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METHODS PAPER

A multiplex set for microsatellite typing and sexing of the European bee-eater (*Merops apiaster*)

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Abstract Microsatellite loci are widely used in ecological and evolutionary studies to assess inbreeding, genetic parentage and population structure. Such loci are often optimised in multiplexes to allow for economical and efficient use. Here, we tested 11 microsatellite loci designed for use in European bee-eaters (*Merops apiaster*), along with 31 loci isolated in other species, for their utility in European bee-eaters sampled on Susak Island, Croatia. Of these 42 loci, 20 were polymorphic in 38 individuals. These polymorphic loci were further assessed in a sub-set of 23 adults, excluding close relatives, and exhibited between three and 13 alleles each. All loci were autosomal, as indicated by the presence of heterozygotes in both males and females. One of the polymorphic loci exhibited low heterozygosity, three loci deviated from Hardy-Weinberg equilibrium and three pairs of loci displayed linkage disequilibrium. The remaining selected eight cross-species loci and seven loci isolated in European bee-eaters were combined with two sex-typing markers and optimised in five multiplexes. A combination of 15 autosomal loci of varying degrees of polymorphism makes this multiplex set particularly suitable for both parentage and spatial genetic analyses. This multiplex set therefore provides a useful toolkit for studying kin selection and population genetics in the cooperatively breeding European bee-eater and, potentially, in other closely related species.

Keywords Cooperative breeding · Cross-species markers · European bee-eater · *Merops apiaster* · Microsatellite markers · Multiplex set

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European bee-eaters (*Merops apiaster*) are a migratory bird species in the Meropidae family (order Coraciiformes). They breed in Southern Europe and the North of Africa and migrate to Southern Africa during winter (Fry 1984). Unlike many cooperatively breeding birds, European bee-eaters are a colonial species (Lessells et al. 1994). Colonies vary in size and can range from two up to several hundred breeding pairs (Fry 1984). Approximately 20 % of broods in this species obtain help from other individuals that have failed in their breeding attempts (Lessells 1990). Females have greater natal dispersal distances than males, although breeding-site fidelity is very common (Lessells et al. 1994). However, less is known about inter-colony dispersal (Dasmahapatra et al. 2004) and associated fine-scale genetic structure, features that may be important if we are to understand the evolution of various life-history traits such as cooperative breeding, coloniality and dispersal. Here, we characterise a set of polymorphic microsatellite loci, obtained via cross-species amplification, and combine these with existing European bee-eater loci (Dasmahapatra et al. 2004) and sexing markers (Dawson 2007) to create a powerful multiplex set. This multiplex set will facilitate the study of dispersal, parentage and kin selection in the European bee-eater and could potentially be of use in similar studies of closely related species.

A total of 42 microsatellite loci were tested for their utility in European bee-eaters, sampled on Susak Island, Croatia (45° 29′ 26″ N, 14° 19′ 46″ E). Eleven of these loci were previously developed for the European bee-eater (Dasmahapatra et al.

Table 1 Five microsatellite multiplex sets tested in adult European bee-eaters (*Merops apiaster*), including eight cross-species markers, seven markers isolated in European bee-eaters and two sex-typing markers

