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Utility of several microsatellite markers for the genetic characterisation of three ex situ populations of threatened caprine taxa (*Capra aegagrus*, *C. cylindricornis* and *C. falconeri*)

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Abstract. Caprines belong to the most endangered group of mammals and artiodactyls suffering from many negative human impacts. Fortunately, many of them are protected and managed by national and international legislation and in situ and ex situ conservation actions. Although many microsatellite markers have been developed for wild and domestic caprines, they remain uninvestigated in respect of their utility for some taxa. We examined the utility of the International Society for Animal Genetics microsatellite set for genetic characterisations of three wild and one domestic *Capra* species from captive or semi-captive ex situ populations in Europe. Our data suggest the utility of this microsatellite set for detecting shared and species-specific alleles, characterising the genetic variability, and determining phylogenetic relationships and intraspecific structures in investigated taxa. We detected a depleted genetic variability in *Capra falconeri* and *Capra cylindricornis* in European ex situ populations; unrelated individuals are therefore needed for improving genetic variability parameters, as they are for the extralimital population of *Capra aegagrus* in the Vřísek game reserve (Czech Republic), for which we identified no genetic introgression from the domestic goat and great dissimilarity with some analysed individuals from European zoos. Current results here indicate some difficulties with the historical evidence, for example with respect to the origin and purity of particular individuals under breeding programmes.

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1 Introduction

Caprines (Caprini *sensu*, Groves and Grubb, 2011) represent a rather evolutionarily young group of bovids, but one that is certainly no less diverse in its morphological, ecological and behavioural features than other groups of ungulates (Groves and Leslie, 2011; Hassanin et al., 2012; Bibi, 2013). Caprines exhibit extraordinary adaptations to many habitats, including the harshest environments of our planet, but despite that, they are not resistant to negative human impacts (e.g. Shackleton, 1997). They suffer from habitat destruction, hunting, competition with domestic livestock, disease from domestic livestock, and also introgression from their domestic counterparts that originated in part from them (Shackleton, 1997; Groves and Leslie, 2011). In effect, Caprini belong to the most endangered group of artiodactyls, with approximate twice as many endangered and critically endangered species as other bovid groups (e.g. Bovini, Antilopini; our counting is based on Groves and Leslie, 2011). Fortunately, many of them are protected and managed by national and international legislation and specific in situ and ex situ conservation actions, and genetic markers are increasingly being used for genetic characterisation (e.g. genetic variability parameters, genetic purity) of caprine species and their particular populations (e.g. Maudet et al., 2002; Hammer et al., 2008; Gebremedhin et al., 2009). To avoid negative effects of outbreeding and inbreeding, management actions should be based upon a large number of purebred individuals; genetic variability parameters, structure and purity are relatively easily and unequivocally detectable with a good resolution by genetic markers analysed by population-genetic and phylogenetic methods (e.g. Allendorf and Luikart, 2007; Frankham et al., 2003). The study of microsatellite loci of *Capra* species is very important for effective scientific management from the prospective of maintaining the wild (sub)species/domestic breed purity and from the perspective of genetic variability parameters.

Microsatellite markers with a high polymorphism are one of the best tools for detecting the genetic fitness of whole populations or even of particular individuals, including paternity determination (e.g. Witzenberger and Hochkirch, 2011). Although many microsatellite markers have been developed for wild and domestic caprines (Arevalo et al., 1994; Bhebhe et al., 1994; Bishop et al., 1994; Crawford et al., 1995; Kemp et al., 1995; Kogi et al., 1995; Ma et al., 1996; Vaiman et al., 1996; Luikart et al., 1999, 2006; Maudet et al., 2001, 2002, 2004; Kumar et al., 2009), they remain uninvestigated in respect of their utility for some taxa. We examined the utility of the ISAG (International Society for Animal Genetics) microsatellite set, available primarily for the domestic goat paternity specification, for genetic characterisations of three wild and one domestic *Capra* species from captive or semi-captive ex situ populations from EAZA (European Association of Zoos and Aquaria) or private institutions in central Europe – specifically *Capra falconeri heptneri*, *C. cylindricornis*, *C. aegagrus* and *C. hircus*. One population, of *Capra aegagrus* in the Vřísek game reserve (near Česká Lípa, northern Bohemia, managed by Forests of the Czech Republic, s. e.), is associated historically with a relatively well-known out-of-range population of this species in the Pálava Biosphere Reserve (southern Moravia), where several (the precise number is unknown) wild goats were released in the 1950s from Prague and Brno zoos (Ernst et al., 2011). The wild goats occupied the steppe and surrounding habitats under some game control management for 43 years, until in 1996 the remaining individuals were captured and transferred to the Vřísek game reserve due to damage to valuable steppe flora in the Pálava reserve (e.g. Heroldová, 1997; Ernst et al., 2011).

