The LINNEAN SOCIETY of London BIOLOGICAL Journal of the Linnean Society

Biological Journal of the Linnean Society, 2010, **100**, 725–736. With 3 figures

Widespread hybridization between the Greater Spotted Eagle *Aquila clanga* and the Lesser Spotted Eagle *Aquila pomarina* (Aves: Accipitriformes) in Europe

ÜLO VÄLI[1,2]*, VALERY DOMBROVSKI[3], RIMGAUDAS TREINYS[4], UGIS BERGMANIS[5], SZILÁRD J. DARÓCZI[6], MIROSLAV DRAVECKY[7], VLADIMIR IVANOVSKI[8], JAN LONTKOWSKI[9], GRZEGORZ MACIOROWSKI[10], BERND-ULRICH MEYBURG[11], TADEUSZ MIZERA[10], RÓBERT ZEITZ[6] and HANS ELLEGREN[1]

[1]*Department of Evolutionary Biology, Evolutionary Biology Centre, Uppsala University, Norbyvägen 18D, SE-75236 Uppsala, Sweden*
[2]*Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Kreutzwaldi 5, EE-51014 Tartu, Estonia*
[3]*Laboratory of Ornithology, Institute of Zoology of National Academy of Sciences, Akademichnaya str. 27, 220072 Minsk, Belarus*
[4]*Institute of Ecology of Nature Research Centre, Akademijos 2, LT–08412 Vilnius, Lithuania*
[5]*Nature Reserve Teiči, Aiviekstes 3, LV-4862 Laudona, Madonas raj, Latvia*
[6]*Milvus Group, Crinului 22, 540343 Tg.-Mures, Romania*
[7]*Rovniková 8, SK-04012 Košice, Slovakia*
[8]*APB-Birds Conservation Belarus, P.O. Box 306, 220050 Minsk, Belarus*
[9]*Museum of Natural History, Wrocław University, ul. Sienkiewicza 21, PL 50-335 Wrocław, Poland*
[10]*University of Life Sciences, Zoology Department, Wojska Polskiego 71c, PL 60-625 Poznań, Poland*
[11]*World Working Group of Birds of Prey, Wangenheimstraße 32, D-14193 Berlin, Germany*

Received 6 November 2009; revised 15 February 2010; accepted for publication 15 February 2010

Hybridization is a significant threat for endangered species and could potentially even lead to their extinction. This concern applies to the globally vulnerable Greater Spotted Eagle *Aquila clanga*, a species that co-occurs, and potentially interbreeds, with the more common Lesser Spotted Eagle *Aquila pomarina* in a vast area of Eastern Europe. We applied single nucleotide polymorphism (SNP) and microsatellite markers in order to study hybridization and introgression in 14 European spotted eagle populations. We detected hybridization and/or introgression in all studied sympatric populations. In most regions, hybridization took place prevalently between *A. pomarina* males and *A. clanga* females, with introgression to the more common *A. pomarina*. However, such a pattern was not as obvious in regions where *A. clanga* is still numerous. In the course of 16 years of genetic monitoring of a mixed population in Estonia, we observed the abandonment of *A. clanga* breeding territories and the replacement of *A. clanga* pairs by *A. pomarina*, whereby on several occasions hybridization was an intermediate step before the disappearance of *A. clanga*. Although the total number of Estonian *A. clanga* × *A. pomarina* pairs was twice as high as that of *A. clanga* pairs, the number of pairs recorded yearly were approximately equal, which suggests a higher turnover rate in interbreeding pairs. This study shows that interspecific introgressive hybridization occurs rather frequently in a hybrid zone at least 1700-km wide: it poses an additional threat for the vulnerable *A. clanga*, and may contribute to the extinction of its populations.

ADDITIONAL KEYWORDS: avian hybridization – extinction – hybrid identification – introgression – microsatellites – raptor – single nucleotide polymorphism – spotted eagles.

*Corresponding author. Current address: Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Kreutzwaldi 5, EE-51014 Tartu, Estonia. E-mail: ulo.vali@emu.ee

INTRODUCTION

Interspecific hybridization, i.e. the interbreeding of individuals from different species, occurs surprisingly frequently in nature (Arnold, 1997). Hybridization could play a role in speciation, and hence provide new material for evolution (Newton, 2003; Price, 2008). Hybridization is often directly associated with rarity: rare species hybridize more easily because the low numbers that lead to a lack of mates is often the main factor to trigger hybridization (Hubbs, 1955; Short, 1969; Randler, 2006). Hybridization is therefore of conservation importance, and presents a serious threat to many endangered species (Rhymer & Simberloff, 1996): it is often associated with the swamping of one species by another (Allendorf *et al*., 2001; Newton, 2003). Although introgression of foreign genes may be inhibited by the inviability or sterility of hybrids of the heterogametic sex (Haldane, 1922), hybridization is a waste of reproductive effort for the rarer species, calling its continuing sustainability into question.

Hybridization is relatively common in birds. Almost one-tenth of bird species have been considered to interbreed in nature and produce hybrid offspring (Panov, 1989; Grant & Grant, 1992), although the proportion may be even twice as high as that (McCarthy, 2006; Aliabadian & Nijman, 2007). However, regular hybridization is rather uncommon in raptors (Panov, 1989; McCarthy, 2006), and there are only relatively few well-studied examples of hybridization occurring in this avian group, such as a hybridizing complex of large falcons (Eastham & Nicholls, 2005; Nittinger *et al*., 2007). The Greater Spotted Eagle *Aquila clanga* Pallas, 1811 and the Lesser Spotted Eagle *Aquila pomarina* Brehm, 1831 are two closely related Eurasian raptors, with overlapping ranges in Eastern Europe, whose numbers have decreased during the last century mainly because of a decline in habitats (Hagemeijer & Blair, 1997). The decline has been particularly dramatic in *A. clanga*, whose vast range across Eurasia is occupied by only a few thousand pairs, with less than a thousand pairs breeding in Europe (BirdLife International, 2004). In contrast, populations of *A. pomarina* are still dense, and in most regions significantly outnumber the sparsely represented *A. clanga* (Hagemeijer & Blair, 1997; BirdLife International, 2004). Both species are listed in Annex I of the EU Directive on the Conservation of Wild Birds (EEC/79/409), as well in the International Union for Conservation of Nature (IUCN) Red List: *A. clanga* as a globally vulnerable species and *A. pomarina* as a species of least conservation concern (IUCN, 2009). The two spotted eagles are relatively young species (Seibold *et al*., 1996; Lerner & Mindell, 2005; Helbig *et al*., 2005a). There is no complete reproductive barrier between them and hybridization has been recorded repeatedly, although mainly in the form of anecdotal evidence of single cases of interbreeding or probable hybrids (Löwis, 1888, 1898; Feldt, 1909; Transehe, 1942, 1965; Bergmanis *et al*., 1997; Bergmanis & Strazds, 2001; Lõhmus & Väli, 2001; Dombrovski, 2002; Gutiérrez & Villa, 2002; Meyburg *et al*., 2005; Treinys, 2005).

