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PRELIMINARY ANALYSIS OF GENETIC VARIABILITY IN MONTAGU'S HARRIER (*CIRCUS PYGARGUS*) USING CROSS-AMPLIFIED MICROSATELLITES

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Abstract.— The aim of our study was to find suitable molecular markers for genetic studies of the population of Montagu's harrier *Circus pygargus*. We used the cross-species amplification strategy to test the usefulness of 24 primer pairs, amplifying the microsatellite loci of several other members of Accipitridae. The analysis was performed on 139 Montagu's harriers from breeding populations in Spain and Poland. We found an amplification success of 50%; however, the level of polymorphism in cross-amplified microsatellites was low, especially in terms of heterozygosity. We did not find significant differences in genetic variability, estimated based on microsatellite markers, between breeding populations from Spain and Poland. The level of genetic differentiation between these two populations was low (F_{ST} = 0.016), although significant. An analysis of genotypes of nestlings in 10 nests suggested one case of extra-pair paternity.

Key words.— microsatellites, cross-species amplification, Montagu's harrier, *Circus pygargus*, genetic variability.

INTRODUCTION

The hawks of the Circinae sub-family are one of the poorest recognized birds of Accipitridae in terms of population genetics and molecular ecology. Until now, only Montagu's harrier (*Circus pygargus*) has gained the attention of researchers. An analysis of mitochondrial genes (*t-RNA Trp*, *ND2* and *COI*) was used mainly to investigate the genetic consequences of demographic changes and to estimate an effective population size, following the glacial and interglacial periods, in the species' European population (Garcia *et al.* 2011), as well as to expand knowledge about the recent evolutionary history and phylogeography of steppe-associated species. The obtained data also suggested that the European breeding population is rather weakly genetically structured, with high dispersal ability and relaxed philopatry as the main reason for this. Simultaneously, a small but significant genetic differentiation between birds breeding in south-western and north-eastern Europe was also suggested.

The Montagu's harrier is interesting not only in terms of phylogeography – this is a rare and endangered raptor, included by the European Committee in the Birds Directive Appendix I (priority species). At least in some countries of central and eastern Europe

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(i.e. Poland), the size of the breeding population and individuals had declined significantly in 1970's and 1980's, but since then, seems to have systematically increased (Tomiałojć and Stawarczyk 2003). However, present trends in population size are difficult to determine. Nonetheless, two demographic trends are presently observed: the disappearance of nesting sites in wetland areas (marshes and river valleys) and a simultaneous increase of the number of birds nesting in cereal crops, which now appear to be the commonest nesting habitat of the species in western Europe, as well as in Poland (Krogulec 1997, Arroyo *et al*. 2002, Zieliński 2007, Krupiński *et al*. 2012). The genetic after-effects of these changes are still unknown, for example: (i) has the genetic variability been reduced in populations which experienced decline in the number of individuals; and, (ii) is there any genetic differentiation between birds nesting in natural humid environments and populations nesting in crop fields?

In Poland, a clear dichotomy in feeding preferences was found. Birds nesting in wetland areas feed mainly on small mammals, birds and invertebrates (mainly *Tetigonidae*), whereas harriers in crop fields prefer birds and insects, which is typical for western European populations (Butet *et al*. 1993, Salamolard *et al*. 2000, Kitowski 2001, Kitowski 2003, Arroyo *et al*. 2004, Mirski *et al*. 2009). It is still unclear if feeding preferences and choice of breeding sites represents phenotypic plasticity within the species or is breeding isolation between populations from different habitats inducing genetic differentiation. Moreover, the species exhibits interesting breeding habits – Montagu's harriers often breed in semi-colonies and it was estimated that extra-pair copulation (EPC) might have consisted of 4–8% of all observed copulations (Arroyo 1999). However, it is still unclear how many EPCs lead to extra-pair fertilizations. Some of these questions were addressed by Garcia *et al*. (2011), who applied mitochondrial DNA, but additionally, bi-parentally inherited markers would be useful for estimating genetic variability within populations, genetic differentiation among them and to characterize some aspects of philopatry and breeding biology.

Molecular markers are widely used in ornithology for wide range of purposes, ranging from conservation and management (Haig *et al*. 2011) to identification of species-specific parasites (Kehlmaier and Quaisser, 2013). Microsatellite markers are one of the most popular molecular tools in population genetics and molecular ecology (reviewed in: Jarne and Lagoda (1996), Chistiakov *et al*. (2006), Selkoe and Toonen (2006). Many strategies have been described for *de novo* isolation of useful microsatellites from genomes of species that are being analysed for the first time (Zane *et al*. 2002), including high-throughput sequencing (e.g. Castoe *et al*. 2012). However, because these procedures still require a skilled molecular biologist and a considerable investment of time and resources, researchers often apply a cross-species amplification strategy that is based on using PCR primers described for microsatellite loci in one species (the source species) to amplify homologous microsatellites in other species (the target species). This concept has been widely used and found to be very useful, for example, in taxa with a low frequency of microsatellites within a genome, such as butterflies or birds (Primmer *et al*. 1996, Galbusera *et al*. 2000, Rutkowski *et al*. 2006, 2009, Mitrus *et al*. 2013). However, the application of cross-species microsatellite amplification has some limitations: the strategy works best for species belonging to the same genus or to recently separated genera (Scribner and Pearce 2000) and, in many cases, a given microsatellite may fail to amplify or may be less or even non-polymorphic in target species (Rubinstein *et al*. 1995, Rutkowski *et al*. 2011). Thus, application of a cross-species strategy requires preliminary research to assess the amplification success of particular markers and their level of polymorphism in the target species, as only polymorphic microsatellites could be successfully used in population or ecological studies.

