTCAACCAGTAGAAGACCC) and ControlK-H_R (AATGGTCA AAATGGCTGAGATTG) adding up to 1752bp for the D-loop region. PCR amplifications were performed in a Eppendorf Mastercycler pro 384 using 25 µL of mix comprising 1× of Buffer 10X (Dream *Taq* Buffer, ThermoFisher Scientific, Waltham, MA), 2.5 mM of $MgCl_2$, 2 µL (2.5 mM) of dNTP, 0.2 mM of BSA, 0.2 µM) of each primer, 0.652 U of Dream *Taq* polymerase (ThermoFisher Scientific) and 5 µL (5–20 ng) of template DNA. PCR conditions were as follow: 95 °C for 5 min; 40 cycles of 94 °C for 30 s, annealing temperature of 55 °C for 1 min, and 72 °C for 1 min 30 s; and a final extension step at 72 °C for 10 min. Raw data of sequences were read, verified, and aligned using CODONCODE Aligner v. 5.1.5 (CODONCODE Corporation, Centerville, MA) and BIOEDIT v. 7.2.5 (Hall 1999). Polymorphic SNPs were confirmed by a second round of PCR amplification with independent sequencing. A total of 94 individuals from different French localities were used for this first step of direct mitochondrial sequencing.

mtDNA SNPs Genotyping Using the KASPar Methodology

We describe here an accessible and cost-effective SNP genotyping method based on KBioscience's competitive allele-specific PCR amplification of target sequences and endpoint fluorescence genotyping (KASPar™) using a LightCycler 480 (Roche Diagnostics). From the initial direct sequencing, 18 polymorphic SNPs were identified and used for competitive allele-specific PCR amplification, Supplementary Table S1. SNP primers design for KASPar genotyping were defined by LGC group (http://www.lgcgroup.com/genomics) from each SNP locus flanking sequence. Two allele-specific oligonucleotides and 1 common oligonucleotide were defined for each locus (Supplementary Table S1). The KASPar system applies 2 fluorescence resonance energy transfer (FRET) cassettes, where fluorometric dye is conjugated to the primer but quenched via resonance energy transfer (reviewed in Semagn et al. 2014). KASPar genotyping is based on competitive allele-specific PCR and enables bi-allelic scoring of SNPs (e.g., Cuenca et al. 2013; Martin et al. 2016). The SNP-specific KASP Assay mix (designed by LGC group, www.lgcgroup.com) and the universal KASP Master Mix were added to DNA samples. A thermal cycling reaction was then carried out, followed by an end-point fluorescent read. Allelic discrimination was finally completed through competitive annealing of 2 allele-specific forward primers, each having a unique tail sequence corresponding to a distinct labeled FRET cassette in the Master Mix, one labeled with FAM™ dye and the other with HEX™ dye. Sequences flanking each SNPs and each specific and common primers that were used are detailed in Supplementary Table S1.

PCR amplification reactions were performed using either the Eppendorf Mastercycler pro 384 (Applied Biosystem) or the LightCycler 480 (Roche Diagnostics), with 4 µL (5–20 ng) of genomic DNA in 8 µL reaction volume containing 1X of universal KASP Master mix (2X) and 0.11 µL of the SNP-specific KASP Assay mix. This SNP-specific KASP Assay mix was composed of the 2 forward primers for allele X and Y, labeled in FAM™ and HEX™ respectively, and the common reverse primer, Supplementary Table S1. Cycling conditions for PCR amplifications were the following for all mtDNA SNPs but

one: 15 min at 94 °C, 10 cycles of 20 s at 94 °C and 60 s at 65 °C to 57 °C with a drop of 0.8 °C per cycle, 40 cycles of 20 s at 94 °C and 60 s at 57 °C, ending by 12 °C for 10 min. Cycling conditions for the remaining mtDNA SNP (Bc_ControlR_9_T/C) were as follows: 15 min at 94 °C, 10 cycles of 20 s at 94 °C and 60 s at 61 °C to 55 °C with a drop of 0.6 °C per cycle, 40 cycles of 20 s at 94 °C and 60 s at 55 °C, and finally 12 °C for 10 min. Fluorescence was detected using a LightCycler® 480 (Roche Diagnostics) and automatic allele calls were checked using the LightCycler 480 SW1.5.0 SP3 version 1.5.0.39 software (Roche Diagnostics GmbH). Lastly, as mitochondrial genome is inherited as a single linkage unit, individual mitochondrial haplotypes were defined as the combination of allelic states for the 18 detected mtDNA polymorphic SNP markers.