From here, the studied taxon *C. falconeri heptneri* is actively managed under the European Endangered Species Programme breeding programme, and *Capra cylindricornis* is managed under the European StudBook breeding programme of the EAZA (Holma, 2007; Fainstein, 2011). The studied taxa meet these International Union for Conservation of Nature criteria: wild goat (*Capra aegagrus*) – vulnerable (Weinberg et al., 2008); East Caucasian tur (*C. cylindricornis*) – near threatened (Weinberg, 2008); markhor (*Capra falconeri*) – endangered (Valdez, 2008). Although the domestic goat (*Capra hircus*) is not threatened on the whole, some specific breeds are threatened or extinct (Taberlet et al., 2008). All wild species analysed here show decreasing population trends (Valdez, 2008; Weinberg, 2008; Weinberg et al., 2008).

This study represents an attempt to analyse the above-mentioned taxa in respect of ISAG microsatellite set utility and to assess detected genetic parameters in the conservation management programmes for these taxa.

2 Materials and methods

2.1 Sample collection

We studied a total of 55 individuals of *Capra aegagrus* (hereinafter referred to as CA), of which $n = 50$ were from the Vřísek game reserve and $n = 5$ from the Olomouc zoo; 26 individuals of *Capra falconeri* (hereinafter referred to as CF), subspecies *C. f. heptneri*, from one breeding institution in Slovakia; 10 individuals of *Capra cylindricornis* (hereinafter referred to as CC) from breeding institutions in the Czech Republic; and 9 individuals of various local breeds of *Capra hircus* (hereinafter referred to as CH) typical for the Czech Republic in order to detect possible genetic introgression to CA. From 2008 to 2012, blood samples were collected from the jugular veins of adult animals by a sampling kit comprising an anticoagulant (150 µL 0.5 M EDTA/5 mL of blood). As for juvenile animals, hair bulbs were sampled and mouth cavity smears were acquired with the oral smear kit, to minimise stress. As for harvested or deceased individuals, the skeletal muscles were used for DNA isolation. All proce-

Table 1. Genetic variation analysed by eight microsatellite markers.