According to our knowledge, no species-specific microsatellite markers have been identified in the genome of Montagu's harrier until now. However, the cross-species experiment indicated that the application of microsatellites from other raptors could be very efficient for this species (Heap *et al*. 2011). Hence, we applied information available for other raptor species to amplify the microsatellites of 139 birds from central Spain and Poland. Apart from selecting a useful set of polymorphic markers, in addition to the microsatellites described by Heap *et al*. (2011), we also wanted to compare the genetic variability of these two breeding populations and estimate the genetic differentiation between them. We expected some differences in the level of genetic variability because the Spanish population of this species is large and has been relatively stable for long time, whereas the Polish breeding population has been recently increasing or has stabilised after some reduction of breeding pairs in the 1980's, at least in some areas (Krupiński 2013). However, short term estimates from 2007–2012 performed as a part of the project: 'Monitoring of Birds of Poland', showed a clear, massive reduction (50%) of the Montagu's harrier population (MBP 2013). Moreover, the Iberian Peninsula could also be a 'genetic stronghold' of the species (Garcia *et al*. 2011). Some level of genetic differentiation could be expected as well. First, the Montagu's harrier in Spain started to nest in crops much earlier (at least 50–60 years) than the population of Poland (Arroyo and Garcia 2002, Zieliński 2007) and, second, some level of genetic differentiation between

south-western and north-eastern populations was suggested (Garcia *et al*. 2011).

Material and methods

We analysed feathers of 74 unrelated individuals from central Spain. The samples were collected in 2010 from nestlings (only one bird per nest was sampled), as well as from adult birds. In Poland, 65 samples (feathers) were collected: 16 feathers from adult birds, presumably unrelated with each other as well as with sampled nestlings; two breeding pairs (4 individuals) and their offspring (6 individuals – 3 from each nest) and nestlings from 14 nests (39 individuals). The sampling was performed in Central Poland (Masovia, 21 samples), Eastern Poland (Podlasie region, 27 samples) and southern Poland (Opolszczyzna and Lower Silesia region, 17 samples). The geographical distribution of sampling sites was presented on Fig. 1. The collected feathers were stored in separate vials, both dry or in 96% alcohol.

The extraction of DNA was performed as described in Rutkowski *et al*. (2010). Then, microsatellite loci described for other Accipitridae species were amplified using polymerase chain reaction (PCR). We tested 24 loci: Age5, Age6, Age11 (Topinka and May 2004), Age07 (Sonsthagen *et al*. 2004), AgCA116, AgCA222, AgCA224, AgCA290, AgCA300, AgCA303, AgCA332, AgCA361, AgCA365, AgCA368, AgCA380 (Takaki *et al*. 2009), BV13, BV20 (Gautschi *et al*. 2000), HF-C1D10, HF-C1E8, HF-C5D4 (Mira *et al*. 2005), Gf3H3 (Mira *et al*. 2002), Aa35 (Martinez-Cruz *et al*. 2002), NVHfr203 (Nesje and Roed 2000), and Fnd1.5 (Padilla *et al*. 2009). In the first step of analysis only unlabeled primers were used. Amplification was performed in 25 μl of reaction mix, containing: 5–6 μl of DNA extract, 12.5 μl REDTaq PCR ReadyMix (Sigma-Aldrich, St. Louis, USA, distribution in Poland: Sigma-Aldrich, Poznań), 7.5 μl of water and 10 pmol of each primer. PCR reaction was performed under the following conditions – initial denaturation: 94°C in 3 min.; 30 cycles: 94°C in 45 s; 52–60°C in 45 s; 72°C in 45 s; final elongation: 72°C in 5 min. Negative PCR controls (one control per set of reactions) were always included. The success of the amplification was evaluated by electrophoresis in agarose gels containing ethidium bromide. When the length of the band was similar to the size of alleles described in the literature, the analysis was classified as a successful amplification (obtaining specific DNA fragment). Next, all successfully amplified loci were amplified using three multiplex-PCR reaction with labelled primers. Following primer mixes were designed: Mix1 (NVH203, AgCA222, AgCA332,