Multiplex	Marker	Source species & EMBL accession number & reference for primer set	Primer sequence (5′–3′)	Fluoro label	*N*	N_a	Allele size range (bp)	H_O	H_E	HWE *p* value	Est. null allele freq.
1	Be2.31	*Merops apiaster* AJ630048 Dasmahapatra et al. 2004	F: CTTCAGGCAAGTGACCACAG R: CAGAGGGACACCAGAGCTTC	6-FAM	21	7	165–181	0.86	0.84	0.886	−0.021
	Be2.33	*Merops apiaster* AJ630050 Dasmahapatra et al. 2004	F: CAGGAATGCTGTTGAACCTG R: ACTGTGCCTTGCTCACATTG	HEX	20	3	176–180	0.50	0.41	0.719	−0.135
	Mor12	*Merops ornatus* DQ304696 Adcock et al. 2006	F: CATTCAAAATACCCCAGTGC R: AGACTGCTTCGTCCTGATGC	HEX	23	3	209–213	0.26	0.27	0.158	0.053
	Mor20	*Merops ornatus* DQ304700 Adcock et al. 2006	F: CCACTTGTCTTTTTGTTCAAACC R: TCTCAGGTCAAGCTGAAACC	6-FAM	21	6	206–216	0.67	0.74	0.283	0.042
	Z002D	Dawson 2007	(Dawson 2007)	6-FAM	18 male	1	125, 125 (Z, Z)	0	0	–	–
					20 female	2	114, 125 (W, Z)	–	–	–	–
2	Bb111-TG	*Bubo bubo* AF432096 (Bb111) Klein et al. 2009	F: CTTTGTCAGTTTTCCCTGTAGC R: ATCTAAGCATTAAAAATGCARAYCTT	HEX	18	6	188–200	0.56	0.63	0.380	0.065
	HvoB1-TTG	*Haliaeetus vociferoides* AJ620441 (HvoB1) Klein et al. 2009	F: AAGCAAGGACTTTCCTTCCAG R: TCTCAAATTGGAACAGAGAAAGG	HEX	23	12	117–142	0.83	0.72	1.000	−0.112
	TG04-061	*Taeniopygia guttata* CK235034 Dawson et al. 2010	F: GACAATGGCTATGAAATAAATTAGGC R: AGAAGGGCATTGAAGCACAC	HEX	18	8	210–218	0.67	0.75	0.087	0.055
	Z002D	Dawson 2007	(Dawson 2007)	6-FAM	18 male	1	125, 125 (Z, Z)	0	0	–	–
					20 female	2	114, 125 (W, Z)	–	–	–	–
3	Be3.24	*Merops apiaster* AJ630052 Dasmahapatra et al. 2004	F: GATCACATTATCCTGCATGTG R: TTATGAAAGTCTACTTATTATGTGTCC	HEX	23	10	141–163	0.78	0.87	0.243	0.043
	Be3.9	*Merops apiaster* AJ630053 Dasmahapatra et al. 2004	F: GGATCTATAAACACATCTGCAT R: AAGGAATTACCTGCCCCTTA	6-FAM	23	13	162–190	0.78	0.89	0.257	0.058
	Mor10	*Merops ornatus* DQ304695 Adcock et al. 2006	F: GGGTGTCTGTACACCTTGTAGC R: AACGTGTTCCCAAAATCTCC	HEX	23	3	198–202	0.61	0.54	0.220	−0.068

Table 1 (continued)

Multiplex	Marker	Source species & EMBL accession number & reference for primer set	Primer sequence (5′–3′)	Fluoro label	*N*	N_a	Allele size range (bp)	H_O	H_E	HWE *p* value	Est. null allele freq.
	Z002D	Dawson 2007	(Dawson 2007)	6-FAM	18 male	1	125, 125 (Z, Z)	0	0	–	–
					20 female	2	114, 125 (W, Z)	–	–	–	–
4	Be1.29	*Merops apiaster* AJ630054 Dasmahapatra et al. 2004	F: TTTTCTCTGGGAGGTGGTTG R: GCTTGAAGGGGATTATGATAGC	HEX	23	11	147–167	0.83	0.85	0.657	−0.007
	Be19.2	*Merops apiaster* AJ630055 Dasmahapatra et al. 2004	F: GTCAAGTGGGCTGTTGGAG R: AAGAGGGGCTACTTCCAAGC	NED	23	7	174–186	0.83	0.83	0.419	−0.013
	Be2.52	*Merops apiaster* AJ630049 Dasmahapatra et al. 2004	F: GGAGTCATCTAGGCCCATCC R: TTTCCCGAGGCAGTGTAAAG	NED	18	13	214–240	0.83	0.90	0.431	0.024
	Z002B	Dawson 2007	(Dawson 2007)	6-FAM	18 male	1	247, 247 (Z, Z)	0	0	–	–
					20 female		241, 247 (W, Z)	–	–	–	–
5	CAM-15	*Taeniopygia guttata* HG518773 Dawson et al. 2013	F: SGACGACTCCTTTATTTCCC R: TTCTGACTTCCYCAGGTAACAC	6-FAM	21	7	261–273	0.81	0.82	0.519	−0.005
	TG13-017	*Taeniopygia guttata* CK313422 Dawson et al. 2010	F: GCTTTGCATCTTGCCTTAAA R: GGTAACTACAACATTCCAACTCCT	HEX	22	4	209–217	0.50	0.58	0.712	0.072
	Z002B	Dawson 2007	(Dawson 2007)	6-FAM	18 male	1	247, 247 (Z, Z)	0	0	–	–
					20 female	2	241, 247 (W, Z)	–	–	–	–

