Isolation and characterization of twenty polymorphic microsatellite markers for the endangered Dupont's lark (*Chersophilus duponti*) and cross-amplification in crested lark (*Galerida cristata*) and thekla lark (*Galerida theklae*)

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Abstract We developed twenty microsatellite markers for the Dupont's lark (*Chersophilus duponti*), one of the most endangered European bird species, and tested in two related, more widely distributed species: crested lark (*Galerida cristata*) and thekla lark (*Galerida theklae*). 14 markers amplified and were polymorphic in Dupont's lark, 11 in crested lark and 6 in thekla lark. Microsatellite variability analyses were carried out on Dupont's lark (N = 23–28 individuals), crested lark (N = 9–10) and thekla lark (N = 14) populations in NE Spain showing moderate/high diversity, ranging from 1 to 13 alleles per locus. The mean allelic richness in Dupont's lark was 6.21 and expected and observed heterozygosities ranged from 0.195 to 0.848 and from 0.071 to 0.889, respectively. Crested and thekla larks showed moderate/high diversity with a mean allelic richness of 4.36 and 4.67, respectively. This microsatellite set could be useful for population genetic studies of lark species widely differing in population fragmentation and conservation status across the Old World.

Keywords Microsatellite · *Chersophilus duponti* · Dupont's lark · Alaudidae · Conservation genetics · Crested lark · Thekla lark

Larks (Family Alaudidae) are among the birds most affected by the degradation, loss and fragmentation of natural steppes in Europe (Tucker and Heath 1994). Particularly, past and current loss of natural steppes (Laiolo and Tella 2006; Vögeli et al. 2010) reduced the distribution of the highly habitat-restrictive and endangered Dupont's lark (*Chersophilus duponti*) to remnant patches of North Africa and Spain, resulting in current population size below 2,000 pairs (Tella et al. 2005). Here, we describe the isolation and characterization of 20 microsatellites developed for *Chersophilus duponti*, which were also tested on two related lark species crested (*Galerida cristata*) and theklae (*Galerida theklae*) larks, less affected by habitat loss and fragmentation (Laiolo and Tella 2006).

Birds were live-trapped in the Ebro Valley, NE Spain (see Vögeli et al. 2010) under license for blood extraction (see Vögeli et al. 2007 for details) and immediately released in the site of capture. DNA extractions were performed from blood samples conserved in absolute ethanol using a "salting-out" protocol (Gemmell and Akiyama 1996). Genomic libraries enriched for microsatellite repeats were constructed using a magnetic bead-based enrichment procedure (Jones et al. 2002). The DNA was incompletely digested with a cocktail of seven blunt-end restriction enzymes (RsaI, HaeIII, Bsr B1, PvuII, StuI, ScaI, Eco RV). Three hundred to 750 bp fragments were adapted with 20 bp oligonucleotides which contained a HindIII site at the 5' end, and subjected to magnetic bead capture using 5'-biotinylated TAGA8, ATG12, GA15, CA15 oligonucleotides as capture molecules. Captured molecules were

amplified using a primer complementary to the adaptor, digested with HindIII to remove the adaptor sequences, and ligated into the HindIII site of pUC19. The plasmids were then electroporated into *Escherichia coli* DH5a. Recombinant clones, identified by blue-white selection, were

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Table 1 Characterization of 20 microsatellites developed for *Chersophilus duponti*