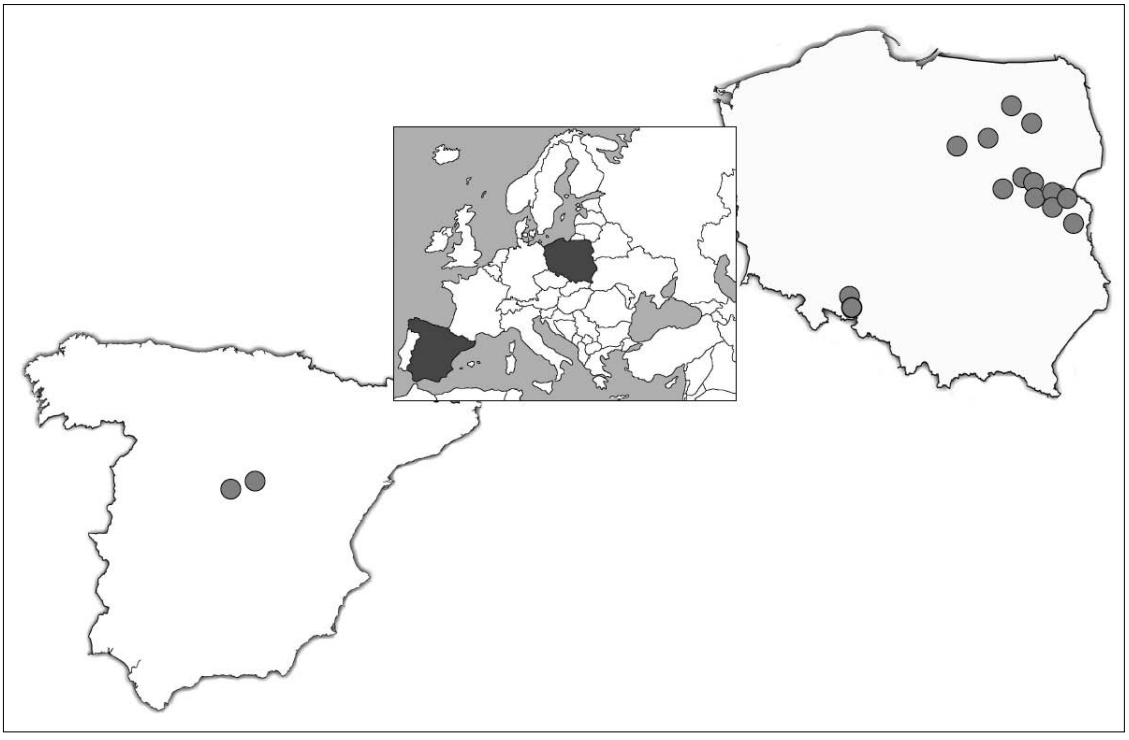

Figure 1. Geographical distribution of Montagu's harrier samples, collected for molecular analysis.

AgCA300); Mix2 (Age11, AgCA224, AgCA290, AgCA365, AgCA368) and Mix3 (Age07, BV13, Fnd1.5, AgCA116,). The reaction mixture contained: 1.5 μl of the mixture of primers ('forward' and 'reverse' for each locus, 2 pmol/μl) 7.5 μl PCR MasterMix (Qiagen, Netherlands/Germany, distribution in Poland: Syngen Biotech, Wrocław), 2.7 μl of water for PCR, and 3–5 μl of DNA. The reaction was performed in the following conditions: 15 min. at 95°C, 39 cycles of 30 s at 94°C, 90 s 57°C, 90 sec 72°C; 1 cycle: 30 s at 94°C, 90 s 57°C, 10 min. at 72°C. The 'forward' primer of each locus was labelled with one of fluorescent dyes (WellRead, Sigma-Aldrich): Dye2; Dye3; Dye4. The genotyping analyses were performed using Beckman Coulter CEQ 8000 sequencer (Beckman Coulter, Inc., Brea, CA, USA; distribution in Poland: Comesa, Warsaw).

The results were analyzed using Beckman Coulter Fragment Analysis Software. Based on obtained results, we excluded from further analysis locus AgCA368 as all analysed individuals had identical heterozygote genotype, suggesting that the PCR product did not contain proper microsatellite sequence.

For each locus, allelic diversity (A), observed heterozygosity (H_O), and unbiased expected heterozygosity (H_E) (Nei 1978) were estimated using GenAlEx version 6.501 (Peakall and Smouse 2001) and FSTAT version 2.9.3.2 (Goudet 2001).

We also tested deviations from Hardy-Weinberg Equilibrium (HWE) for each locus, as well as for all loci combined, using Genepop on the Web version 4.2 (Raymond and Rousset 1995, Rousset 2008). Additionally, for each locus, the fixation index (F_{IS}) was calculated and its significance was tested by 240 randomizations with Bonferroni correction for multiple comparisons (Bonferroni corrected P-value at $\alpha = 0.05$ was 0.00417). The probability of genotypic linkage disequilibrium between all pairs of loci was also calculated.

Then, we repeated the analysis for Spanish and Polish breeding populations separately. Basic indices of microsatellite polymorphism were calculated as described above, but we also calculated overall heterozygosity and F_{IS}, mean A, mean allelic richness A_R (Petit *et al*. 1998), mean number of private alleles (P) and private allelic richness (P_R) in each population. Additionally, in the case of Polish population, set of samples containing only unrelated individuals was analysed, namely: we included in the analysis adult birds (presumably unrelated) and one chick per nest. From two nests, where both parents and all chicks were sampled, we include in this analysis only parents. The analysis were performed using GenAlEx version 6.501 (Paekal and Smouse 2001), FSTAT version 2.9.3.2 (Goudet 2001) and HP-RARE 1.0 (Kalinowski 2005).

We also made an attempt to identify extra-pair paternity in clutches of Montagu's harrier. In this analysis, samples from 10 nests from Polish population were included. In the case of two nests the samples were collected from all chicks, as well as from social parents. From other 8 nests we had samples only from chicks. This analysis also allowed to check whether amplified microsatellites are inherited according to Mendelian pattern.

Results

Agarose gel electrophoresis indicated the successful amplification of 14 among 24 tested loci. However, analysis using the automatic sequencer showed that cross-species amplification yielded proper microsatellite loci in only 13 cases (Table 1). Additionally, one locus (Age07) was monomorphic in 139 tested individuals. Hence, we obtain 12 polymorphic microsatellites (50% of tested loci, 92% of successfully amplified). In general, successful cross-amplification was high for microsatellites designed originally for members of the Accipitrinae sub-family: northern goshawk *Accipiter gentilis* (10 out of 15 tested primer pairs yielded a microsatellite product, and 9 of them were then confirmed to be polymorphic), but also for two falcons: saker *Falco rusticolus* and lesser kestrel *F. naumanni* (two microsatellites were tested and both were amplified and polymorphic). One out of two tested loci from the bearded vulture *Gypaetus barbatus* was amplified and appeared to be polymorphic. No amplification success was obtained in the case of primers designed for amplification of microsatellite loci in Bonelli's eagle *Hieraaetus fasciatus* and Spanish imperial eagle *Aquila adalberti*.