N is the number of genotyped adult European bee-eater (*Merops apiaster*) individuals from Susak Island, Croatia, N_a is the number of alleles, H_O is the observed heterozygosity, H_E is the expected heterozygosity and *Est. null allele freq.* is the estimated null allele frequency

2004), and we assessed these loci for their utility in our study population. The remaining 31 loci were selected based on their high utility in multiple other species (Dawson et al. 2010, 2013), their utility in species belonging to the same order as European bee-eaters ($N = 7–23$; Appendix 1) or both. Five of these loci were originally isolated from the closely related Rainbow bee-eater (*Merops ornatus*; Adcock et al. 2006), and 26 were of high cross-species utility, including 24 that were previously characterised in a related but more distant Coraciiform species, the European roller (*Coracias garrulous*; Martín-Gálvez et al. 2014).

Blood samples (ca 50 μl) were collected by puncturing the brachial vein of 38 European bee-eaters from 15 colonies on Susak Island in 2014. These samples were stored in 1 ml of 96 % ethanol in rubber-sealed screw-topped microfuge tubes. We extracted genomic DNA via an ammonium acetate precipitation method (Nicholls et al. 2000) and performed PCR amplification in a 2-μl volume. Each PCR reaction contained approximately 15 ng of genomic DNA, 0.2 μM of each primer (except for *Be2.52* [0.6 μM], *Be3.9* [1.0 μM], *CAM-15* [0.4 μM], *Mor14* [0.6 μM] and *TG13–017* [0.6 μM]), HotStar*Taq* DNA polymerase, $MgCl_2$ and dNTPs supplied at the concentrations stated in the manufacturer's buffer (QIAGEN Multiplex PCR Master Mix). Forward primers were fluorescently labelled and PCR products were amplified using a DNA Engine Tetrad PTC-225 Peltier thermal cycler (MJ Research).

Loci of high cross-species utility and those tested in the European roller were amplified in singleplexes across all 38 individuals with the following PCR profile: 95 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 56 °C for 90 s and 72 °C for 60 s and a final step of 60 °C for 30 min. The Rainbow and European bee-eater loci were initially assessed in multiplex reactions (designed using Multiplex Manager 1.2) in seven adults that were not close relatives ($R \leq 0.126$; GenAlEx 6.41, Peakall and Smouse 2006) with the same PCR constituents, including 0.2 μM of each primer (except for *Be2.52* [0.6 μM], *Be3.9* [1.0 μM] and *Mor14* [0.6 μM]), and the same PCR profile. PCR products were separated on an ABI 3730 DNA Analyser with an ABI ROX500 size standard and alleles scored using GENEMAPPER 3.7 (Applied Biosystems).