Locus (reference)	Primer sequence (5′ → 3′) Forward (F)/Reverse®	FM	PC	T	IA	*n*	Ng	Na	MAF	He	Ho	PIC	*F*
HSC (Glowatzki-Mullis et al., 2007)	F:CTGCCAATGCAGAGACACAAGA R:GTCTGTCTCCTGTCTTGTCATC	FAM	480	CA	271, **283**, *285*, **287**, **293**, **301**	55	6	6	0.6727	0.4766	0.4545	0.404	0.0467
				CF	271, 273, ***281***	26	4	3	0.6923	0.4796	0.5385	0.4212	−0.125
				CC	***269***, 271	10	2	2	0.9500	0.1000	0.1000	0.0905	0.0000
				CH	**267**, 273, *285*	9	5	3	0.5556	0.6013	0.5556	0.4889	0.0805
INRA0063 (Vaiman et al., 1994)	F:GACCACAAAGGGATTTGCACAAGC R:AAACCACAGAAATGCTTGGAAG	FAM	160	CA	**166**, 172, 174, *176*	55	5	4	0.5909	0.5024	0.5818	0.391	−0.16
				CF	174, 176, ***178***	26	5	3	0.4808	0.6237	0.6923	0.5327	−0.113
				CC	*172*, 176	10	3	2	0.6500	0.4789	0.5000	0.3515	−0.0465
				CH	172, 174, *176*	9	3	3	0.7778	0.3856	0.4444	0.3267	−0.1636
SRCRSP0024 (Yeh et al., 1997)	F:AGCAAGAAGTGTCCACTGAACAG R:TCTAGGTCCATCTGTGTTATTGC	FAM	320	CA	**144**, **146**, **152**, 158, 160, *164*	55	10	6	0.3636	0.7014	0.7636	0.6364	−0.09
				CF	158, *160*, 164	26	3	3	0.6923	0.4638	0.6154	0.3914	−0.336
				CC	*164*	10	1	1	1.0000	0.0000	0.0000	0.0000	NI
				CH	**150**, *158*, 160	9	4	3	0.5556	0.6209	0.7778	0.5174	−0.2727
ILSTS19 (Kemp et al., 1995)	F:AGGGACCTCATGTAGAAGC R:ACTTTTGGACCCTGTAGTGC	HEX	320	CA	***140***, 148, 150, **152**	55	4	4	0.9091	0.172	0.0545	0.1652	0.6848
				CF	148, *150*	26	3	2	0.6731	0.4487	0.5	0.3432	−0.117
				CC	***172***	10	1	1	1.0000	0.0000	0.0000	0.0000	NI
				CH	**146**, 148, *150*, **154**	9	5	4	0.5000	0.6601	0.5556	0.5567	0.1667
INRA005 (Bishop et al., 1994)	F:TTCAGGCATACCCTACACCACATG R:AAATATTAGCCAACTGAAAACTGGG	HEX	80	CA	114, 116, *118*	55	4	3	0.7182	0.4135	0.3455	0.3329	0.1659
				CF	114, **122**, **136**, ***138***	26	5	4	0.5769	0.5943	0.6154	0.5249	−0.036
				CC	*114*	10	1	1	1.0000	0.0000	0.0000	0.0000	NI
				CH	**112**, *114*, 116, 118	9	6	4	0.4444	0.6732	0.6667	0.5652	0.0103

Table 1. Continued.

Locus (reference)	Primer sequence (5′ → 3′) Forward (F)/Reverse®	FM	PC	T	IA	*n*	Ng	Na	MAF	He	Ho	PIC	*F*
MAF0065 (Bishop et al., 1994)	F:AAAGGCCAGAGTATGCAATTAGGAGGAG R:CCACTCCTCCTGAGAATATAACATG	NED	480	CA	***110***, 120, **122**, 124, 130, **134**	55	8	6	0.6455	0.5344	0.4545	0.4833	0.1507
				CF	**116**, *118*	26	3	2	0.6923	0.4344	0.3846	0.3353	0.1166
				CC	***108***	10	1	1	1.0000	0.0000	0.0000	0.0000	NI
				CH	118, 120, *124*, 130, **136**	9	5	5	0.4444	0.7124	1.0000	0.6173	−0.4400
SRCRSP005 (Arevalo et al., 1994)	F:GGACTCTACCAACTGAGGTACAAG R:TGAAATGAAGCTAAAGCAATGC	NED	800	CA	160, ***162***, 166, 170, **172**, **176**	55	7	6	0.9	0.1892	0.109	0.1833	0.4255
				CF	**168**, *170*, **178**	26	5	3	0.6346	0.5181	0.3846	0.4354	0.2614
				CC	*170*	10	1	1	1.0000	0.0000	0.0000	0.0000	NI
				CH	160, 166, *170*, **174**	9	5	4	0.3889	0.7516	0.7778	0.6571	−0.0370
SRCRSP008 (Bhebhe et al., 1994)	F:TGCGGTCTGGTTCTGATTTCAC R:CCTGCATGAGAAAGTGGATGCTTAG	NED	800	CA	221, *229*, 239	55	6	3	0.4455	0.6027	0.6364	0.5108	−0.057
				CF	*225*, 229	26	2	2	0.8077	0.3167	0.3846	0.2624	−0.22
				CC	not identified	NI	NI	NI	NI	NI	NI	NI	NI
				CH	***219***, 221, 225, 229, 239	9	5	5	0.5000	0.6928	0.8889	0.6035	−0.3061
Mean				CA		55	6.25	4.75	0.6556	0.449	0.425	0.3884	0.054
				CF		26	3.75	2.75	0.6563	0.4849	0.5144	0.4058	−0.062
				CC		10	I.25	1.125	0.8250	0.1938	NI	0.1802	−0.0385
				CH		9	4.75	3.88	0.5208	0.6373	0.7083	0.5416	−0.1193