Population Sampling and Statistical Analyses

We tested the polymorphism of isolated suitable microsatellite markers and we validated the 18 SNP assays using KASPar genotyping chemistry on a diversity panel of 95 individuals coming from 5 geographically distinct populations located in France (Supplementary Figure S1). Names, sample sizes and coordinates (WGS84) of sampled populations are the following: "North coastline" (N = 20; Latitude: 1.5832, Longitude: 50.4869), "North mining area" (N = 30, Lat.: 2.7967, Long.: 50.4038), "Picardie" (N = 13; Lat.: 3.9334, Long.: 49.5534), "Lorraine" (N = 10, Lat.: 6.1050, Long.: 48.5509), and "Brittany" (N = 22; Lat.: −1.9920, Long.: 48.0160). Classic parameters of genetic diversity and genetic differentiation across the 5 surveyed populations were estimated for each loci using FSTAT version 2.9.3 (Goudet 1995).

Results and Discussion

Of the 47 nuclear microsatellite loci initially tested, 22 had easily readable PCR amplification products with no stutter peaks or dubious electrophenograms. Twenty-one markers out of the 22 tested were polymorphic. In order to reduce genotyping costs and efficiency, primer pairs were successfully combined into 3 multiplexes, each including 6 to height markers (Table 1). Out of 210 comparisons and after Bonferroni corrections, no linkage disequilibrium was observed for any pairs of loci. Moderate to high polymorphism was observed with clear sizing PCR products. Excluding the locus BC38 which was monomorphic, the observed number of alleles varied from 2 to 14 across loci (mean = 5.77), for a total of 127 alleles on the studied samples (Table 1). The mean observed (H_o) and mean expected heterozygosity (H_e) values ranged from 0.035 to 0.714 and 0.035 to 0.720, respectively (mean = 0.509 and 0.504). Mean F_{IS} estimates across populations and across loci (intrapopulation fixation index, measuring departures from panmixia) were all conform to Hardy–Weinberg expectations (i.e., not statistically significantly different from 0), with F_{IS} estimates varying from −0.258 to 0.074 for a mean multilocus value of −0.015. Except for locus BC35, most loci detected population genetic structuring as F_{ST} estimates (interpopulation fixation index, measuring the extent of population genetic differentiation) were highly significant (all at $P < 0.001$) and ranged from 0.130 to 0.450 with a mean multilocus estimate of 0.222. Estimates of R_{ST}, a similar statistic based on models which take into account the microsatellite evolution, especially the variance in allele length (Slatkin 1995), were also all significant (ranging from 0.094 to 0.593, with a mean estimate of 0.273) making these microsatellite loci markers excellent tools to detect phylogeographical signals.

KASPar genotyping data conformed to previous Sanger sequencing data and, out of the 18 mtDNA SNP primers tested, only 2 were monomorphic (Bc_ControlR_6_T/C and Bc_ControlR_19_C/A) across the 5 surveyed populations. The combination of allelic states for polymorphic mtDNA SNPs yielded a total of 10 different haplotypes. The within-population number of mtDNA haplotypes ranged from 2 to 5 (mean = 3), toad populations located in human-modified habitats ("North mining area" and "Picardie" populations) being the most diverse (Supplementary Figure S1). mtDNA genetic differentiation among populations was highly significant, with a G_{ST} estimate (estimator designed for mitochondrial variation and strongly related to F_{ST} index, see Pons and Petit 1996) of 0.407 ($P < 0.001$). For each mtDNA haplotype, the Supplementary Table S2 and the Supplementary Figure S1 give 1) their detailed allelic combinations, 2) their geographical distributions over the surveyed populations, and 3) their genealogical relationships based on a median-joining network. Finally, the KASPar technology proved to be very fast and cost-effective as compared to classical microsatellite genotyping and direct Sanger sequencing. Indeed, the genotyping cost per individual was over 4-fold and 14-fold cheaper for the KASPar SNP genotyping (runtime of 2 min) than for microsatellite genotyping (runtime of 45 min) and direct Sanger sequencing (runtime of 2 h 30 min), respectively.

Overall, the newly developed nuclear microsatellite markers show high levels of polymorphism and can be applied for low-cost high throughput genotyping. The same holds for mtDNA SNP markers that exhibited different maternal lineages within populations. Therefore, they will be markers of choice for fine and large-scale population genetics studies designed to infer inbreeding levels, effective population sizes and patterns of gene flow among populations, and to trace back phylogeographical patterns. In addition, they are proving useful for ongoing parentage analyses assessing male and female reproductive success, which is of immediate value for monitoring and conservation actions.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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Data Availability

Nuclear DNA sequences used to design the 22 microsatellite primers have been assigned to the following GenBank numbers: KX237573–KX237594. Mitochondrial DNA sequences used to define the 18 polymorphic SNPs have been assigned to the following GenBank number: KX237595–KX237630. Microsatellite and mtDNA genotypes are deposited in the Dryad repository: http://dx.doi.org/10.5061/dryad.6t2c4.

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