Locus	Repeat motif	Accession no.	Primer sequences (5 ⁰ -3 ⁰)
A112	(GT) ₁₂	HM749625	F: GCTGGTGAAGTTAGGCTCTG R: AGTTTGTCTGCTGGAAGAC
A113	(CA) ₁₃	HM749626	F: AGGCTGAACTAAGTTGCATCC R: GTCTTCAAAATTGGGCATATTCATACC
A7	(CA) ₁₄	HM749627	F: TCCATACTGAACCCCTCTCAC R: TTGATAAATGCTGCCTAAGAG
B10	(TC) ₁₄	HM749629	F: AGGGAGATACATTGCTTGGAG R: CAGGGCTAAGAGAGAGAGGAC
B107	(GA) ₁₈	HM749630	F: TGCATCTGCTGTATCAAGTTC R: TGGGAAGCATAATTTTCTCTG
B118	(CT) ₁₃	HM749632	F: ACAGGATCTCTTTCATTGTCTT R: GTGTCTTGCACCTTGTTCAGACTCCTAC
B9	(TC) ₁₉	HM749634	F: AGGCTTGTGCTCAGTGTGTTGT R: CTAACGGACGGATTACAGTGG
C105	(TCA) ₆	HM749635	F: ACTGTGCATCACTTCCTCTGTA R: CAAAACCTTGTGGGTTGAGATG
C112	(TGA) ₁₀	HM749636	F: TTTAGCCCAAGATAAGTGACC R: GTGTCTTCATTCCACAGATGATCCTTTTC
C119	(ATC) ₈	HM749637	F: CTGAGCATCTTGAAAATCACTC R: AAACCTGTGCCTGAACTCTCTG
D10	(ATCT) ₁₆	HM749640	F: GCCAAGACTGAGTTGTAAAACA R: TCCCCACTGAAATAGACAGAC
D109	(GATA) ₁₆	HM749641	F: GGTCGATATTTGGTGGATAATG R: GTGTCTTTTGAAACCCACACTTCCTAGTC
D112	(GATA) ₁₅	HM749642	F: GGTGAGGGTGACTGAAGTTC R: TCAAAGCAATCAAGTGTGTC
D115	(GATA) ₁₆	HM749643	F: AAGAGCCTTTCAGGGACAC R: GCTGCCTTTATCTGTTTATCG
A109	(TG) ₁₃	HM749624	F: TTCTCCAAACCCTGAAACTG R: TCTTGATGTGAGGCACTGAC
A8	(TG) ₁₃ (GA) ₁₃	HM749628	F: TTCTTTCTGCTCTGTTCACAG R: TTGGACTTCCAGATGAATGTA
B7	(CT) ₁₅	HM749633	F: CACAATGGAGTGGTGTCTG R: GCAAAAATGGCTCAGTCTC
B111	(GA) ₁₃	HM749631	F: TGTGGTTACCAGAAGACTTC R: GCTCAGAACTGCACTGTAA
C2	(TGA) ₆	HM749639	F: AACCCAGTCCTTCGAGTG R: CCTAATGGACCCCAATTAAC
C120	(TCA) ₉	HM749638	F: GCACAACACCAAATCCAG R: TGGAATGCCTTGTCACAT

chosen arbitrarily for sequencing on an ABI 373, using ABI Prism Taq dye terminator cycle sequencing methodology.

Clones that contained at least six tandem repeats and long enough flanking regions were selected for primer design. Twenty primer pairs were designed using PRIMER 3 (Rozen and Skaletsky 2000). Forward primers were

synthesized with an M13 tail at their 5⁰-end (Sigma-Genosys Ltd) so that amplification products could be fluorescently labelled using M13 primers (Boutin-Ganache et al. 2001). (See Table 1 for primers details). PCR reactions were performed in a final volume of 25 μ l, which include 50–80 ng of DNA, 1.5–2.5 mM of MgCl₂, 0.25 μ M reverse primer, 0.017 μ M forward primer,

0.25 **1M** universal fluorescently labeled M13 primer, 0.25 mM dNTPs, 19 reaction buffer (7.5 mM Tris-HCl, 2.0 mM (NH₄)₂SO₄), 0.1 **1g/1L** of bovine serum albumin (BSA; Biomol) and 0.5 units of Taq polymerase (BIOTAQ, Bioline). Reactions conditions were as follows: an initial denaturation step of 2 min at 94°C, 16 cycles consisting of 30 s at 92°C, 30 s at 66 or 55°C (depending on the marker and the species, see Table 2) annealing temperature

decreasing one degree/cycle, and 30 s at 72°C, followed by 27 additional cycles consisting of 30 s at 92°C, and a final step of 5 min at 72°C. Products were analysed on an ABI 3100 DNA Genetic Analyser and alleles were scored with GeneMapper 4.0 (Applied Biosystems, Inc.).