In analysing polymorphic microsatellites in Montagu's harrier, we identified 56 microsatellite alleles (0.40 alleles per individual). In six loci, the polymorphism was moderate – from 6 to 9 alleles were identified, whereas in another six loci, we found very low levels of polymorphism (from 2–4 alleles only, Table 1). The observed heterozygosity was low, only in three cases (Age11, AgCA222, AgCA300) did the value exceed 0.60. Six out of twelve analysed microsatellites were not in the Hardy-Weinberg Equilibrium (HWE, Table 1). However, after the Bonferroni correction, F_{IS} values significantly differed from zero in only four cases: AgCA116, AgCA224, AgCA361, indicating significant heterozygote deficiency, whereas AgCA300 presented significant heterozygote excess. No significant linkage disequilibrium among the tested pairs of loci was found after the Bonferroni correction (corrected P-value at $\alpha = 0.05$ was 0.001, 1100 permutations were performed).

In the breeding population from Spain, locus BV13 appeared to be monomorphic. From 2 to 7 alleles were

Table 1. The results of cross-species amplification of 24 microsatellite loci in 139 Montagu's harriers. The names of microsatellite loci are given according to original publications. The references relating particular loci are given in Material and methods section. Shaded squares indicated presence of appropriate PCR product, detected using electrophoresis ('–' analysis using sequencer indicated, that all individuals had identical, heterozygote genotype, than the loci was excluded from further analysis). Size range – length in base pairs of PCR product; A – number of alleles; H_O – observed heterozygosity; H_E – expected heterozyogsity; HWE – *P*-values for HWE exact test for heterozygote deficiency/excess; F_{IS} – fixation index; * – significant F_{IS} value after Bonferroni correction (Bonferroni corrected *P*-value at $\alpha = 0.05$ was 0.00417).

Polymorphism detected using sequencer								
Locus	Source species		A	Size range	H_O	H_E	HWE	F_{IS}
Age5	*Accipiter gentilis*							
Age6	*Accipiter gentilis*							
Age11	*Accipiter gentilis*		6	236–254	0.647	0.680	0.044	0.051
Age07	*Accipiter gentilis*		1	155	–	–	–	–
AgCA116	*Accipiter gentilis*		2	254–256	0.122	0.187	<0.001	0.349*
AgCA222	*Accipiter gentilis*		9	108–140	0.761	0.749	0.071	-0.013
AgCA224	*Accipiter gentilis*		6	140–158	0.308	0.503	<0.001	0.390*
AgCA290	*Accipiter gentilis*		7	188–226	0.360	0.370	0.016	0.029
AgCA300	*Accipiter gentilis*		2	182–184	0.629	0.449	<0.001	-0.407
AgCA303	*Accipiter gentilis*							
AgCA332	*Accipiter gentilis*		6	214–224	0.381	0.494	0.047	0.232*
AgCA361	*Accipiter gentilis*		2	85–93	0.239	0.211	0.219	-0.132
AgCA365	*Accipiter gentilis*		3	169–177	0.266	0.275	0.708	0.034
AgCA368	*Accipiter gentilis*	–						
AgCA380	*Accipiter gentilis*							
BV13	*Gypaetus barbatus*		3	142–158	0.014	0.014	1.000	-0.002
BV20	*Gypaetus barbatus*							
HF-C1D10	*Hieraaetus fasciatus*							
HF-C1E8	*Hieraaetus fasciatus*							
HF-C5D4	*Hieraaetus fasciatus*							
Gf3H3	*Hieraaetus fasciatus*							
Aa35	*Aquila adalberti*							
NVH203	*Falco rusticolus*		4	172–186	0.108	0.117	0.130	0.080
Fnd1.5	*Falco naumanni*		6	194–206	0.460	0.497	0.579	0.077

found in the remaining loci (Table 2). Three loci were not in the HWE due to heterozygote deficiency, but only one F_{IS} value significantly differed from zero (AgCA332). Locus AgCA300 indicated high and significant heterozygote excess, supported by a significant and negative F_{IS}.

The analysis of all samples from Poland (including related individuals) indicated significant heterozygote deficiency in three loci, however, the F_{IS} value was significant only in the case of AgCA224 (Table 2). In locus AgCA300, similarly to results from Spain, high and significant heterozygote excess was found. All loci were polymorphic with 2 to 8 alleles. When only unrelated individuals were analysed, heterozygote deficiency was found only in two loci, but heterozygote excess was still evident in the case of AgCA300 (Table 2).

The comparison of genetic variability, estimated based on microsatellite polymorphism, did not indicate a clear difference. The number of alleles seemed to be slightly higher in Poland, as well as the mean number of private (unique) alleles per locus, especially when we applied measures taking into account differences in sample size (private allelic richness, P_R) (Table 3). Similarly, the overall observed heterozygosity in Spain was lower than in Poland for both data sets ('Total' and 'Unrelated', Table 3). The analysis indicated significant deviation from the Hardy-Weinberg equilibrium in both analysed breeding populations, but the F_{IS} value for the Polish breeding population was low and did not significantly differ from zero. Moreover, the data set including only unrelated individuals in Poland showed no significant deviation from the HWE (Table 3).