We found 20 loci to be polymorphic in seven to 38 individuals. Five loci were monomorphic and 17 failed to amplify a product (Appendix 1). The five monomorphic loci in this population may be variable and so of utility in other populations. Locus *Be72* is the same as *Be3.9*, which we tested under the latter name and found polymorphic (Adcock et al. 2006). Polymorphic loci were amplified as multiplex PCRs across all 38 individuals using the PCR profile stated above. Each multiplex set included a marker of known utility for sexing European bee-eaters; *Z002B* or *Z002D* (Dawson 2007). Loci were then characterised across a sub-set of 23 European bee-eaters, by selecting one male and one female adult from each colony to minimise overlapping generation and social structure effects. All 20 loci were autosomal as heterozygotes occurred in both sexes (18 males and 20 females assessed). One autosomal locus (*TG01–000*) amplified successfully in singleplex but failed to amplify in multiplex PCR (Appendix 1). Another locus (*Be48*) was polymorphic with only three alleles and little genetic variation in the Susak Island individuals ($H_O = 0.09$, Appendix 1). Despite the relatively low variability among Susak Island individuals, this locus may be suitable for population structure analyses as heterozygosity was higher ($H_O = 0.29$) in a French European bee-eater population (Dasmahapatra et al. 2004).

We used data from the 23 adult European bee-eaters to test each locus for deviation from Hardy-Weinberg Equilibrium (HWE) and for linkage disequilibrium among groups of loci (GENEPOP 4.2; Rousset 2008). Three loci (*Be2.16*, *Mor14* and *Mor15*) deviated from HWE and were not included in the final multiplex set (Appendix 1). Loci *Be2.16* and *Mor15* also displayed high null allele frequency estimates (>0.10; CERVUS 3.0.03, Kalinowski et al. 2007). After correcting for multiple testing (Benjamini and Hochberg 1995), three pairs of loci (*TG04–061 & CAM-15*, *Mor10 & Be19.2* and *Mor10 & Bb111-TG*) displayed linkage disequilibrium ($p < 0.05$). We therefore omitted data from *CAM-15* and *Mor10* from the analyses of relatedness. The final 15 autosomal loci (eight cross-species loci combined with seven loci isolated in European bee-eaters) were checked and confirmed unique using BLAST software (Altschul et al. 1997). These 15 loci, optimised in five multiplexes, will be useful in future studies, especially when combined with the two sex-typing markers (Table 1). In particular, the marker set will facilitate both fine-scale and large-scale population genetic structure analyses, and facilitate parentage assignment and relatedness estimation for kin selection studies. More specifically, this multiplex set will provide an efficient and economical method for investigating the social dynamics of European bee-eaters and, potentially, closely related species.

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Compliance with ethical standards

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Ethical approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

This article does not contain any studies with human participants performed by any of the authors.

Appendix

Appendix 1 Details of the 42 microsatellite markers tested in the European bee-eater (*Merops apiaster*). Markers in italics and underlined were included in the final multiplex set

Marker	Source species & EMBL accession number & locus name (when different)	Reference for primer set	Reason for testing	Fluoro label	Plex used in initial test	*N*	N_a	Allele size range (bp)	Outcome
ApCo46-ZEST	*Aphelocoma coerulescens* AF520885 (ApCo46)	Stenzler et al. 2002	1, 2	6-FAM	Singleplex	7	–	–	PCR failed
ApCo80-CEST	*Aphelocoma coerulescens* AF520879 (ApCo80)	Stenzler et al. 2002	1, 2	HEX	Singleplex	7	–	–	PCR failed
Bb111-TG	*Bubo bubo* (AF432096, Bb111), *Taeniopygia guttata*, *Gallus gallus*	Klein et al. 2009	1, 2	HEX	Singleplex	18	6	188–200	Polymorphic, see Table 1
Be1.29	*Merops apiaster* AJ630054	Dasmahapatra et al. 2004	3	HEX	Multiplex	23	11	147–167	Polymorphic, see Table 1
Be19.2	*Merops apiaster* AJ630055	Dasmahapatra et al. 2004	3	NED	Multiplex	23	7	174–186	Polymorphic, see Table 1
Be2.16	*Merops apiaster* AJ630047	Dasmahapatra et al. 2004	3	NED	Multiplex	23	5	189–195	Polymorphic but rejected due to high estimated null allele frequency (0.251) and deviation from HWE ($p = 0$)
Be2.31	*Merops apiaster* AJ630048	Dasmahapatra et al. 2004	3	6-FAM	Multiplex	21	7	165–181	Polymorphic, see Table 1
Be2.33	*Merops apiaster* AJ630050	Dasmahapatra et al. 2004	3	HEX	Multiplex	20	3	176–180	Polymorphic, see Table 1
Be2.46	*Merops apiaster* AJ630051	Dasmahapatra et al. 2004	3	6-FAM	Multiplex	7	1	187	Monomorphic
Be2.52	*Merops apiaster* AJ630049	Dasmahapatra et al. 2004	3	NED	Multiplex	18	13	214–240	Polymorphic, see Table 1
Be24	*Merops apiaster* AJ630044	Dasmahapatra et al. 2004	3	HEX	Multiplex	8	–	–	PCR failed; did not amplify as singleplex or multiplex
Be3.24	*Merops apiaster*		3	HEX	Multiplex	23	10	141–163	Polymorphic, see Table 1

