J Ornithol (2005) 146: 226–234
DOI 10.1007/s10336-005-0083-8

ORIGINAL ARTICLE

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Genetic differentiation and hybridization between greater and lesser spotted eagles (Accipitriformes: *Aquila clanga, A. pomarina*)

Received: 16 December 2004 / Revised: 14 February 2005 / Accepted: 15 February 2005 / Published online: 28 April 2005

Abstract Greater and lesser spotted eagles (*Aquila clanga, A. pomarina*) are two closely related forest eagles overlapping in breeding range in east-central Europe. In recent years a number of mixed pairs have been observed, some of which fledged hybrid young. Here we use mitochondrial (control region) DNA sequences and AFLP markers to estimate genetic differentiation and possible gene flow between these species. In a sample of 83 individuals (61 *pomarina*, 20 *clanga*, 2 F1-hybrids) we found 30 mitochondrial haplotypes which, in a phylogenetic network, formed two distinct clusters differing on average by 3.0% sequence divergence. The two species were significantly differentiated both at the mitochondrial and nuclear (AFLP) genetic level. However, five individuals with *pomarina* phenotype possessed *clanga*-type mtDNA, suggesting occasional gene flow. Surprisingly, AFLP markers indicated that these "mismatched" birds (originating from Germany, E Poland and Latvia) were genetically intermediate between the samples of individuals in which mtDNA haplotype and phenotype agreed. This indicates that mismatched birds were either F1 or recent back-cross hybrids. Mitochondrial introgression was asymmetrical (no *pomarina* haplotype found in *clanga* so far), which may be due to assortative mating by size. Gene flow of nuclear markers was estimated to be about ten times stronger than for mtDNA, indicating a sex-bias in hybrid fertility in accordance with Haldane's rule. Hybridization between the two species may be more frequent and may occur much further west than hitherto assumed. This is supported by the recent discovery of a mixed pair producing at least one fledgling in NE Germany.

Keywords Amplified fragment length polymorphism · Haldane's rule · Hybridization · Hypervariable control region 1 · Mitochondrial introgression

Communicated by F. Bairlein

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Introduction

Among the hawk-like birds of prey (Accipitriformes) only a few cases of species forming a "zone of overlap and hybridization" (sensu Short 1969) are known, and even fewer of them have been well documented. One example is the contact zone of western and eastern marsh harrier (*Circus aeruginosus, C. spilonotus*) in Siberia (Fefelov 2001). In Europe, a possible case where the frequency of hybridization may have been underestimated concerns the greater spotted eagle (*A. clanga*) and the lesser spotted eagle (*A. pomarina*), two similar species of Palearctic forest eagles. *A. pomarina* is restricted as a breeding bird to temperate parts of Central and eastern Europe and winters in Africa. *A. clanga* breeds from eastern Europe to eastern Asia and winters mainly on the Indian subcontinent and in SE Asia, but also in the Middle East and NE Africa (Cramp et al. 1980). Breeding ranges of the two species overlap in an

area several hundred kilometres in width between the Baltic republics, eastern Poland, Belarus, and Ukraine (Hagemeijer and Blair 1997). In the entire area of sympatry, *A. clanga* is much rarer than *A. pomarina* (Meyburg et al. 2001). However, both an assessment of the distribution and relative abundance of the two species as well as documentation of possible hybridization have been hampered by difficulties of field identification. Reliable characters for distinguishing the species in the field have only become known since about the mid-1970s (Porter et al. 1974; Svensson 1975; for an up-to-date summary, see Forsman 1999).

Studies of museum specimens have indicated that, in the zone of sympatry between the two species, a considerable proportion of individuals may show intermediate phenotypic characters (Zhezherin 1969). Whether this is due to hybridization and whether such hybridization leads to gene flow (back-crossing), remained unknown. Only in recent years have a few cases of mixed pairs and successful hybridization been described (Bergmanis et al. 2001, Lõhmus and Väli 2001; Dombrovski 2002; Väli and Lõhmus 2004). These were discovered mainly during the course of ringing work at the nests, an activity that started in Latvia in 1985 (nestlings) and 1994 (adults) and was intensified in Germany from 1990 onwards (nestlings and adults), partly in the course of satellite telemetry studies (Meyburg et al. 1995). In eastern Poland, mixed pairs were first discovered when attempts were made to capture adults for satellite telemetry (from 1992 onwards). Recent surveys in Estonia showed that hybridization probably occurs regularly, but the extent of possible back-crossing is still unknown (Väli and Lõhmus 2004).