Studies to assess the extent of hybridization have been hindered by the difficulties posed by hybrid identification. Not all hybrids can be identified by morphology and, if they are fertile and the hybridization is introgressive, detection of backcrosses is even more challenging (e.g. Benedict, 1999; Eastham & Nicholls, 2005; Gaubert *et al*., 2005; McCarthy, 2006). Hybrid detection is particularly difficult when interbreeding species are similar, and spotted eagles undoubtedly belong to this category (Randler, 2004) because many of their morphological identification characters show overlapping variation (Bergmanis, 1996; Forsman, 1999; Väli & Lõhmus, 2004; Dombrovski, 2006). However, genetic methods have recently provided new tools for hybridization studies. In spotted eagles, after the first applications of maternally inherited mitochondrial DNA (Väli & Lõhmus, 2004; Helbig *et al*., 2005b), multilocus amplified fragment length polymorphism (AFLP) markers were used in order to study differentiation and gene flow among species (Helbig *et al*., 2005b), but these markers remained relatively ineffective for individual assignments. Recently, Väli *et al*. (2010) applied a set of single nucleotide polymorphism (SNP) markers that, in combination with microsatellites, provided enough resolution power to efficiently identify pure species, F_1 hybrids (offspring of interbreeding *A. clanga* and *A. pomarina*), and backcrosses to parental species. Here, we apply this marker set in a spatiotemporal analysis of spotted eagle hybridization in Europe. On the spatial scale, we search for hybridization events across Europe and ask how widespread hybridization is and whether it poses a significant threat for the vulnerable *A. clanga*. On the temporal scale, we ask whether hybridization increases the risk of extinction in declining *A. clanga* populations, and verify this by results from the genetic monitoring of a mixed spotted eagle population in Estonia.

MATERIAL AND METHODS

SAMPLES

In order to estimate the current distribution and frequency of hybridization, a total of 738 spotted eagles were sampled in the wild between 1994 and 2009 (after 2001 in most European populations). Samples studied represent 408 breeding pairs of

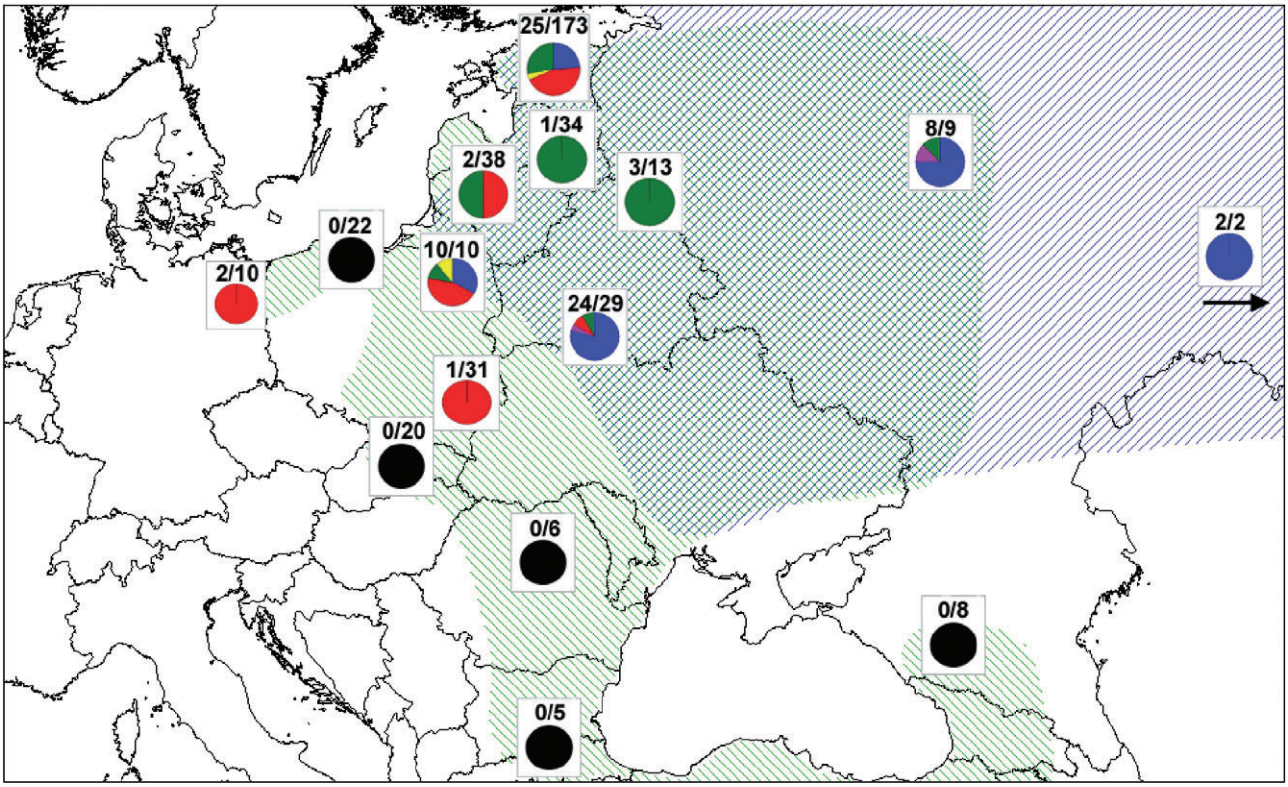

Figure 1. Distribution and frequency of spotted eagle hybridization in Europe. We have focused on *Aquila clanga* and hybridizing pairs, and excluded *Aquila pomarina* pairs, in all populations except those where only *A. pomarina* was found (in black). Other pie charts show the relative proportion of *A. clanga* × *A. clanga* (blue), *A. clanga* × *A. pomarina* (red), F_1 hybrid × *A. clanga* (violet), F_1 hybrid × *A. pomarina* (green), and $F_1 \times F_1$ (yellow) in populations after excluding *A. pomarina*. The numbers above each pie chart indicate the sample size both including *A. pomarina* (before slash) and excluding *A. pomarina* (after slash). The distribution range of *A. clanga* is shaded in blue and that of *A. pomarina* is in green (mainly according to Cramp & Simmons, 1980 and Hagemeijer & Blair 1997), but new field observations and museum data from the eastern limit (Melnikov *et al*., 2001; Mischenko *et al*., 2010; V. Belik, V. Dombrovski & M. Dzmitranok, unpubl. data) have been taken into account as well.

spotted eagles from 14 populations across Europe, covering both sympatric and allopatric regions of the distribution ranges (Fig. 1; Table 1), as well as two reference individuals from the Asian part of Russia, and thus remote from all *A. pomarina* populations. Instead of 'individuals', we prefer to use 'breeding pair' as a study unit because: (1) this is the common standard in the monitoring of raptor populations where non-territorial individuals are difficult to count; (2) samples were collected at nest sites; and (3) we made a consensus decision based on the assignment results of offspring and one or two adults (see below). In the spatial analysis, each pair was used only once in the final calculation of hybridization frequency.

A temporal study was conducted in Estonia, at the north-western limit of both species' distribution ranges, where spotted eagles have been continuously monitored (nests checked, birds scrutinized, and genetic samples collected) since the mid 1990s. According to estimates by Lõhmus (1998) from that period, *A. clanga* was present in some 5% of Estonian spotted eagle breeding pairs, i.e. in 20–30 out of 500–600 pairs. We included a total of 376 individuals from Estonia (a subsample of the total sample), representing 286 breeding attempts in 173 breeding territories (between 1994 and 2009, but mostly from 1999–2009; Appendix; Table 1). In the temporal study, assignment decisions were made on an annual basis, and several individuals of these long-lived species were studied repeatedly, which enabled us to verify the accuracy of genotyping.