Table 2. Polymorphism of cross-amplified microsatellite markers in populations of Montagu's harrier from Spain (75 samples) and Poland (64 samples). In the case of Poland two sample sets were analysed: 'Total' – 65 samples, including related individuals; 'Unrelated' – 35 samples from 20 presumably unrelated adult birds and 16 unrelated chicks (one per sampled nest). Monomorphic Age07 was excluded from analysis. A – number of alleles; H_O – observed heterozygosity; H_E – expected heterozygosity; HWE – *P*-values for HWE exact test for heterozygote deficiency/excess (ns – $P > 0.05$; * – $P < 0.05$; *** – $P < 0.01$, *** $P < 0.001$); F_{IS} – fixation index; * – significant F_{IS} value after Bonferroni correction (Bonferroni corrected *P*-value at $\alpha = 0.05$ was 0.00208); na – not estimated due to monomorphism

	Age11	AgCA116	AgCA222	AgCA224	AgCA290	AgCA300	AgCA332	AgCA361	AgCA365	BV13	NVH203	Fnd1.5
Spain (*N* = 74)												
A	6	2	7	4	6	3	6	2	3	1	3	4
H_O	0.676	0.054	0.811	0.319	0.236	0.708	0.311	0.192	0.257	0.000	0.081	0.419
H_E	0.692	0.078	0.723	0.533	0.347	0.471	0.491	0.173	0.266	0.000	0.078	0.441
HWE	ns	ns	ns	***	***	**	***	ns	ns	na	ns	ns
F_{IS}	0.030	0.311	-0.114	0.408*	0.326	-0.468*	0.373*	-0.099	0.043	na	-0.029	0.058
Poland 'Total' (*N* = 65)												
A	5	2	8	5	4	2	6	2	3	3	4	6
H_O	0.615	0.200	0.703	0.297	0.500	0.603	0.462	0.292	0.277	0.031	0.138	0.508
H_E	0.663	0.291	0.759	0.447	0.390	0.441	0.487	0.250	0.284	0.030	0.159	0.541
HWE	ns	*	ns	**	***	***	ns	ns	ns	ns	ns	ns
F_{IS}	0.080	0.320	0.082	0.342*	-0.274	-0.360*	0.060	-0.164	0.032	-0.004	0.139	0.070
Poland 'Unrelated' (*N* = 35)												
A	5	2	8	4	4	2	5	2	3	1	3	5
H_O	0.457	0.143	0.765	0.229	0.382	0.588	0.514	0.257	0.229	0.000	0.143	0.514
H_E	0.648	0.224	0.773	0.334	0.338	0.323	0.492	0.224	0.205	0.000	0.185	0.545
HWE	*	ns	ns	ns	*	***	ns	ns	ns	na	ns	ns
F_{IS}	0.308	0.375	0.026	0.329	-0.117	-0.288*	-0.030	-0.133	-0.099	na	0.241	0.071

The genetic differentiation between Polish and Spanish breeding populations, measured with $F_{ST,}$ was very low (F_{ST} = 0.016, 95%CI 0.005–0.028), although the permutation test indicated that differentiation was significant.

The analysis of genotypes in two clutches, where both parents and all chicks were sampled, indicated that amplified microsatellites were inherited according to the Mendelian pattern of inheritance (Table 4). In these two nests, we did not find evidence of extra-pair paternity – in the genotypes of nestlings, we found only alleles which were also present in the genotype of the fathers. Similarly, in the majority of analysed nests where all chicks (but not parents) were sampled, no evidence of EPP was detected – not more than four alleles (the largest possible number of alleles transmitted to the next generation by one pair of individuals) were found in specific nests and the pattern of homozygosity and heterozygosity corresponded to the assumption that markers were transmitted to zygotes by one parental pair. However, in one case (nest number 4, Table 4), we found some inconsistency between locus Age11 and Fnd1.5: the chicks of one nest had genotypes 242/242, 239/239 and 242/245 in one locus (locus Age11, Nest 4, Table 4) and 198/198, 202/202 and 198/204 (Fnd1.5), which is not possible assuming one parental pair.

Discussion

Cross-amplification of microsatellite loci in Montagu's harrier

Our experiment, involving several Accipitridae as a source species for microsatellite primers and Montagu's harrier as a target species, indicated a successful amplification rate of polymorphic markers of 50%. This is almost an identical value to that found by

Table 3. Comparison of genetic variability, estimated based on polymorphism in 12 microsatellite loci (monomorphic Age07 was excluded from analysis), between Montagu's harrier populations from Spain and Poland. In the case of Poland two sample sets were analysed: 'Total' – 65 samples, including related individuals; 'Unrelated' – 35 samples from 20 presumably unrelated adult birds and 15 unrelated chicks (one per sampled nest). A – mean number of alleles per locus; A_R – mean allelic richness; P – mean number of private alleles; P_R – private allelic richness; H_O – heterozygosity observed; H_E – heterozygosity expected; HWE – significance of HWE exact test for heterozygote deficiency/excess (*** $P < 0.001$); F_{IS} – inbreeding coefficient (* – values significant after Bonferroni correction, 480 randomization, adjusted P-value = 0.00208). [1] – values for Spain, estimated in relation to Poland 'Unrelated'.