Here, we investigate the genetic differentiation and potential gene flow between the two eagle species using sequences of the mitochondrial control region and nuclear amplified fragment polymorphism (AFLP) markers. In particular, we were interested to see if unisexual (female) hybrid sterility might restrict mitochondrial gene flow in the contact area. If interspecific hybrids show reduced viability or fertility compared to the parental species, the heterogametic sex (female in birds) usually suffers more than the homogametic sex, a pattern known as "Haldane's rule" (Haldane 1922). In birds, several cases conforming to this pattern have been documented (Tegelström and Gelter 1990; Wu and Davis 1993; Helbig et al. 2001).

If unisexual hybrid sterility was operating among spotted eagles, we would expect mitochondrial gene flow to be restricted or completely interrupted between the taxa, because sterility should preferentially occur in females, i.e. the sex transmitting the mitochondrial genome. If, alternatively, segregation of mitochondrial haplotypes between the two taxa is found to be incomplete, this could either be due to ancestral polymorphism or ongoing gene flow. To decide between these alternatives, we investigated anonymous nuclear genomic markers by AFLP (Vos et al. 1995), a method which has previously been used to distinguish populations within a species and to identify hybrids (Bensch et al. 2002a, 2002b; Wang et al. 2003). If ancestral polymorphism was responsible for the lack of complete segregation between mtDNA and phenotype, we would expect "mismatch" birds (individuals with phenotype of one, but mt haplotype of the other taxon) to be clearly assignable, genetically, to one or the other taxon. Alternatively, if such mismatches were due to recent hybridization, AFLP markers should indicate a genetically intermediate position of mismatched individuals.

Methods

Sampling

Blood samples were taken of 61 *A. pomarina* individuals (adults and nestlings) in Germany (13 in Mecklenburg-Vorpommern, 11 from Hakel forest, Sachsen-Anhalt), eastern Poland (3), Belarus (4) and Latvia (30). *Aquila clanga* was sampled on the breeding grounds in Poland (9) and Belarus (4) as well as on migration or wintering grounds in Turkey (2), Saudi Arabia (2) and Israel (3). Only individuals not directly related through the maternal line were included in this analysis (i.e. no known mother-offspring pairs or siblings). In addition, two putative F1 hybrids were included, i.e. offspring of mixed pairs whose mothers were of the *clanga* phenotype: one from near Greifswald, Mecklenburg-Vorpommern, NE Germany (year 2003; this individual was not scored for AFLP) and one from NE Poland (Biebrza, year 2001). Since eagles are known to be faithful to their nest-sites over many years (Meyburg et al. 2004), we sampled each territory only once in order to avoid the inclusion of siblings or half-siblings.

Nestlings were sampled shortly before fledging and were identified based on plumage features (size of pale spots on median wing-coverts, width of barring on remiges) and structural measurements (bill height, length of middle toe, emargination on seventh primary) using criteria outlined by Bergmanis (1996) and Forsman (1999). Care was also taken to safely identify both adults attending a nest.

mtDNA

Total DNA was isolated from the blood samples using a standard salting-out procedure (Miller et al. 1988). A large fragment of mitochondrial DNA including the entire control region (cr), ND6 gene, and pseudo-control region was amplified on a Perkin Elmer thermocycler (Geneamp 4200) with primers UUL (Liebers et al. 2001) and DDL (Helbig and Seibold 1999) using the Expand TM Long Template PCR System (Boehringer Mannheim) as described in Liebers et al. (2001). Primer UUL binds to the tRNA-Thr gene immediately upstream of the cr, DDL binds to the 12sRNA gene