Blood or feather samples were obtained during the breeding season at nest sites, and in their vicinity, from both nestlings and adults. The morphology of the nestling (in spotted eagles usually only one offspring fledges) was scrutinized thoroughly during sampling, and parental birds were described when

Table 1. Classification of breeding pairs studied in 14 populations. In addition to the five groups included in the main analysis, intermediates and potential $F_1 \times F_1$ individuals are also shown

No.	Population	*N*	*A. cla.*	*A. cla.*/$F_1 \times$ *A. cla.*	$F_1 \times$ *A. cla*	*A. cla* × *A. pom*	$F_1 \times F_1$	$F_1 \times$ *A. pom.*	*A. pom.*/$F_1 \times$ *A. pom.*	*A. pom.*
1	Estonia	173	6	0	0	11	1	7	4	144
2	North-western Russia	9	6	0	1	0	0	1	0	1
3	Latvia	34	0	0	0	0	0	1	1	32
4	Lithuania	38	0	0	0	1	0	1	1	35
5	North-eastern Belarus	13	0	0	0	0	0	3	0	10
6	North-eastern Germany	10	0	0	0	2	0	0	0	8
7	Northern Poland	22	0	0	0	0	0	0	0	22
8	North-eastern Poland	10	3	0	0	4	1	1	1	0
9	Southern Belarus	29	19	1	1	2	0	2	0	4
10	South-eastern Poland	31	0	0	0	1	0	0	0	30
11	Slovakia	20	0	0	0	0	0	0	0	20
12	Romania	6	0	0	0	0	0	0	0	6
13	Greece	5	0	0	0	0	0	0	0	5
14	Caucasian Russia	8	0	0	0	0	0	0	0	8
	Total	408	35	1	2	20	2	16	6	326

they were present (see Forsman, 1999; Väli & Lõhmus, 2004 for a comprehensive description of the morphological traits we used). If an *A. pomarina* nestling had characteristics typical of its species, and at least one of its parents was unequivocally assigned to the species by appearance, only the nestling sample was normally used in the genetic analysis; the adult's feathers were used only occasionally to confirm the assignment of its nestling. However, territories inhabited by *A. clanga* or potential hybrids were usually studied more carefully, and whenever possible we included samples from adults. To exclude the possibility of sampling nest-visiting adults from other pairs (Meyburg, Meyburg & Franck-Neumann, 2007), we also checked the parentage in adult–nestling pairs by comparing individual genotypes (data not shown). Additional field observations on species identification and pair bonds were used to complement the genetic data, especially in any unsuccessful breeding years of the temporal study. Field records were particularly informative in the case of males, which usually do not shed feathers at nest sites (Lõhmus & Väli, 2004). On the other hand, males are easier to observe while hunting, and are more protective at nest sites, and therefore easier to trap and study in the hand.

Laboratory analysis

DNA was extracted from blood cells and freshly plucked feathers using proteinase-K treatment followed by salting (Aljanabi & Martinez, 1997) or the phenol-chloroform purification method. From small moulted contour feathers, and some large flight feathers, we extracted DNA from the basal tip using the DNEasy tissue kit (Qiagen). Usually, however, DNA was extracted from the blood clot in the superior umbilicus of flight feathers following the methodology of Horvath *et al*. (2005). This blood clot is secure from contamination and the quantity of DNA is relatively large, and thus the simple phenol-chloroform protocol could be used to obtain a high yield of good-quality DNA.

In order to assign individuals to species, or to one of the hybrid classes, birds were genotyped by 30 nuclear markers. Twenty-two microsatellites, developed for other eagle species, were amplified and genotyped on a MegaBACE 1000 automated capillary sequencer (Amersham Biosciences), and eight nuclear SNP markers were analysed by restriction enzyme digestion and electrophoresis on 3% agarose gel (Table 2), as described by Väli *et al*. (2010). However, in order to ensure amplification success in poorer feather samples as well, we designed new primers for all SNP markers (only two 5′ primers were retained) for amplification of shorter DNA fragments (200–300 bp) (Table 2). Although not used directly in the assignment process, information on the maternal lineage of the hybrid offspring studied was obtained from the mitochondrial DNA pseudo-control region, which differs by some 4% between the two species (Väli, 2002). Species-specific lineage was detected either by sequencing or by restriction enzyme analysis using the *Cac8I* enzyme, which has a specific recognition site in *A. clanga*. In order to sex adult birds, a portion of the *CHD1* gene was amplified according to the methodology of Griffiths *et al*. (1998)

Table 2. Genetic markers used in the assignment of individuals. For each marker, its type [single nucleotide polymorphism (SNP) or microsatellite (Ms)], primer sequence, and reference are presented. Primers marked with an asterisk are the same as in Väli *et al*. (2010). [References: this study; Martinez-Cruz *et al*. (2002); Hailer, Gautschi & Helander (2005); Busch *et al*. (2005).]

Marker	Type	Forward primer (5′–3′)	Reverse primer (5′–3′)	Ref.
1.26928	SNP	GACCTTCCAGAAGCTATTGC*	ATTTAGCATGGGTGAGCCTA	1
4.12303	SNP	TGTTCAAATGTCCTTGGTTG	CTGCACGAGACATGCTTAAC	1
4.FIB	SNP	TGGGTCCTGAGGAAAGACAG	TTCCCCAATCTAAACAATTCC	1
5.15691	SNP	AATGGTCCAACTTTGAAATCT	TCAGCTTATTTGTTGCTCCA	1
7.04557	SNP	ACTAACTTGCTTGCCATGTG	GCTGTCTGCCCTTCAGTAAT	1
8.17388	SNP	CAGCGTAATGTACCAAATGC	CCCAGTCTTTTCAGCTTAGG	1
13.12260	SNP	AAGCAGAAGCTGTCTTCCG*	GCCCTTCACAAGTCAGGTAA	1
17.14657	SNP	ACTTGGCCACAGTGAGTATG	TCACTAATGTTGCCTGGAGA	1
Aa02	Ms	CTGCAGATTTCACCTGTTCTG	CTTCCAGGTCTTGCAGTTTACC	2
Aa12	Ms	TCATCAACCTGACCCTTTCC	TGCACTGAAGTTTCTCGGC	2
Aa15	Ms	TCACTGACCTGCCCTCTACA	CCAACCCTCTAGTCGTCCAC	2
Aa26	Ms	GCAAAGGTAAACTGCATCTGG	ATGCACTATTGGTAAACAGGCA	2
Aa27	Ms	GAGATGTCTTCACAGCTTGGC	AAGTCTCAGAGACTGACGGACC	2
Aa35	Ms	GCAGCAGAAAGTGCATACGA	GACCAAATGAAATGCGCC	2
Aa39	Ms	TTCTGTTTTTCCACTTGCTTG	TATTGAGCTCACAAAAACAAAGG	2
Aa43	Ms	CCACACTGAGAAACTCCTGTTG	TTCCTGAGAGCTCTTCCTGC	2
Aa49	Ms	AGGAGGTGCCAGTTTTCTCC	AGCGGGTCTGTGGCTCAT	2
Aa53	Ms	ATCGCTTCCATGAGCTGATT	GAGTGCGGAGAGCTCTGC	2
Aa57	Ms	AACATTAAGGCAGATGTGGACA	TACTGTGGACACGGACAGGA	2
Hal1	Ms	GAATACACCCAGAACAGCAACC	CCCAGCTGTGCTCATAACATAC	3
Hal4	Ms	TAAGGCTTTTCTTCGCGTGT	TCAACAACCCCTCCGTAGAC	3
Hal7	Ms	TTCAGAAGGTGCATGCAGTAG	GGGATGTGCAAAGAAATCTACC	3
Hal9	Ms	TGAGCTTTGTAGTAGCAGTGGTG	TGCAAAAATAGAGCCAATACCC	3
Hal13	Ms	CCACTCAGTAAGGAGCTTTGC	CCTTGTGTTTGCTGCAGATG	3
Hal18	Ms	GACAGGGAGCGAGTTAGTGG	CCAGCCACAAAGGTACTAAGG	3
IEAAAG04	Ms	GCATGTAACAAGTTTAATGTTGATGG	GTTGGAAACAGGACATGTTAAGC	4
IEAAAG12	Ms	GCTGCTGCTAAGAATCACTCATGTAC	GTGGGAAGGTGGGTTGTCAG	4
IEAAAG13	Ms	GAATACCACAATAAGAGGCAGAGTG	GTCTAAAATGAAGTGAATCTGTAAGACAG	4
IEAAAG14	Ms	GTCCAGATTCCCTGCTAGAAAGC	GTTGGAGAGTCTAAGCACTGAATCAG	4
IEAAAG15	Ms	GAGAATAATTTTTGAAAAGCAGTAGG	GCTTAGTTGTTCAGAGGACGGTAAG	4