Appendix 1 (continued)

Marker	Source species & EMBL accession number & locus name (when different)	Reference for primer set	Reason for testing	Fluoro label	Plex used in initial test	*N*	N_a	Allele size range (bp)	Outcome
		Dasmahapatra et al. 2004							
	AJ630052								
Be3.9	*Merops apiaster*	Dasmahapatra et al. 2004	3	6-FAM	Multiplex	23	13	162–190	Polymorphic, see Table 1
	AJ630053								
Be48	*Merops apiaster*	Dasmahapatra et al. 2004	3	6-FAM	Multiplex	23	2	151–153	Polymorphic but rejected due to low heterozygosity (H_O = 0.09)
	AJ630045								
CAM-06	*Taeniopygia guttata* (HG518764), *Gallus gallus*	Dawson et al. 2013	1	HEX	Singleplex	7	–	–	PCR failed
CAM-12	*Taeniopygia guttata* (HG518770), *Gallus gallus*	Dawson et al. 2013	1, 2	6-FAM	Singleplex	7	–	–	PCR failed
CAM-13	*Taeniopygia guttata* (HG518771), *Gallus gallus*	Dawson et al. 2013	1, 2	HEX	Singleplex	7	–	–	PCR failed
CAM-15	*Taeniopygia guttata* (HG518773), *Gallus gallus*	Dawson et al. 2013	1, 2	6-FAM	Singleplex	21	7	261–273	Polymorphic, see Table 1
CAM-16	*Taeniopygia guttata* (HG518774), *Gallus gallus*	Dawson et al. 2013	1, 2	HEX	Singleplex	14	–	–	PCR failed
CAM-18	*Taeniopygia guttata* (HG518776), *Gallus gallus*	Dawson et al. 2013	1, 2	HEX	Singleplex	7	–	–	PCR failed
CAM-20	*Taeniopygia guttata* (HG518778), *Gallus gallus*	Dawson et al. 2013	2	HEX	Singleplex	7	–	–	PCR failed
CAM-24	*Taeniopygia guttata* (HG518782), *Gallus gallus*	Dawson et al. 2013	1,2	HEX	Singleplex	7	–	–	PCR failed
HvoB1-TTG	*Haliaeetus vociferoides* (AJ620441, HvoB1), *Taeniopygia guttata, Gallus gallus*	Klein et al. 2009	1, 2	HEX	Singleplex	23	12	117–142	Polymorphic, see Table 1
Mor10	*Merops ornatus* DQ304695	Adcock et al. 2006	2	HEX	Multiplex	23	3	198–202	Polymorphic, see Table 1

Appendix 1 (continued)