downstream of the pseudo-control region. Note that *Aquila* eagles possess mitochondrial gene order C as described by Mindell et al. (1998). The PCR products were of variable lengths due to variable numbers of tandem repeats in the pseudo-control region (cf. Väli 2002). The hypervariable part of the control region (HVR-1), in most birds consisting of approximately 300–450 nucleotides near the 3' end of the cr, was sequenced using the primer HLB (Liebers et al. 2001). This primer binds to the D-box which is relatively highly conserved among birds (Baker and Marshall 1996). A total of 448 nucleotides was sequenced in each individual, either manually using ^{33} P-labelled dATP or on a Li-cor 4200 automated sequencer using fluorescently labelled primers. For a subsample of individuals ($n = 22$) part of, or the entire, cytochrome b gene was sequenced using methods and primers described by Helbig et al. (2005). Particular precautions were taken to ensure that sequences derived from genuine mtDNA rather than possible nuclear copies of the control region. Target sequences were compared between long-PCR amplicons obtained from the same DNA with up to five different primer combinations (for details see Liebers et al. 2001). We found no discrepancies indicating coamplification of non-mitochondrial sequences.

AFLP

We used the AFLP-protocol of Vos et al. (1995) with minor modifications, described in detail by Bensch et al. (2002a). Briefly, total genomic DNA (250 ng) was restricted for 1 h with 2.5 units each of *Eco*RI (Amersham) and *Tru*I (an isoschizomer of *Mse*I; Tamro Laboratories), after which a ligation mix containing 0.5 μM of E-adaptor, 5 μM of M-adaptor and 0.5 units of T4 DNA ligase was added (see Bensch et al. 2002a for adaptor sequences). A preselective amplification was performed with an additional T at the 3' end of the E-primer and an additional C at the 3' end of the M-primer. The diluted product of the preselective amplification was used in a series of selective PCR amplifications aimed at testing various combinations of selective primers, each containing three additional nucleotides at the 3' end. The fragments were separated in 6% polyacrylamid gels and detected by fluorescein labelled E-primers in a Vistra FluorImager. Based on the number of easily scorable, potentially informative polymorphic bands, we chose three selective primer combinations, A ($E_{TCT} \times M_{CGA}$), B ($E_{TGA} \times M_{CGT}$) and C ($E_{TAG} \times M_{CAT}$), which were applied to all individuals in our sample. Only variable bands that could be scored unequivocally for all samples were analyzed: 6 for primer combination A, 9 for primer combination B and 26 for primer combination C. Individual bands were scored as either present or absent, i.e. we treated the AFLP markers as being dominant, not separating between heterozygotes and homozygotes.

Analysis

From the 30 HVR-1 haplotypes a median-joining network (Bandelt et al. 1999) was constructed using the program NETWORK 4.1 (Röhl 2004). Genetic differentiation between species (Φ_{ST}) was calculated using ARLEQUIN v. 2.0 (Schneider et al. 2000), which was also used to perform an analysis of molecular variance (AMOVA) with Kimura 2-parameter distances. Likelihood mapping (Strimmer and Haeseler 1997) was performed with TREE-PUZZLE 5.0 (Schmidt et al. 2000) using the HKY model and gamma distributed rate variation between sites. For the AFLP data we also used ARLEQUIN to calculate F_{st} values between the species. Note that this method of calculating F_{st} makes no assumption regarding Hardy-Weinberg equilibrium. For the separation of the two species and classification of individuals of potential hybrid origin, we employed an assignment test (Paetkau et al. 1997; Waser and Strobeck 1998) based on the presence / absence of fragments using the Doh assignment calculator (http://www2.biology.ualberta.ca/jbrzusto/Doh.php). Because we scored the AFLP markers as dominant, the assignment calculator was set at ploidy = 1. The prediction is that pure genotypes will be identified by a larger difference in the calculated likelihood of origin than hybrids, which, on the other hand, will be revealed by having similar likelihood scores for the two taxa as a result of their mixed ancestry. We also illustrated the genetic variation among individuals using principal coordinate analyses (PCO) using the R package (http://www.fas.unmontreal.ca/BIOL/legendre/). We used a matrix of Euclidean pairwise distances between individuals, from which the PCO-analysis is extracting the major axes of variation in the data set.