or Fridolfsson & Ellegren (1999), and Z and W chromosome-specific fragments were separated by electrophoresis on 2% agarose gel (the first of the two protocols also required restriction by the *VspI* enzyme, which has two recognition sites in W-specific fragments. but only one in the Z-specific fragments; Väli, 2004).

Data analysis

Individuals were assigned to groups using Bayesian model-based Markov chain Monte Carlo (MCMC)-simulation approaches in STRUCTURE 2.2 (Pritchard, Stephens & Donnelly, 2000) and NewHybrids 1.1 (Anderson & Thompson, 2002). In STRUCTURE 2.2, the number of assumed groups K was assumed to be two, as two interbreeding species were being studied. The analysis was run for 100 000 burn-in and 500 000 analytical iterations. For each individual, the probability q of belonging to one of the identified populations was calculated using correlated allele frequency models, assuming gene flow between species. Comparatively, all analyses, with no significant differences in results, were conducted using the independent allele frequency model. As the occurrence of two species was presumed, an admixture model without preliminary information on species ancestry was used in order to avoid any potential influence on results. In order to assign an individual to a group, we used the error rate 0.1 from the expected value, which resulted in the following ranges of q (indicated as a probability of recent *A. clanga* ancestry): *A. pomarina*, $q = 0$–0.1; backcross $F_1 \times A.$ *pomarina*, $q = 0.15$–0.35; F_1, $q = 0.4$–0.6; backcross $F_1 \times A.$ *clanga*, $q = 0.65$–0.85; *A. clanga*, $q = 0.9$–1.0. Individuals with probabilities that were intermediate

between the two groups were treated as intermediate according to this analysis. Structure was also used for estimating allele frequencies in the two species in order to present the assignment power of and gene flow in various markers.

NewHybrids 1.1 was developed specially for identifying hybrids between two species, and it aims to assign each individual into one of the categories: pure species, F_1 or later generation hybrids, or backcrosses. Power analysis by synthetic genotypes shows that our marker sets may have problems with identifying F_2 hybrids (Väli *et al*., 2010), and there is a tendency to assign false positives in this class (E.C. Anderson, pers. comm.). We therefore included only five potential classes in the analyses: *A. clanga*, *A. pomarina*, F_1 hybrids, *A. clanga* × F_1, and *A. pomarina* × F_1. Individuals with all probabilities below 0.7 also showed, in most cases, a relatively high value in terms of belonging to another group, and were thus assigned as intermediate between these groups. However, we also performed simulations with a sixth, F_2 class (data not shown), and in cases when subsequent probabilities for F_2 were high ($q_n > 0.8$), and in which the analysis of the five classes gave an unclear result ($q_n < 0.7$ in all groups) in this analysis, we assigned individuals to the F_2 class. Each run lasted for at least 25 000 sweeps during burn-in and 100 000 sweeps during the analysis stage. Simulations were performed numerous times using both uniform and Jeffreys-like priors. Except for a few intermediate individuals, no great differences were found between the different runs and approaches.

Finally, each individual was assigned to a species or to a hybrid group, taking all assignment results into account. Assignments of a nestling and adults (if available) were combined, and the most plausible decision was made for each breeding pair. To depict individual assignments, genotypes of all birds were transformed into values in a multidimensional space by the Factorial Correspondence Analysis (using GENETIX 4.05) (Belkhir *et al*., 2004). We also estimated genetic differentiation between species by calculating F_{st} values between *A. clanga* and *A. pomarina* groups (hybrids and backcrosses excluded) using GENALEX 6.1 (Peakall & Smouse, 2006).

RESULTS

Analysis of the European populations

Most of the 738 individuals genotyped by SNPs and microsatellites could be assigned with confidence to one of the five presumed classes – *A. clanga*, *A. pomarina*, F_1 hybrids between the two species, *A. clanga* × F_1, or *A. pomarina* × F_1 – by both Bayesian methods (Table 1). Results from two approaches usually coincided completely, and in a few cases high probability from one method helped to clarify doubts raised by moderate probability from another method. Only seven samples were identified as intermediate by both approaches (Table 1). Even the less powerful factorial correspondence analysis (FCA) separated the two species clearly, whereas previously assigned hybrids and backcrosses appeared between the two species in an FCA plot (Fig. 2). Differentiation between the two species was evident in microsatellites ($F_{st} = 0.12$), SNPs ($F_{st} = 0.44$) and in both marker types combined ($F_{st} = 0.20$).

Hybridization between *A. clanga* and *A. pomarina* was detected in six populations across the sympatric

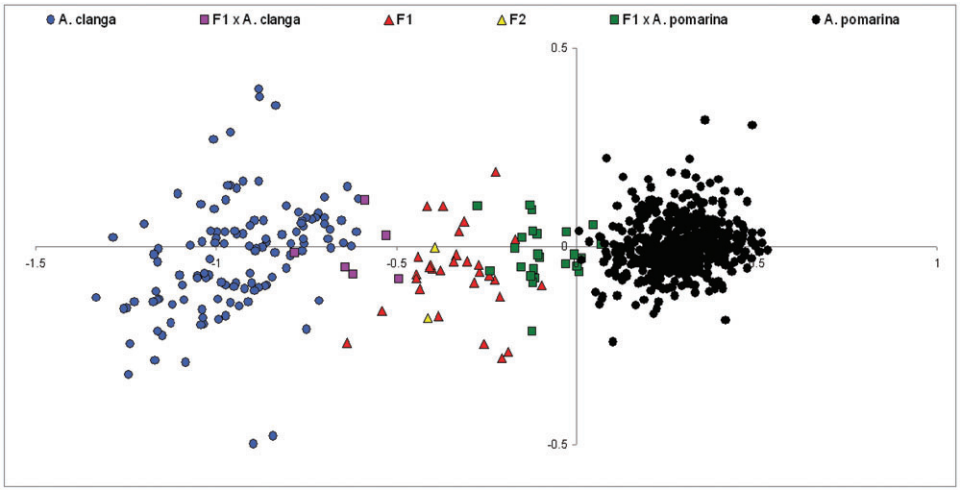

Figure 2. Distribution of the 738 spotted eagle individuals studied on the two-dimensional factorial correspondence analysis (FCA) plot. Prior to plotting, birds were classified according to the consensus of genotype-based Bayesian assignments.

area (including Germany and south-eastern Poland, regions previously considered as being outside the *A. clanga* distribution range). A combination of field data, molecular sexing of adults, and mtDNA analysis indicated that *A. clanga* × *A. pomarina* pairs were made up of male *A. pomarina* and female *A. clanga* in 17 cases, whereas the reverse situation occurred in four cases. In seven populations we found mixed pairs of F_1 backcrossing to *A. pomarina*, the more common species in the western part of the sympatric area, whereas introgression (backcrossing) to *A. clanga* was detected only at the eastern limits of the distribution of *A. pomarina* in Russia and in southern Belarus, both of which hold a relatively large population of *A. clanga*. Altogether, 8.6% of the breeding pairs studied were composed of two pure *A. clanga* individuals, with hybridizing *A. clanga* × *A. pomarina* pairs making up 4.9%, and second-generation backcrossing pairs (F_1 × pure species) making up 4.4%.