Abbreviations: FM – fluorescence marking, PC – primer concentration (nM), T – taxon, IA – identified alleles (species-specific ones are in bold, alleles with the highest frequency are in italic), *n* – sample size, Ng – numbers of genotypes, Na – number of alleles, MAF – major allele frequency, He – expected heterozygosity, Ho – observed heterozygosity, PIC – polymorphic information content, *F* – inbreeding coefficient.

dures were carried out in accordance with the laws and ethical guidelines established in the Czech Republic and during the course of regular veterinary work with these animals. All biological samples were stored at −85 °C except for hair bulbs, which were stored in paper bags at temperatures not exceeding 24 °C.

2.2 Microsatellite genotyping and analyses

Genome DNA was isolated by the QIAamp DNA blood kit. Target DNA sequences comprising potential polymorphic loci were amplified by the polymerase chain reaction (PCR). We tested a modified multiplex typing panel of microsatellite loci according to panels of goat markers for parentage verification tested using the 2001/2002 ISAG comparison test by the ISAG Standing Committee on "Applied Genetics in Sheep and Goats". The panel allows the co-amplification and four-colour detection of eight microsatellite markers (multiplex1 proposed by LGS, Cremona, Italy); for more details see Table 1.

A Veriti thermal cycler (Applied Biosystems) was used for the PCR 8-plex. The total volume of the amplification mixture amounted to 6.25 µL. The reagency concentration

amounted to 1.2× PCR buffer (comprising 15 mM $MgCl_2$), 336 µM dNTP mixture, 0.9 U Taq Gold polymerase (AmpliTaq Gold™, Applied Biosystems), 3 % DMSO, and 10–100 ng/µL DNA. Primers of selected microsatellite markers were used in concentrations ranging from 80 to 800 nM (see Table 1). Primer concentration, marking and sequence are listed under Table 1. The following cycling conditions were applied: initial denaturation 95 °C for 4 min, in 31 cycles; 94 °C/30 s, 55 °C/30 s, 72 °C/60 s, final elongation 72 °C 60 min, and termination at 4 °C.

A total of 0.5 µL of ROX 500 size standard (Applied Biosystems) and 11.5 µL Hi-Di™ unionised formamide (Applied Biosystems) were added to the amplification product of 0.5 µL. Samples were denaturised for 5 min at 95 °C and then laid on ice for 5 min. Polymorphism of studied loci was detected with ABI PRISM 310® genetic analyser (Applied Biosystems, Foster City, CA, USA). Fluorescent markers were evaluated by GeneScan® 3.7 NT and Genotypern® 3.7 NT (Applied Biosystems) software.

Basic genetic parameters (see Table 1) were calculated using the PowerMarker v3.25 (Liu and Muse, 2005), exclusion probabilities and combined exclusion probabilities (CEPs) according to Jamieson and Taylor (1997). Deviations from the Hardy–Weinberg and linkage equilibrium were analysed with GENEPOP v.4.2.1 (Rousset, 2008), while phylogenetic relationship and population structuring of studied taxa were detected using MEGA5 (Tamura et al., 2011) and STRUCTURE version 2.3.4 (Pritchard et al., 2000; see also Falush et al., 2003, 2007; Hubisz et al., 2009).