PCR amplification were tested and optimized using eight individuals from different populations of Dupont's, crested and theklae larks. The polymorphism was assessed

Table 2 Cross-species amplification of the 20 microsatellite loci in *C. duponti*, *G. cristata* and *G. theklae*

Locus	Spp.	N	Status	NA	Size range (bp)	H _o	H _e	Fis	MgCl ₂ (mM)	T °C
A112	<i>C. duponti</i>	27	P	6	136–151	0.667	0.689	0.034	2	66–50
	<i>G. theklae</i>	14	P	4	135–141	0.643	0.732	0.126	2	55–40
	<i>G. cristata</i>	9	P	6	135–159	0.556	0.621	0.111	2	55–40
A113	<i>C. duponti</i>	23	P	12	234–263	0.782	0.879	0.112	2.5	66–50
	<i>G. theklae</i>	10	N	–	–	–	–	–	–	–
	<i>G. cristata</i>	10	N	–	–	–	–	–	–	–
A7	<i>C. duponti</i>	27	P	3	319–325	0.407	0.343	–0.192	2.5	66–50
	<i>G. theklae</i>	10	N	–	–	–	–	–	–	–
	<i>G. cristata</i>	10	N	–	–	–	–	–	–	–
B10	<i>C. duponti</i>	27	P	6	252–264	0.556	0.651	0.149	2.5	66–50
	<i>G. theklae</i>	10	M	1	250	–	–	–	–	–
	<i>G. cristata</i>	10	M	1	250	–	–	–	–	–
B107	<i>C. duponti</i>	25	P	6	210–220	0.640	0.721	0.114	1.5	55–40
	<i>G. theklae</i>	10	N	–	–	–	–	–	–	–
	<i>G. cristata</i>	10	N	–	–	–	–	–	–	–
B118	<i>C. duponti</i>	28	P	2	135–137	0.071	0.070	–0.019	2.5	66–50
	<i>G. theklae</i>	10	N	–	–	–	–	–	–	–
	<i>G. cristata</i>	10	N	–	–	–	–	–	–	–
B9	<i>C. duponti</i>	28	P	7	146–161	0.786	0.801	0.019	2	66–50
	<i>G. theklae</i>	14	P	6	133–151	0.429	0.439	0.025	2	55–40
	<i>G. cristata</i>	10	P	2	133–135	0.500	0.521	0.042	2	55–40
C105	<i>C. duponti</i>	28	P	2	158–161	0.393	0.363	–0.084	2.5	66–50
	<i>G. theklae</i>	10	N	–	–	–	–	–	–	–
	<i>G. cristata</i>	10	N	–	–	–	–	–	–	–
C112	<i>C. duponti</i>	28	P	5	166–182	0.2143	0.203	–0.055	2	66–50
	<i>G. theklae</i>	24	M	1	167	–	–	–	2	55–40
	<i>G. cristata</i>	10	P	3	169–179	0.600	0.563	–0.069	2	55–40
C119	<i>C. duponti</i>	28	P	3	189–198	0.679	0.663	0.026	2.5	66–50
	<i>G. theklae</i>	24	M	1	187	–	–	–	2	55–40
	<i>G. cristata</i>	10	P	2	187–193	0.300	0.395	0.250	2	55–40
D10	<i>C. duponti</i>	28	P	10	158–195	0.571	0.780	0.271*	2.5	66–50
	<i>G. theklae</i>	14	P	4	141–187	0.286	0.381	0.257	2.5	55–40
	<i>G. cristata</i>	10	P	4	141–187	0.300	0.437	0.325	2.5	55–40
D109	<i>C. duponti</i>	27	P	8	324–348	0.815	0.848	0.039	2.5	66–50
	<i>G. theklae</i>	10	N	–	–	–	–	–	–	–
	<i>G. cristata</i>	10	N	–	–	–	–	–	–	–
D112	<i>C. duponti</i>	27	P	10	296–329	0.815	0.804	–0.014	1.5	66–50
	<i>G. theklae</i>	10	M	1	–	–	–	–	–	–
	<i>G. cristata</i>	10	M	1	–	–	–	–	–	–