Results

Mitochondrial DNA

The complete HVR-1 alignment consisted of 83 sequences, each 448 nucleotides in length. Twenty-six positions showed nucleotide variation defining a total of 30 haplotypes (Table 1). All variation was in the form of nucleotide substitutions and no length variation was found within the stretch sequenced. Variable sites were highly concentrated in the central portion of the HVR-1. Transition differences were found at 24 sites, transversions at two sites (C-A and C-G, respectively). Overall, the HVR-1 region proved more variable than the mitochondrial pseudo-control region studied by Väli (2002) and Väli et al. (2004).

In the median-joining network (Fig. 1), the haplotypes formed two distinct clusters whose mutual monophyly was supported at 100% by likelihood mapping. Haplotypes of cluster A occurred exclusively in *A. pomarina*, those of cluster B occurred on both species, but predominantly in *A. clanga*. The two offspring of

mixed pairs (one each from Poland and Germany) had cluster B haplotypes as expected, since their putative mothers were of the *clanga* phenotype. Haplotypes of cluster A and B differed on average by 13.4 nucleotides (3.0% sequence divergence), 10 nucleotide differences (2.2%) were fixed between the clusters. This was reflected in a clearly bimodal frequency distribution of pairwise sequence divergence values (Fig. 2).

Five of the 61 phenotypically pure lesser spotted eagles carried cluster B ("*clanga*-type") haplotypes (sub-

Table 1 Mitochondrial (HVR-1) haplotypes found in this study of greater and lesser spotted eagles (*Aquila clanga, A. pomarina*). Variable nucleotide positions are shown with reference to haplotype 01 (dots indicate identity with HT_01). Haplotype frequencies per species (number of phenotypically identified individuals carrying resp. haplotype) and EMBL accession numbers are given

Ht no.	variable nucleotide sites 11111111111222222233 14892455678888889012223311 80381945913456786650340612	frequency *clanga*	 *pomarina*	acc. no.
HT_01	ACACAGCGGTTCCTAGGTAACTGTTC	9	2	AJ875190
HT_02	.....A...	2	-	AJ875191
HT_03	.....C.....	4	-	AJ875192
HT_04	.....T.....T.AC..	1	-	AJ875193
HT_05	.....C.....A...	1	-	AJ875194
HT_06	.....A.....	-	1	AJ875195
HT_07	.....A.....A.....	2	1	AJ875196
HT_08	.....A.....A.....A...	1	-	AJ875197
HT_09	.....AA.....A...	1	-	AJ875198
HT_10	.T.....AAC.....A.....A...	1	1	AJ875199
HT_11	G.....A.ACC.TCGAC.....AC..	-	3	AJ875200
HT_12	G.....A.ACC.TCGAC.....AC.T	-	4	AJ875201
HT_13	G.....A.ACC.TCGAC.....ACC.	-	1	AJ875202
HT_14	G.....A.ACC.TCGAC....CAC..	-	2	AJ875203
HT_15	G.....A.ACC.TCGAC....CACC.	-	6	AJ875204
HT_16	G.....A.ACC.TCGAC...T.AC..	-	8	AJ875205
HT_17	G.....A.ACC.TCGAC...T.AC.T	-	9	AJ875206
HT_18	G.....A.ACC.TCGAC...TCAC..	-	4	AJ875207
HT_19	G.....A.ACC.TCGAC...TCAC.T	-	3	AJ875208
HT_20	G.....A.ACC.TCGAC..GT.AC..	-	1	AJ875209
HT_21	G.....A.ACC.TCGAC.G...AC..	-	4	AJ875210
HT_22	G.....A.ACC.TCGAC.G...AC.T	-	1	AJ875211
HT_23	G.....A.ACC.TCGAC.G.T.AC..	-	1	AJ875212
HT_24	G.....A.ACC.TCGAC?..TCAC..	-	1	AJ875213
HT_25	G.....A.ACC.TCGAT..G..AC..	-	2	AJ875214
HT_26	G....AA.ACC.TCGAC...TCACC.	-	1	AJ875215
HT_27	G...G.A.ACC.TCGAC...TCAC.T	-	1	AJ875216
HT_28	G..T..A.ACC.TCGAC.....AC.T	-	1	AJ875217
HT_29	G..T..A.ACC.TCGAC..G..AC..	-	1	AJ875218
HT_30	G.G...A.ACC.TCGAC...T.AC..	-	2	AJ875219