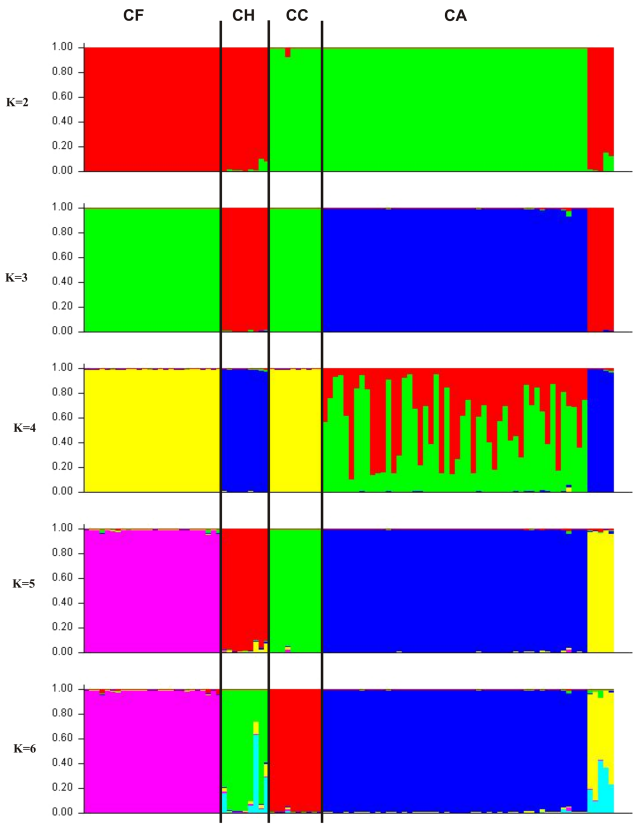

Figure 1. Results of the STRUCTURE analysis of the *Capra* microsatellite markers studied here.

3 Results and discussion

Our data proved the utility of the used microsatellite set for the understanding of genetic variability and fitness of analysed populations (for details see Table 1). For example, we found polymorphism at all loci of CA, CF and CH, but at only two loci of CC (one locus was not amplified). CA exhibits the most species-specific alleles, as well as number of alleles per locus and number of genotypes, followed by CH, CF and CC. Our study enlarges the number of known alleles, for example in CA (Maudet et al., 2004; Glowatzki-Mullis et al., 2007). On the contrary, however, Pokorádi et al. (2006) detected more alleles for CF and Luikart et al. (1999) for CH, probably due the higher sample size. As CH comprise a lot of breeds (Taberlet et al., 2008), our sample of local Czech breeds cannot reflect the diversity of this one properly. Alleles with the highest frequency do not correspond in most cases with species-specific alleles, and the highest average frequency of main alleles was found in CC; it was almost identical in CF and CA, and the lowest frequency was recorded in CH.

As the heterozygosity is considered one of the main indices of viability and adaptability of animal populations, the average heterozygosity and PIC were highest in the CH (comparable with Jandurová et al., 2004, and Luikart et al., 1999, specifically in the white short-haired, brown short-haired and Saanen goat breeds) followed by CF (higher than Pokorádi et al., 2006), CA (much higher than CA from the game park in Berne; Saitbekova et al., 1999), and the lowest was recorded in CC (lower than Maudet et al., 2004). Limited genetic variability in CF and CC is certainly associated with a limited number of founders of the captive populations (see Holma, 2007; Fainstein, 2011) and is comparable with populations of *Capra* species with historical bottlenecks and associated depleted genetic variability such as *C. ibex* (detected heterozygosity 0.13–0.4; Maudet et al., 2002; Biebach and Keller, 2009) or *C. walie* (0.35; Gebremedhin et al., 2009). New founders and/or their gametes using the AI procedure are of great conservation importance for their long-term ex situ management.

When assessing the population, the Hardy–Weinberg balance was calculated for CA $\chi^2 = \infty$, df = 16, $P <$ Highly sign.; CF $\chi^2 = 11.5950$, df = 16, $P < 0.7714$; CH $\chi^2 = 7.6063$, df = 16, $P < 0.9597$. However, it was impossible to calculate it for CC. The probabilities of paternity exclusion, one parental genotype unavailable, and parentage exclusion were, for a panel of eight microsatellites, 99.49, 94.81 and 99.99 % respectively in all goat samples.