Further introgression beyond the second generation apparently also occurs, as we discovered *A. clanga* mtDNA in four adults and four nestlings (from eight different pairs) that were assigned by nuclear markers to *A. pomarina*. Backcrossing was also suggested by similar probabilities for the assignment of seven individuals to two classes (pure species and backcrosses; Table 1). Some of these uncertainties may arise from the inadequate power of the analysis, but on at least one occasion we confirmed the nestling's mother, and on a further occasion the father, as a backcross F_1 × *A. pomarina*; in another case one parent was an F_1 × *A. clanga*. Two nestlings were assigned to the F_2 group because the probability of belonging to that class was found by NewHybrids 1.1 (with six classes) to be exceptionally high (0.89 and 0.90), whereas analysis with only five classes gave inadequate probabilities for all classes. Moreover, STRUCTURE 2.2 gave probabilities close to 0.50, as can be expected for F_2 individuals, but not for backcrosses.

Monitoring of the Estonian population

Most of the individuals studied in the Estonian population were assigned rather unequivocally to one of the classes (Appendix; Table 1). Ten out of 11 *A. clanga* × *A. pomarina* pairs were made up of male *A. pomarina* and female *A. clanga*, whereas in one case the reverse was observed. We detected backcrossing to *A. pomarina*, but not to *A. clanga*. Additionally, one adult was identified as a backcross between F_1 and *A. pomarina* in four pairs, and *A. clanga* mtDNA was found in two nestlings (from two pairs) that were assigned by nuclear markers to *A. pomarina*.

Up to 2003, the intensive survey continuously revealed previously unknown *A. clanga* breeding territories (one or two territories per year), which obscured the possible changes in *A. clanga* population. After that, we noted a slight decrease in the numbers of *A. clanga* pairs and hybridizing pairs, although not in backcrossing pairs, which do not show a clear trend (Fig. 3). The small size of the sample, however, does not permit broader conclusions to be made. When we analysed separately the occupation history of each territory where an *A. clanga*, or an F_1 hybrid, had been recorded, the prevalent abandonment of territories, or replacement of rare *A. clanga* with the common *A. pomarina*, was noticeable (Table 3). In contrast, the opposite development was rare – we found an adult *A. pomarina* replaced by an

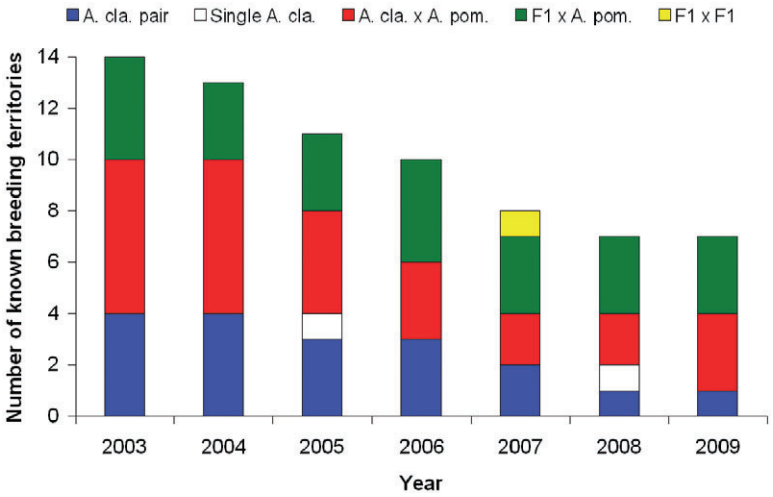

Figure 3. Number of breeding territories occupied by *Aquila clanga* and hybrids in Estonia in 2003–09. Genetic data are complemented with field observations.

Table 3. Occupation history of spotted eagle breeding territories in Estonia, where at least one parent was an *Aquila clanga* or an F_1 hybrid. Genetic data are complemented with field observations

No. of territory	Study years	Assignment of breeding pairs to a group
1	2003–2009	*A. cla* pair
2	2003–2009	*A. cla* pair → single *A. cla* → *A. pom* pair
3	2002–2009	*A. cla* pair → *A. pom* pair
4	1994–2009	*A. cla* pair → single *A. cla* → abandoned since 2006
5	1998–2009	*A. cla* (pair?) → *A. cla* × *A. pom* → F1 × *A. pom* → *A. pom* pair → abandoned since 2008
6	1997–2009	*A. cla* × *A. pom*
7	2004–2009	*A. cla* × *A. pom*
8	1994–2009	*A. cla* × *A. pom* → *A. cla* pair → *A. cla* × *A. pom* → F_1 × *A. pom*
9	2001–2009	*A. cla* × *A. pom* → F_1 × *A. pom* → $F_1 \times F_1$ → F_1 × *A. pom*
10	2004–2009	*A. cla* × *A. pom* → *A. pom* pair
11	2001–2009	*A. cla* × *A. pom* → *A. pom* pair
12	2000–2008	*A. cla* × *A. pom* → *A. pom* pair
13	2002–2007	*A. cla* × *A. pom* → abandoned since 2004
14	1999–2007	*A. cla* × *A. pom* → abandoned since 2003
15	2006–2008	F1 × *A. pom* → *A. pom* pair
16	2002–2007	F1 × *A. pom* → *A. pom* pair
17	1999–2006	F1 × *A. pom* → *A. pom* pair
18	2004–2008	F1 × *A. pom* → *A. pom* pair
19	1998–2001	F1 × *A. pom* → *A. pom* pair
20	1999–2009	*A. pom* pair → *A. cla* × *A. pom*
21	1999–2007	*A. pom* pair → F_1 × *A. pom*

A. clanga or a hybrid on only two occasions, and once by a backcross F_1 × *A. pomarina*. In one other case a hybridizing pair was replaced by an *A. clanga* pair, but this changed back over a 7-year period, with ultimately the substitution of an F_1 × *A. pomarina* pair. By 2009, four *A. clanga* territories were abandoned, and now the species is only found in four breeding territories (interbreeding with *A. pomarina* in three of them). Three territories were occupied by an adult hybrid interbreeding with *A. pomarina*, and altogether ten previous *A. clanga* or hybrid territories were occupied by *A. pomarina* pairs.