	Spain	Poland	
		'Total'	'Unrelated'
N	74	65	35
A	3.75	4.08	3.58
A_R	3.66/3.26[1]	4.07	3.57
P	0.50/0.58[1]	0.83	0.42
P_R	0.45/0.31[1]	0.87	0.62
H_O	0.280	0.335	0.303
H_E	0.319	0.359	0.331
HWE	***	***	ns
F_{IS}	0.129*	0.073	0.099

Primmer *et al*. (1996) (46%), Galbusera *et al*. (2000) (39%), Rutkowski *et al*. (2006) (46%) and Mitrus *et al*. (2013) in cross-species experiments involving a wide range of bird species. The experiment focused on three *Circus* species (*C. macrourus*, *C. cyaneus* and *C. pygargus*) also indicated similar rates (Heap *et al*. 2011). In the case of Montagu's harrier, the authors successfully amplified 19 among 27 tested loci from other raptors, and 15 of them appeared to be polymorphic. As stated by Galbusera *et al*. (2000), the strict pre-selection of markers allows for both a highly successful amplification rate and a relatively high level of polymorphism. Primmer *et al*. (2005) suggest that the success of the cross-amplification strategy in birds can by interlinked to the genetic distance between source and target species, and this pattern was also confirmed for other animal groups (e.g. fish Carreras-Carbonell *et al*. 2008). Although the genetic distance between Montagu's harrier and other Accipitridae has not been estimated until now, a phylogenetic analysis of Buteonine, including other members of the genus *Circus* (marsh harrier *C. aeruginosus* and African marsh harrier *C. ranivorous*), suggested a close genetic relationship between *Accipiter* and *Circus* (Lerner *et al*. 2008). This could explain the high cross-amplification success between *Circus pygargus* and *Accipiter gentilis* and the complete lack of amplification of microsatellite primers designed originally for Aquilinae members: *Aquila adalberti* and *Hieraaetus fasciatus*, as members of these genus form a distant clade in relation to Accipitrinae in the phylogeny tree of Accipitridae (Lerner and Mindel 2005). Surprisingly, we demonstrated very high cross-amplification success of the polymorphic microsatellite from Falconidae – we tested two microsatellites and both of them appeared to amplify and be polymorphic. Indeed, some microsatellite loci seem to be preserved in a wide range of species and show polymorphism even in distant evolutionary lineages (e.g. Primer *et al*. 1996, Rutkowski *et al*. 2006). Moreover, apart from genetic distance, other factors may influence cross-amplification success and maintenance of polymorphism in microsatellite loci, for example, the length of the microsatellite repeat region (Primmer *et al*. 2005).

Two primer pairs tested in our study (Age5 and Age11), developed for *Accipiter gentilis* were also cross-amplified by Heap *et al*. (2011) in Montagu's harrier and two other *Circus* species. Surprisingly, divergent results were obtained. Whereas both markers were found to be polymorphic by Heap *et al*. (2011), we failed to amplify Age5 in our samples of Montagu's harrier, despite applying a wide range of annealing temperatures. Locus Age11 was shown to be polymorphic in Montagu's harrier in both studies. This locus was also amplified by Topinka and May (2004) in five out of six studied species and was polymorphic in only three of them. This can suggest that the success of the cross-species amplification strategy is difficult to estimate based on the results of other studies.

Nuclear microsatellites are known to have high frequencies of null alleles or alleles that consistently do not amplify during PCR. The mutations within sequences flanking microsatellite loci, specifically within the region binding the primers, are the main explanation for null alleles (Callen *et al*. 1993). As a result, null alleles are not detected in heterozygous genotypes and artificially increase the frequency of homozygotes (Dakin and Avise 2004). Undetected alleles can lead to biased estimates of genetic variability or genetic differentiation among populations. However, simulations indicate that ignoring low-frequency null alleles (5–8%) will only slightly bias estimates of population differentiation using F_{ST} (Chapuis and Estoup 2007). More severe effects can be observed in parentage analyses, where null alleles may lead to the false exclusion of a true parent (Dakin and Avise 2004). The results of our study suggest that the frequency of alleles in locus AgCA224, in particular, can be burdened with the presence of null alleles – it was the only locus showing heterozygote deficiency in both studied populations, and F_{IS} values were high and significant. Indeed, microsatellite primers developed for other, even closely related species are more likely to display null alleles than species-specific primers. This is

Table 4. The analysis of Mendelian pattern of inheritance in cross-amplified microsatellite loci and paternity in ten nests of Montagu's harrier from Poland. Only nests where parental birds had been sampled or more than two chicks had been collected, were chosen for this analysis. M – male; F – female, N1-*n* – nestlings. In bold – the nestlings from nest, where multiple-paternity was suggested