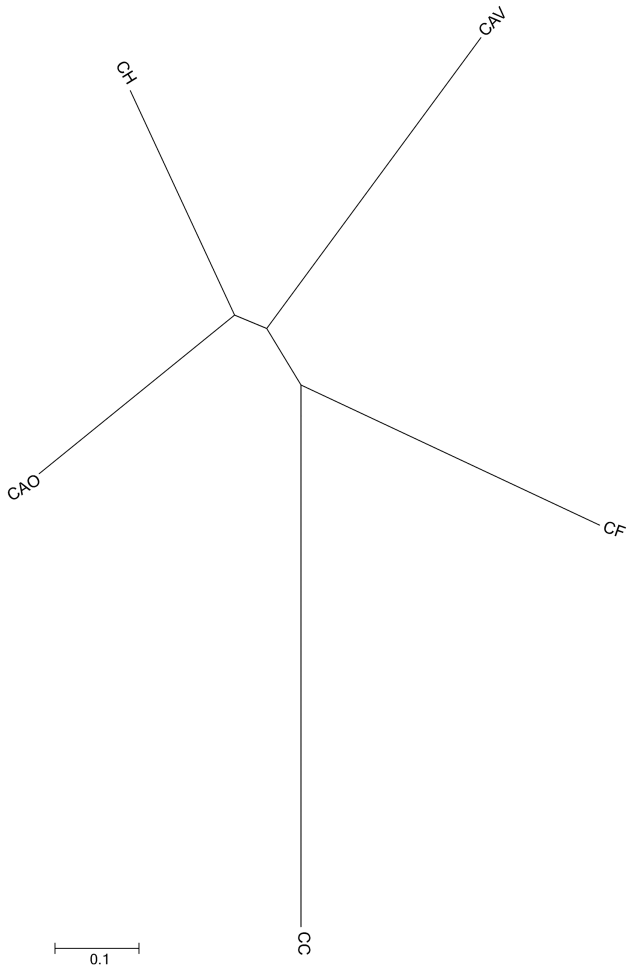

Figure 2. Topology of neighbour-joining tree showing the genetic relationship among four *Capra* populations using genetic distances for eight microsatellite loci.

All analysed taxa were differentiated by STRUCTURE (Fig. 1) with $K = 5$. Moreover, the CA set is split up in two independent sets: CA from the Vřísek game reserve and CA from the Olomouc zoo. When analysing the polymorphism of studied microsatellite loci, no influence of CH on the CA population from the Vřísek game reserve ($n = 50$) was established. On the contrary, CA from the Olomouc zoo ($n = 5$) shows a distinctive genetic link to CH, as demonstrated by $K = 2$, $K = 3$, $K = 4$, and $K = 6$, although this stock could, based on some historic evidence, have the same link to the Pálava-founding CA population. Based on our evidence, though, this link seems improbable. Our study also indicates that CA from the Pálava population at the Vřísek game reserve is genetically valuable and has no genetic introgression from the CH as was assumed by Anděra and Červený (2009). It is appropriate to point out that the material is incomplete (e.g. in relation to the study of Naderi et al., 2008) and that the whole issue is more complex than work on very different species and on wild species with no domesticated counterparts, as both species are historically related with possible mutual gene flow. Our study also detected no introgression of domestic goats into the CF stock in the European zoos (Hammer et al., 2008), which points to a high breeding-value of the analysed portion of CF.

The neighbour-joining tree (Fig. 2) shows the differentiation of two lines, namely CA together with CH and CC together with CF. All species exhibit comparable divergences from the ancestral stock, except for CC, which shows more changes unique to that group. The genus *Capra* is relatively young, with speciation still in progress (Hassanin et al., 2012). In effect, the phylogenetic relationships of species within the group depend on the genetic markers used (Luikart et al., 2001, 2006), possibly due to incomplete lineage sorting and/or also former natural hybridisation events (Pidancier et al., 2006). Our results are more similar to the taxa relationships presented in Luikart et al. (2001).

In general, the information presented here indicates some difficulties with historical evidence, for example with respect to the origin and purity of particular individuals, and underlines the need to use genetic methods for valuable science-based conservation management of ex situ populations.

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All procedures were carried out in accordance with the laws and ethical guidelines established in the Czech Republic and during the course of regular veterinary work with these animals. The authors declare that they have no conflict of interest.

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