Table 2 continued

Locus	Spp.	N	Status	NA	Size range (bp)	H _o	H _e	Fis	MgCl ₂ (mM)	T °C
D115	C. duponti	27	P	7	268–292	0.889	0.832	–0.070	2.5	66–50
	G. thecklae	10	M	1	229	–	–	–	–	–
	G. cristata	10	M	1	229	–	–	–	–	–
A109	C. duponti	–	–	–	–	–	–	–	–	–
	G. thecklae	148	P	6	261–285	0.428	0.817	0.485*	2	55–40
	G. cristata	10	P	7	261–289	0.200	0.868	0.779*	2	55–40
A8	C. duponti	–	–	–	–	–	–	–	–	–
	G. thecklae	14	P	3	315–321	0.286	0.262	–0.095	2	55–40
	G. cristata	9	P	3	305–327	0.556	0.660	0.167	2	55–40
B7	C. duponti	–	–	–	–	–	–	–	–	–
	G. thecklae	24	M	1	296	–	–	–	2	55–40
	G. cristata	10	P	4	306–318	0.500	0.658	0.250	2	55–40
B111	C. duponti	–	–	–	–	–	–	–	–	–
	G. thecklae	14	P	6	199–225	0.714	0.780	0.088	2	55–40
	G. cristata	10	P	13	199–257	1	0.910	–0.104	2	55–40
C2	C. duponti	–	–	–	–	–	–	–	–	–
	G. thecklae	24	M	1	145	–	–	–	2	55–40
	G. cristata	10	P	2	141–145	0.200	0.189	–0.059	2	55–40
C120	C. duponti	–	–	–	–	–	–	–	–	–
	G. thecklae	24	M	1	304	–	–	–	2	55–40
	G. cristata	13	P	2	304–306	0.000	0.148	1	2	55–40

In column status: – no amplification, M monomorphic, P polymorphic, N number of individuals, NA number of alleles, H_o observed heterozygosity, H_e expected heterozygosity under Hardy–Weinberg equilibrium, Fis inbreeding coefficient

* Significant at 0.05

in 23, 9 and 14 individuals Dupont's crested and thekla larks respectively from the same population.

We calculated observed and expected heterozygosities and number of alleles per locus with Cervus ver. 3.0 (Marshall et al. 1998) and linkage disequilibrium and Hardy–Weinberg equilibrium with GENEPOP on the web (Raymond and Rousset 1995; Rousset 2008) and Genetix (Belkhir et al. 1996–1998). Sequential Bonferroni corrections were applied using a nominal P value of 0.05 (Rice 1989). MICROCHECKER version 2.2.3 (Van Oosterhout et al. 2004) and INEst10 (Chybicki and Burczyk 2009) were used to identify possible null alleles, large allele dropout, scoring errors, and typographic errors. We also performed individual jackknife procedures in order to identify sample-specific errors on Hardy–Weinberg equilibrium (Morin et al. 2009).

Fourteen loci were found polymorphic in Dupont's lark. The observed number of alleles, heterozygosities and inbreeding coefficients for each locus are listed in Table 2. None of the markers deviated from Hardy–Weinberg equilibrium, but marker D10 showed a significant deficit of heterozygotes and a significant inbreeding coefficient and was detected as a candidate for having null alleles,

although parallel analyses did not support this conclusion (Authors, unpubl. data). We found no evidences for linkage disequilibrium, and none of the samples was identified as influential on Hardy–Weinberg equilibrium.

Polymorphism and inbreeding were also examined in two Galerida species using the same methodology. Eleven and six loci amplified on crested and thekla lark respectively with the number of alleles ranging from 1 to 13 (Table 2). Only A109 marker showed a significant heterozygote deficit in both species, and was detected as a candidate for having null alleles.

We are confident that this set of markers will become an important tool for assessing the effects of a process of habitat loss and fragmentation on the population genetics of lark species differing in their susceptibility to habitat transformations.

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