	Age11		AgCA116		AgCA222		AgCA224		AgCA290		AgCA300		AgCA332		AgCA361		AgCA365		BV13		NVH203		Fnd1.5	
Nest 1																								
M	242	242	254	256	116	120	142	144	198	200	182	184	214	216	93	93	169	177	158	158	182	182	198	198
F	239	239	256	256	116	122	144	144	200	200	182	184	214	216	93	93	177	177	158	158	180	182	198	202
N1	239	242	256	256	116	116	144	144	198	200	182	182	214	216	93	93	169	177	158	158	182	182	198	202
N2	239	242	256	256	116	116	144	144	198	200	182	182	214	216	93	93	169	177	158	158	182	182	198	198
N3	239	242	254	256	116	120	144	144	198	200	182	184	214	216	93	93	177	177	158	158	182	182	198	198
Nest 2																								
M	239	239	256	256	108	114	144	144	200	200	182	184	214	216	93	93	169	177	158	158	182	182	204	204
F	239	242	254	256	116	116	144	146	198	200	182	182	216	216	85	93	177	177	158	158	182	182	198	198
N1	239	239	254	254	108	116	144	146	198	200	182	184	214	216	85	93	177	177	158	158	182	182	198	204
N2	239	242	254	256	114	116	144	144	198	200	182	184	216	216	93	93	169	177	158	158	182	182	198	204
N3	239	239	254	254	114	116	144	146	198	200	182	184	216	216	85	93	177	177	158	158	182	182	198	204
Nest 3																								
N1	239	242	254	254	116	116	144	146	198	200	182	182	216	216	93	93	177	177	158	158	182	182	198	200
N2	239	245	254	256	116	116	144	146	198	200	182	182	216	216	85	93	177	177	158	158	182	182	200	204
N3	239	239	254	256	116	116	146	146	198	200	182	182	216	216	85	93	169	177	158	158	182	182	198	204
N4	242	245	254	256	116	120	144	144	200	200	182	182	216	218	85	93	177	177	158	158	182	182	198	198
Nest 4																								
N1	242	242	256	256	116	122	144	144	200	200	182	182	216	216	85	93	177	177	158	158	182	182	198	204
N2	242	245	256	256	116	122	144	146	198	200	182	182	216	216	85	93	169	177	158	158	182	182	198	204
N3	239	242	256	256	112	114	142	144	198	200	182	182	216	216	85	93	169	169	158	158	182	182	202	202
N4	239	239	256	256	114	116	146	146	200	200	182	184	214	216	93	93	177	177	158	158	182	182	198	198
Nest 5																								
N1	239	245	256	256	116	116	142	144	200	200	182	184	216	216	85	93	177	177	158	158	182	186	198	198
N2	239	245	256	256	116	116	144	144	200	200	182	184	214	214	93	93	177	177	158	158	182	186	198	198
N3	239	245	256	256	116	116	144	144	200	200	182	184	216	216	93	93	177	177	158	158	182	186	198	198
Nest 6																								
N1	239	245	256	256	114	122	144	144	198	200	182	184	214	214	93	93	175	177	158	158	182	182	198	206
N2	239	245	256	256	116	116	144	144	198	200	182	184	216	216	93	93	169	177	158	158	182	182	198	206
N3	239	245	256	256	116	118	144	144	198	200	182	184	214	216	93	93	177	177	158	158	182	182	198	206
Nest 7																								
N1	239	242	256	256	116	120	144	144	200	200	182	184	216	216	93	93	177	177	158	158	182	182	198	202
N2	239	242	256	256	116	120	144	144	200	200	182	182	216	216	93	93	177	177	158	158	182	182	198	202
N3	239	242	256	256	116	120	142	144	200	200	182	184	216	216	93	93	177	177	158	158	182	182	198	198

Table 4. Continued

	Age11		AgCA116		AgCA222		AgCA224		AgCA290		AgCA300		AgCA332		AgCA361		AgCA365		BV13		NVH203		Fnd1.5	
Nest 8																								
N1	245	245	256	256	120	122	146	146	200	200	182	184	216	216	93	93	169	177	158	158	182	182	198	198
N2	242	245	256	256	120	122	146	146	200	200	184	184	216	216	93	93	169	177	158	158	172	182	198	202
N3	242	245	256	256	116	122	142	146	198	200	182	182	214	216	93	93	169	177	148	158	172	182	198	202
Nest 9																								
N1	239	245	256	256	118	122	144	144	200	200	184	184	214	214	93	93	177	177	158	158	182	182	198	200
N2	239	242	254	256	118	118	142	144	198	200	184	184	216	216	93	93	177	177	158	158	182	182	198	200
N3	239	242	256	256	118	118	146	146	198	200	182	184	214	214	93	93	177	177	158	158	182	182	198	200
Nest 10																								
N1	239	245	256	256	120	122	146	158	200	200	182	184	216	216	93	93	169	177	158	158	182	182	198	198
N2	239	239	254	256	116	116	146	146	198	200	182	184	216	216	85	93	169	177	158	158	182	182	198	204
N3	239	242	254	256	114	114	144	144	198	200	182	184	216	216	85	93	177	177	158	158	182	182	198	198

because nucleotide substitution within flanking regions should be more frequent between species than within species. There are several methods of dealing with genetic data containing null alleles (Oddou-Muratorio *et al.* 2009), from estimating their frequency and correcting genotypes (e.g. Chapuis and Estoup 2007), to simply removing loci bearing null alleles from further analysis. The last solution would be especially advisable in the case of species with a high number of available microsatellites. As the number of polymorphic microsatellites for Montagu's harrier is still low, we suggest that genotypes in AgCA224 be corrected to estimate genetic variability and differentiation, and to carefully analyse data in this locus during paternity studies.

Locus AgCA300, independently of the analysed data set, showed a high and significant heterozygote excess. Although the chromatograms shown present characteristic PCR products after the amplification of microsatellite loci, and fragment lengths estimated in our study (182–184 base pairs) correspond well to results obtained in the source species (Takaki *et al.* 2009), we cannot exclude the possibility of artificially increased heterozygosity resulting from high frequency artefacts obtained during the amplification of this locus. Takaki *et al.* (2009) excluded AgCA300 from analysis, suggesting the presence of null alleles. Our experiment indicated that in the case of Montagu's harrier, the problem with AgCA300 could be linked rather to non-specific amplification then to the presence of null alleles. Nonetheless, we also advise the exclusion of this locus from studies of Montagu's harrier.