DISCUSSION

Extent and frequency of hybridization

The current study confirmed extensive introgressive hybridization between the two spotted eagle species in Europe, and highlights the significance of hybridization as an additional threat factor to the globally vulnerable *A. clanga*. Hybridization occurs over a very large area. Two interbreeding pairs of *A. clanga* and *A. pomarina* were discovered in Germany, whereas in central European Russia breeding of an *A. pomarina* pair and two backcrossing pairs were detected (Table 1). Given that only nine pairs were sampled in this Russian population, we believe that interbreeding also takes place here, and that the backcrosses detected do not just reflect gene flow from west to east. This suggests a hybrid zone of at least 1700 km in width. Even when only the extensively hybridizing population in the west (north-eastern Poland) is taken into account (excluding well-studied Germany, where only two cases are known), the zone width is at least 1100 km, which is much wider than previously recorded hybrid zones in birds (Price, 2008: 326–328).

We undoubtedly underestimated the proportion of *A. pomarina*, as sampling in most populations was not random but was biased towards rarer species and hybrids. Comparisons made among these latter groups are more reliable, but are still likely to be somewhat biased, as most of the hybridizing populations studied originated from the western limit of sympatry, where *A. clanga* is rare. However, the fact that hybridizing *A. clanga* × *A. pomarina* pairs and backcrossing pairs were together more numerous than *A. clanga* pairs (Table 1) nevertheless calls for vigilance. In the case of the more closely monitored Estonian population, the proportion of interbreeding pairs was almost equal to the proportion of *A. clanga* pairs when annual average estimates were considered, and was even twice as high when all breeding territories detected during the study period were con-

sidered together (Appendix; Fig. 3). This probably does not indicate a lack of selection for conspecifics in spotted eagles; rather it shows the shortage of *A. clanga* individuals and the fragility of the reproductive barrier separating the species. On the other hand, the difference between these estimates may indicate a higher exchange of individuals in hybridizing pairs.

Asymmetry of hybridization and introgression

We established sexually asymmetrical hybridization, as in 81% of cases a male *A. pomarina* mated with a female *A. clanga*. Field data from Belarus suggest a more even sex ratio in hybridizing pairs (V. Dombrovski, unpubl. data), but the observed asymmetry is noteworthy, even if it is limited to the northwestern regions where we collected most of our samples. It can be accounted for in a number of ways. The sex bias could occur because females from a rare species tend to interbreed with males from common species, but not vice versa, because of the selectiveness of females (Wirtz, 1999). However, there is no clear evidence to support the validity of this theory in birds (Randler, 2002), and in some species males are more selective than females (Saether, Fiske & Kalas, 2001). It has also been suggested that the reason for asymmetry could be the reversed sexual dimorphism in raptors: males are smaller than females, leading to an advantage for the males of the smaller species, and the females of the larger species, thus maximising the size factor (Helbig *et al*., 2005b). Indeed, there is a fitness-related advantage of large body size in *A. pomarina* females (Lõhmus & Väli, 2004), and this may act as one of the triggers in hybridization because *A. pomarina* males prefer and compete for *A. clanga* females that are larger than *A. pomarina* females. Finally, the hybridization mechanism could simply be based on sexual differences in territory selection pattern. In many animals, including raptors, males select a territory whereas females select their male partner (Wirtz, 1999). *Aquila clanga* males can fail to establish a breeding territory because of a loss of suitable habitat, and therefore often remain vagrant, whereas *A. clanga* females are prepared to select *A. pomarina* males occupying a territory.

The crucial question in the conservation of hybridizing species is the fertility of hybrids and introgression of genes from one species to another (Allendorf *et al*., 2001). Our study shows that hybrids of spotted eagles are fertile, as one could expect from their relatively recent divergence (Price & Bouvier, 2002). This applies to both males and females, and means that Haldane's rule (Haldane, 1922) is not fully followed. Earlier studies have reported that unidirectional introgression occurs, as *A. clanga* mtDNA has been found in *A. pomarina* or in intermediate birds, but not vice versa (Väli & Lõhmus, 2004; Helbig *et al*., 2005b). However, as hybridization is asymmetrical between female *A. clanga* and male *A. pomarina*, backcrossing to *A. clanga* would remain unnoticed using mtDNA analysis, as there is no difference between mtDNA molecules originating from hybrids or from pure species. Here, we also confirmed the same pattern using the more informative nuclear markers. There may be several explanations for an asymmetry towards *A. pomarina*. Backcrossing to the more common species is simply more probable, as hybrids have a lesser probability of meeting and mating with other hybrids, or with the rare species, than with individuals of the common species. Alternatively, Helbig *et al*. (2005b) suggested that asymmetrical introgression in sexually dimorphic spotted eagles could also result from size-oriented sexual selection, as hybrid females would mate with *A. pomarina* males, which are smaller than themselves, but not with *A. clanga* males that are larger or of equal size.

Future prospects

The main factor for the separation of the two species in the breeding grounds is probably the difference in habitat selection. *Aquila clanga* prefers the vicinity of wetlands, whereas *A. pomarina* occupies less moist habitats (Cramp & Simmons, 1980). However, habitat use is rather flexible as at least some *A. clanga* are able to inhabit drained habitats less typical for the species (Dombrovski & Ivanovski, 2005), and these biotopes could also be occupied by *A. pomarina* (Lõhmus & Väli, 2005). Habitat nevertheless remains the key factor, as its loss leads to a decline in numbers. In essence, the smaller the numbers of *A. clanga*, the higher the probability of hybridization, as rarity is the most important factor triggering interbreeding (Hubbs, 1955; Short, 1969; Wirtz, 1999; Randler, 2006). As *A. clanga* numbers have been declining during the last century (Hagemeijer & Blair, 1997), a simultaneous increase of hybridization can be expected. Indeed, Dombrovski (unpubl. data) has studied the morphology of museum specimens, and reported the increase of hybrids during the second half of the 20th century. The accumulation of hybridization records in recent years, mentioned above, probably also highlights the increased frequency of interbreeding (but it must also be attributed in part to increased awareness). There are still *A. clanga* populations in Eastern Europe, such as those in Belarus and central European Russia, where the species is not outnumbered by hybridizing pairs

(see also Dombrovski, 2005 for field data). Hybridization pressure on these populations is probably growing as the numbers of *A. clanga* decline.

The analysis of the Estonian population indicated the decline of *A. clanga*, but did not directly confirm a recent increase in hybridization. However, if the history of each breeding pair involving an *A. clanga* or a hybrid (Table 3) is studied, it clearly shows that the decline of *A. clanga* in Estonia is concomitant with extensive hybridization with *A. pomarina*, and hybridization is often an intermediate step on the way to the disappearance of *A. clanga*. Such a rapid decline and changing population structure of a long-lived bird is alarming. The Estonian *A. clanga* population has few males, and there has been hardly any replacement among females (Ü. Väli, unpubl. data). Following the disappearance of an *A. clanga* individual, the territory is either abandoned or occupied by *A. pomarina*. Hybridization has been a significant threat for several species and subspecies (Rhymer & Simberloff, 1996). It could therefore be an important factor contributing to the decline, and potentially even the extinction, of *A. clanga* populations in Europe. This should be borne in mind when proposing measures for *A. clanga* conservation.