Marker	Source species & EMBL accession number & locus name (when different)	Reference for primer set	Reason for testing	Fluoro label	Plex used in initial test	*N*	N_a	Allele size range (bp)	Outcome
Mor12	*Merops ornatus* DQ304696	Adcock et al. 2006	2	HEX	Multiplex	23	3	209–213	Polymorphic, see Table 1
Mor14	*Merops ornatus* DQ304697	Adcock et al. 2006	2	NED	Multiplex	23	5	195–207	Polymorphic but rejected due to deviation from HWE ($p = 0.044$)
Mor15	*Merops ornatus* DQ304698	Adcock et al. 2006	2	NED	Multiplex	22	9	158–180	Polymorphic but rejected due to high estimated null allele frequency (0.254) and deviation from HWE ($p = 0.002$)
Mor20	*Merops ornatus* DQ304701	Adcock et al. 2006	2	6-FAM	Multiplex	21	6	206–216	Polymorphic, see Table 1
Pte24-CEST	*Pomatostomus temporalis* DQ234870 (Pte24)	Blackmore et al. 2006	2	HEX	Singleplex	14	–	–	PCR failed
SAP47-ZEST	*Sayornis phoebe* AY823673 (SAP47)	Beheler et al. 2007	1, 2	6-FAM	Singleplex	7	–	–	PCR failed
Tc.11B4E-CEST	*Telespiza cantans* AF036266 (Tc.11B4E)	Tarr et al. 1998	1, 2	6-FAM	Singleplex	7	1	153	Monomorphic
TG01-000	*Taeniopygia guttata* (CK314156), *Gallus gallus*	Dawson et al. 2010	1, 2	6-FAM	Singleplex	14	2	205–207	Polymorphic, but, although it amplified in singleplex, failed in multiplex
TG01-040	*Taeniopygia guttata* (DV576233), *Gallus gallus*	Dawson et al. 2010	1, 2	6-FAM	Singleplex	7	1	288	Monomorphic
TG02-078	*Taeniopygia guttata* (CK305233), *Gallus gallus*	Dawson et al. 2010	1, 2	HEX	Singleplex	14	–	–	PCR failed
TG03-002	*Taeniopygia guttata* (DV575298), *Gallus gallus*	Dawson et al. 2010	1, 2	6-FAM	Singleplex	14	1	119	Monomorphic
TG03-098	*Taeniopygia guttata* (DV573670), *Gallus gallus*	Dawson et al. 2010	1, 2	HEX	Singleplex	14	–	–	PCR failed

Appendix 1 (continued)

Marker	Source species & EMBL accession number & locus name (when different)	Reference for primer set	Reason for testing	Fluoro label	Plex used in initial test	*N*	N_a	Allele size range (bp)	Outcome
TG04-012	*Taeniopygia guttata* (CK306810), *Gallus gallus*	Dawson et al. 2010	1, 2	HEX	Singleplex		–	–	PCR failed
TG04-061	*Taeniopygia guttata* (CK235034), *Gallus gallus*	Dawson et al. 2010	1, 2	HEX	Singleplex	18	8	210–218	Polymorphic, see Table 1
TG05-053	*Taeniopygia guttata* (CK314425), *Gallus gallus*	Dawson et al. 2010	1, 2	6-FAM	Singleplex	14	–	–	PCR failed
TG11-011	*Taeniopygia guttata* (CK308096), *Gallus gallus*	Dawson et al. 2010	1, 2	6-FAM	Singleplex	7	–	–	PCR failed
TG13-017	*Taeniopygia guttata* (CK313422), *Gallus gallus*	Dawson et al. 2010	1, 2	HEX	Singleplex	22	4	209–217	Polymorphic, see Table 1
Tgu06	*Taeniopygia guttata* CK307697	Slate et al. 2007	1, 2	HEX	Singleplex	7	1	172	Monomorphic

N is the number of European bee-eater (*Merops apiaster*) individuals tested/genotyped from Susak Island, Croatia, *Na* is the number of alleles. Please note, many of these markers were donated from earlier studies and failure to amplify could be a result of the age of the primers.

Reason for testing:

1: Marker of known high cross-species utility (Dawson et al. 2010, 2013, DAD unpublished data)

2: Marker possessed proven utility in a related Coraciiform species: Rainbow bee-eater (*Merops ornatus*; Adcock et al. 2006) or European roller (*Coracias garrulous*; Martín-Gálvez et al. 2014)

3: Locus isolated from study species European bee-eater (*Merops apiaster*, Dasmahapatra et al. 2004)

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