Genetic variability of Montagu's harrier and differentiation between Spanish and Polish breeding populations

The polymorphism of microsatellite loci in Montagu's harrier, obtained using the cross-species amplification strategy, should be described as low. The most polymorphic locus, AgCA222, had 9 alleles; three other moderately polymorphic loci had from 6 to 7 alleles; and half of the polymorphic loci had only from 2 to 4 alleles. Also heterozygosity was low, exceeding 0.60 in only two loci. These could be explained either by a low level of genetic variability in the studied species, or by an outcome resulting from the use of microsatellites from other species. Indeed, many experiments applying cross-species amplification of microsatellites indicated a lack of amplification, monomorphism and/or lower level of polymorphism in the target species as compared to the source species (Rubinstein *et al.* 1995, Jarne and Lagoda 1996, Rutkowski *et al.* 2006). However, it was also shown that in declining bird species, the estimate of genetic variability based

on microsatellite polymorphism is frequently low (e.g. Segelbaher *et al*. 2003, Martínez-Cruz *et al*. 2004, Rutkowski *et al*. 2005, Johnson *et al*. 2009, see Evans and Sheldon (2008) for meta-analysis). Indeed, the population of Montagu's harriers has declined in central Europe during the 20th century as a consequence of extensive habitat destruction and human persecution (Clarke 1996, Krogulec 1997). Since then, the species has been red-listed in many European countries as declining or threatened. Hence, the low number of microsatellite alleles and low heterozygosity could suggest the effects of decreasing genetic variability due to population decline. On the other hand, genetic variability in many Accipitridae species, estimated by using microsatellite markers, seems to be generally low. For example, the British golden eagle *Aquila chrysaetos* population has declined in the late nineteenth and early twentieth century, but a comparison of present and historic samples did not indicate a clear reduction either in the number of microsatellites or in the level of heterozygosity (Bourke *et al*. 2010). Even in the 'predeclining' period (based on nearly 80 museum samples, dated from 1790–1940), the genetic variability of the golden eagle population seems to be low (A = 4.4; $H_O = 0.50$). Similarly, an analysis of microsatellite polymorphism in six Japanese and Central Asia populations of the widespread Northern Goshawk indicated allelic diversity in a range of 3.9–4.8, and heterozygosity higher than 0.60 in only one of six studied populations (Takaki *et al*. 2009). Hence, we can speculate that low microsatellite variability is characteristic of Accipitridae populations, because these predatory birds have low effective population sizes, and/or the number of individuals has been fluctuating in subsequent seasons, causing serial bottlenecks.

As it was shown by a previous study of Montagu's harrier, the Spanish breeding population constitutes a western European stronghold of genetic variability for the species (Garcia *et al*. 2011). The size of the breeding population in Spain was recently estimated at 5000 breeding pairs (Garcia and Arroyo 2003). Simultaneously, the Polish population had significantly decreased in number and size during the 1980's (Clarke 1996, Tomiałojć and Stawarczyk 2003). Hence, we expected to find some differences in the level of genetic variability between these two breeding populations. Surprisingly, microsatellite polymorphism did not indicate such differences, moreover – genetic variability in Poland seemed to be slightly higher than in Spain (however, if only unrelated individuals were analysed in Poland, this pattern is evident only in the case of the number of private alleles). The study of Garcia *et al*. (2011) suggests higher genetic variability, compared to other European breeding populations, in the coastal areas of Spain. In this region, Montagu's harriers still breed in the natural habitats of wetlands and shrub lands (Garcia and Arroyo 2003), whereas in the central part of the country, the birds use cereal crop fields as nesting sites (Garcia and Arroyo 2003). It was shown that population productivity is higher when natural vegetation is used as breeding habitat – the nests are not disturbed by crop harvesting (Pandolfi and Giacchini 1991, Arroyo *et al*. 2002). Similarly, samples from Poland were exclusively collected from the crop-breeding population. Hence, we can assume that in both compared populations, genetic variability was reduced to a similar level by human impact. However, this statement should be verified in future studies that compare breeding populations from natural and agricultural environments.

We found a small but significant genetic differentiation between the species' breeding populations of Poland and Spain. Western European populations (including Spain) winter primarily in western Africa (Limiñana *et al*. 2007, 2012), while central and eastern European harriers (including Poland) winter further east (Trierweiler *et al*. 2006). If the philopatry was strong within the species, the division of wintering sites should induce genetic differentiation among the breeding populations of Europe. However, it was also suggested that 'relaxed philopatric behaviour', described for Montagu's harrier (Limiñana *et al*. 2012), prevents genetic differences from occurring throughout Europe. Indeed, an analysis of mitochondrial genes suggested an intensive gene flow within the European population of the species (Garcia *et al*. 2011). Our data support this observation. Moreover, a weakly marked genetic structure seems to be a frequent characteristic among migratory predators (e.g. Sonsthagen *et al*. 2004, Le Gouar *et al*. 2008, Takaki *et al*. 2009, Rutkowski *et al*. 2010). Hence, small genetic differentiation, estimated by us based on microsatellite markers could reflect a high gene flow interlinked with 'relaxed philopatry' and substantial dispersal ability of the species.

An analysis of microsatellite genotypes in ten clutches of Montagu's harrier suggested that in one of the investigated nests, more than two fathers contributed to the nestlings' genetic pool. Colonial breeding of Montagu's harrier within clumps, characterized by short, near-neighbour nest distances in open habitats, increases the risk of extra-pair copulation (EPC). This was also confirmed for other species nesting in high densities (Westneat and Sherman 1997). In the case of Montagu's harrier, males spend a majority of their time outside of the territory (Mougeot 2004, Wiącek 2008). Mate-guarding, along with intensive courtship feeding during the period of female fertility in Montagu's harrier are very difficult to carry out. Wiącek (2008) also showed that copulation frequency was higher in semi-colonial than in solitary nests, suggesting a higher risk for EPC (to dilute sperm from rival males). He also observed that males from clumped

nests spend more time in the territory than males in separated territories, which may indicate a certain rudimentary form of mate-guarding. However, it seems that this behavior does not completely prevent extra pair paternity in Montagu's harrier.

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