ACKNOWLEDGEMENTS

We thank Sven Aun, Igor Babkin, Viktor Belik, Marina Dzmitranok, Raivo Endrekson, Tarmo Evestus, Mikhail Ivanov, Roman Kiselev, Kristo Lauk, Asko Lõhmus, Anton Makarov, Riho Männik, Joachim Matthes, Vladimir Melnikov, Gennady Mindlin, Renno Nellis, Rein Nellis, Kostas Poirazidis, Svetlana Romanova, Vitaliy Ryabtsev, Pauli Saag, Lauri Saks, Wolfgang Scheller, Gunnar Sein, Urmas Sellis, Indrek Tammekänd, Jaak Tammekänd, Aarne Tuule, Eet Tuule, Joosep Tuvi, Dmitriy Zhuravlev, and Nikolay Yakovets for their help with sample collection. Frank Hailer kindly provided primers for the microsatellite analysis. Comments of four anonymous reviewers greatly improved the manuscript. Financial support was provided by APB-BirdLife Belarus, the Estonian State Nature Conservation Centre, the Estonian Science Foundation (grant nos ETF6050 and ETF7593), the Frankfurt Zoological Society, the Ornithological Society of the Middle East, the Royal Society for the Protection of Birds, and the Marie Curie Intra-European Fellowship for ÜV within the 6th European Community Framework programme.

REFERENCES

Aliabadian M, Nijman V. 2007. Avian hybrids: incidence and geographic distribution of hybridization in birds. *Contributions to Zoology* **76:** 59–61.

Aljanabi SM, Martinez I. 1997. Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. *Nucleic Acids Research* **25:** 4692–4693.

Allendorf FW, Leary RF, Spruell P, Wenburg JK. 2001. The problems with hybrids: setting conservation guidelines. *Trends in Ecology & Evolution* **16:** 613–622.

Anderson EC, Thompson EA. 2002. A model-based method for identifying species hybrids using multilocus genetic data. *Genetics* **160:** 1217–1229.

Arnold ML. 1997. *Natural hybridization and evolution*. Oxford, New York: Oxford University Press.

Belkhir K, Borsa P, Chikhi L, Raufaste N, Bonhomme F. 2004. *GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations. Laboratoire Génome, Populations, Interactions, CNRS UMR 5000*. Montpellier, France: Université de Montpellier II.

Benedict RA. 1999. Morphological and mitochondrial DNA variation in a hybrid zone between short-tailed shrews (*Blarina*) in Nebraska. *Journal of Mammalogy* **80:** 112–134.

Bergmanis U. 1996. On the taxonomy of the Lesser Spotted Eagle *Aquila pomarina* and Greater Spotted Eagle *A. clanga*. In: Meyburg BU, Chancellor RD, eds. *Eagle studies*. Berlin, London & Paris: World Working Group of Birds of Prey, 199–207.

Bergmanis U, Strazds M. 2001. Another possible hybridization case of Lesser and Greater Spotted eagle in Latvia. *Putni Daba* **11.2:** 6–7.

Bergmanis U, Petrinš A, Strazds M, Krams I. 1997. Possible case of hybridization of the Lesser Spotted eagle and the Greater Spotted eagle in Eastern Latvia. *Putni Daba* **3:** 2–6.

BirdLife International. 2004. *Birds in Europe: Population estimates, trends and conservation status*. Cambridge, UK: BirdLife International.

Busch JD, Katzner TE, Bragin E, Keim P. 2005. Tetranucleotide microsatellites for *Aquila* and *Haliaeetus* eagles. *Molecular Ecology Notes* **5:** 39–41.

Cramp S, Simmons KEL. 1980. *The birds of the Western Palearctic*. Oxford: Oxford University Press.

Dombrovski VC. 2002. Hybridization of Lesser and Greater Spotted Eagles (*Aquila pomarina et A. clanga*) in Belarus: rule or exception? *Subbuteo* **5:** 23–31.

Dombrovski VC. 2005. Hybridation entre Aigles criard *Aquila clanga* et pomarin *A. pomarina* en Biélorussie: conséquence taxonomique. *Nos Oiseaux* **52:** 27–30.

Dombrovski VC. 2006. Morphological characteristics and diagnostic features of the Greater Spotted (*Aquila clanga*), Lesser Spotted (*A. pomarina*) eagles, and their hybrids. *Ornithologia* **33:** 29–41.

Dombrovski VC, Ivanovski VV. 2005. Numbers, distribution and breeding ecology of the Greater spotted Eagle (*Aquila clanga*). *Belarus. Ornithologia* **32:** 57–70.

Eastham CP, Nicholls MK. 2005. Morphometric analysis of large *Falco* species and their hybrids with implications for conservation. *Journal of Raptor Research* **39:** 386–393.

Feldt. 1909. Ueber Raubvögel, Vogelschutz, Naturbeobachtung. *Neue Baltische Waidmannsblätter* **1909:** 266–270.

Forsman D. 1999. *The raptors of Europe and the Middle East: a handbook of field identification*. Poyser: London.

Fridolfsson AK, Ellegren H. 1999. A simple and universal method for molecular sexing of non-ratite birds. *Journal of Avian Biology* **30:** 116–121.

Gaubert P, Taylor PJ, Fernandes CA, Bruford MW, Veron G. 2005. Patterns of cryptic hybridization revealed using an integrative approach: a case study on genets (*Carnivora*, *Viverridae*, *Genetta* spp.) from the southern African subregion. *Biological Journal of the Linnean Society* **86:** 11–33.

Grant PR, Grant R. 1992. Hybridization of bird species. *Science* **256:** 193–197.

Griffiths R, Double MC, Orr K, Dawson RJG. 1998. A DNA test to sex most birds. *Molecular Ecology* **7:** 1071–1075.

Gutiérrez R, Villa S. 2002. A possible hybrid Spotted Eagle × Lesser Spotted Eagle in Spain. *Birding World* **15:** 104–105.

Hagemeijer EJM, Blair MJ. 1997. *The EBCC atlas of European breeding birds*. London: Poyser.

Hailer F, Gautschi B, Helander B. 2005. Development and multiplex PCR amplification of novel microsatellite markers in the White-tailed Sea Eagle, *Haliaeetus albicilla* (*Aves*: *Falconiformes*, *Accipitridae*). *Molecular Ecology Notes* **5:** 938–940.

Haldane JBS. 1922. Sex-ratio and unisexual sterility in hybrid animals. *Journal of Genetics* **12:** 101–109.

Helbig AJ, Kocum A, Seibold I, Braun MJ. 2005a. A multi-gene phylogeny of aquiline eagles (Aves : Accipitriformes) reveals extensive paraphyly at the genus level. *Molecular Phylogenetics and Evolution* **35:** 147–164.

Helbig AJ, Seibold I, Kocum A, Liebers D, Irwin J, Bergmanis U, Meyburg BU, Scheller W, Stubbe M, Bensch S. 2005b. Genetic differentiation and hybridization between Greater and Lesser Spotted Eagles (*Accipitriformes*: *Aquila clanga*, *A. pomarina*). *Journal of Ornithology* **146:** 226–234.

Horvath MB, Martinez-Cruz B, Negro JJ, Kalmar L, Godoy JA. 2005. An overlooked DNA source for non-invasive genetic analysis in birds. *Journal of Avian Biology* **36:** 84–88.

Hubbs CL. 1955. Hybridization between fish species in nature. *Systematic Zoology* **4:** 1–20.

IUCN. 2009. *IUCN Red List of Threatened Species. Version 2009.2*. Available at http://www.iucnredlist.org. Downloaded on 6 November 2009.

Lerner HRL, Mindell DP. 2005. Phylogeny of eagles, Old World vultures, and other Accipitridae based on nuclear and mitochondrial DNA. *Molecular Phylogenetics and Evolution* **37:** 327–346.

Lõhmus A. 1998. Numbers of the Greater and the Lesser Spotted Eagle in Estonia. *Hirundo* **11:** 24–34.

Lõhmus A, Väli Ü. 2001. Interbreeding of the Greater *Aquila clanga* and Lesser Spotted Eagle *A. pomarina*. *Acta Ornithoecologica* **4:** 377–384.

Lõhmus A, Väli Ü. 2004. The effects of habitat quality and female size on the productivity of the Lesser Spotted Eagle *Aquila pomarina* in the light of the alternative prey hypothesis. *Journal of Avian Biology* **35:** 455–464.

Lõhmus A, Väli Ü. 2005. Habitat use by the vulnerable Greater Spotted Eagle *Aquila clanga*, interbreeding with the Lesser Spotted Eagle *Aquila pomarina* in Estonia. *Oryx* **39:** 170–177.

Löwis O. 1888. Die baltischen Raubvögel. *Baltische Monatsschrift* **35:** 527–556, 689–717.

Löwis O. 1898. *Diebe und Räuber in der Baltischen Vogelwelt*. Riga: J. Deubner.

Martinez-Cruz B, David VA, Godoy JA, Negro JJ, O'Brien SJ, Johnson WE. 2002. Eighteen polymorphic microsatellite markers for the highly endangered Spanish Imperial Eagle (*Aquila adalberti*) and related species. *Molecular Ecology Notes* **2:** 323–326.

McCarthy EM. 2006. *Handbook of avian hybrids of the world*. Oxford, New York: Oxford University Press.

Melnikov VN, Solovkov DA, Kostin BA, Egorova NA, Bogomolov DV. 2001. Nesting of Lesser Spotted Eagle (Aquila pomarina) in Ivanovo region (Russia). *Acta Ornithoecologica* **4:** 287–289.

Meyburg B-U, Mizera T, Matthes J, Graszynski K, Schwanbeck JP, Maciorowski G. 2005. Hybridization of Greater *Aquila clanga* and Lesser Spotted Eagle *A. pomarina* in Poland and Germany. In: Mizera T, Meyburg BU, eds. *International Meeting on Spotted Eagles (Aquila clanga, A. Pomarina and A. Hastata) – Research and Conservation*. Osowiec: Biebrza National Park, 115–117.

Meyburg BU, Meyburg C, Franck-Neumann F. 2007. Why do female Lesser Spotted Eagles (*Aquila pomarina*) visit strange nests remote from their own? *Journal of Ornithology* **148:** 157–166.

Mischenko A, Väli Ü, Treinys R, Bergmanis U, Domaschevski S. 2010. The Lesser Spotted Eagle *Aquila pomarina* C.L. Brehm, 1831. In: Galushin VM, Belik VP, Melnikov VN, Bragin EA, Numerov AD, eds. *Birds of Russia and surrounding regions: Falconiformes*. In press.

Newton I. 2003. *The speciation and biogeography of birds*. London: Academic Press.

Nittinger F, Gamauf A, Pinsker W, Wink M, Haring E. 2007. Phylogeography and population structure of the saker falcon (*Falco cherrug*) and the influence of hybridization: mitochondrial and microsatellite data. *Molecular Ecology* **16:** 1497–1517.

Panov EN. 1989. *Hybridization and ecological isolation in birds*. Nauka: Moscow.

Peakall R, Smouse PE. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* **6:** 288–295.

Price T. 2008. *Speciation in birds*. Greenwood Village, CO: Roberts and Company.

Price TD, Bouvier MM. 2002. The evolution of F-1 postzygotic incompatibilities in birds. *Evolution* **56:** 2083–2089.

Pritchard JK, Stephens M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. *Genetics* **155:** 945–959.

Randler C. 2002. Avian hybridization, mixed pairing and female choice. *Animal Behaviour* **63:** 103–119.

Randler C. 2004. Frequency of bird hybrids: does detectability make all the difference? *Journal of Ornithology* **145:** 123–128.

Randler C. 2006. Behavioural and ecological correlates of natural hybridization in birds. *Ibis* **148:** 459–467.

Rhymer JM, Simberloff D. 1996. Extinction by hybridization and introgression. *Annual Review of Ecology and Systematics* **27:** 83–109.

Saether SA, Fiske P, Kalas JA. 2001. Male mate choice, sexual conflict and strategic allocation of copulations in a lekking bird. *Proceedings of the Royal Society of London Series B-Biological Sciences* **268:** 2097–2102.

Seibold I, Helbig AJ, Meyburg BU, Negro JJ, Wink M. 1996. Genetic differentiation and molecular phylogeny of European *Aquila* eagles according to cytochrome b nucleotide sequences. In: Meyburg B-U, Chancellor RD, eds. *Eagle studies*. Berlin, London, Paris: World Working Group of Birds of Prey, 1–15.

Short LL. 1969. Taxonomic aspects in avian hybridization. *Auk* **86:** 84–105.

Transehe N. 1942. Das Vogelleben des Angerschen Sees. *Korrespondenzblatt des Naturforscher-Vereins zu Riga* **64:** 101–124.

Transehe Nv. 1965. *Die vogelwelt lettlands*. Hannover-Döhren: Harro von Hirschheydt.

Treinys R. 2005. The Greater Spotted Eagle Aquila clanga: previous, current status and hybridization in Lithuania. *Acta Zoologica Lituanica* **15:** 31–38.

Väli Ü. 2002. Mitochondrial pseudo-control region in old world eagles (genus *Aquila*). *Molecular Ecology* **11:** 2189–2194.

Väli Ü. 2004. Sex ratio of Lesser Spotted Eagle *Aquila pomarina* nestlings in good and poor breeding years. *Bird Study* **51:** 189–191.

Väli Ü, Lõhmus A. 2004. Nestling characteristics and identification of the Lesser Spotted Eagle *Aquila pomarina*, Greater Spotted Eagle *A. clanga*, and their hybrids. *Journal of Ornithology* **145:** 256–263.

Väli Ü, Saag P, Dombrovski V, Meyburg B-U, Maciorowski G, Mizera T, Treinys R, Fagerberg S. 2010. Microsatellites and single nucleotide polymorphisms in avian hybrid identification: a comparative case study. *Journal of Avian Biology* **41:** 34–49.

Wirtz P. 1999. Mother species–father species: unidirectional hybridization in animals with female choice. *Animal Behaviour* **58:** 1–12.

APPENDIX

Number of breeding pairs of spotted eagles studied with genetic markers in 1994–2009 in Estonia, assigned to different groups. The last row includes all territories registered, not the sum of annual results. As a result of the changes in occupation history (Table 3), some territories are included in several groups.

	N	*A. cla* pair	*A. cla* × *A. pom*	$F_1 \times F_1$	$F_1 \times$ *A. pom*	$F_1 \times$ *A. pom*/ *A. pom*	*A. pom* pair
1994	3	1	1	0	0	0	1
1995	1	1	0	0	0	0	0
1996	1	1	0	0	0	0	0
1997	5	2	0	0	0	0	3
1998	6	0	0	0	0	0	6
1999	31	2	3	0	1	1	24
2000	27	2	2	0	1	0	22
2001	30	2	3	0	0	1	24
2002	53	3	4	0	1	1	44
2003	12	4	2	0	0	0	6
2004	17	1	4	0	1	0	11
2005	34	2	1	0	2	1	28
2006	38	3	2	0	2	0	31
2007	28	1	1	1	1	0	24
2008	20	1	2	0	2	0	15
2009	28	1	3	0	2	0	22
Total no. of studied pairs	173	6	11	1	7	4	144