sequently called "mismatch" birds): 3 originated from Mecklenburg-Vorpommern, NE-Germany, 1 from the Biebrza region in eastern Poland and 1 from Latvia. Contrary to expectation, mismatched individuals did not appear to be more frequent in the eastern part of the zone of sympatry (Latvia, E Poland, Belarus: 2 out of 37) than to the west of this zone (Germany: 3 out of 24). No eagle identified phenotypically as *A. clanga* contained cluster A haplotypes (but note smaller sample size).

As a control, we also sequenced the cytochrome b gene of 10 *A. pomarina* (among them 3 mismatch birds as defined by their HVR-1 haplotype), 1 putative F1 hybrid and 11 *A. clanga* individuals. Cytochrome b haplotypes of *clanga* and *pomarina* (AJ604487, AJ604490) differed on average by 20 nucleotides (1.75% sequence divergence). As expected from the clonal inheritance of mtDNA, all 3 *pomarina* with a HVR-1 mismatch also had a *clanga* cyt b haplotype.

Despite some unidirectional mitochondrial introgression, the two eagle species were significantly differentiated at the mitochondrial-genetic level (AMOVA on HVR-1 data: $\Phi_{CT} = 0.8136$; $p < 0.001$). This differentiation corresponds to an average of 0.11 female migrant per generation (N_m) between the species. We sampled *A. pomarina* populations in eastern Europe (E Poland, Belarus and Latvia) and at the westernmost edge of the species' range in Germany. These populations are separated by a minimum distance of 650 km, but were not significantly differentiated ($\Phi_{SC} = -0.00089$; $p > 0.25$; $N_m = \text{infinite}$). The three-way AMOVA indicated that 81.3% of the total mitochondrial-genetic variation was distributed between the species, 18.6% was within populations and only 0.1% was between the eastern and western part of the *pomarina* range.

AFLP

None of the 41 polymorphic AFLP markers could be used alone to separate the two species. The marker showing the most extreme differentiation was present in 74% of *clanga* and 5% of *pomarina*. Despite the lack of highly informative markers the assignment test correctly classified all the supposedly pure genotypes, except 1 *clanga* and 4 *pomarina*. The assignment test indicated that the 5 individuals identified as *pomarina*, but with *clanga* mtDNA, were more similar to *clanga* than the supposedly pure *pomarina* (Fig. 3). Excluding the doc-

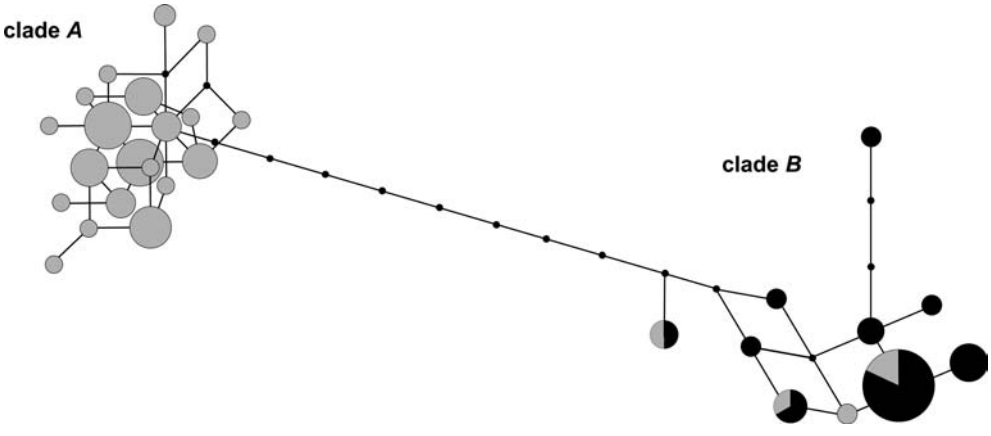

Fig. 1 Median joining network of HVR-1 haplotypes found in greater and lesser spotted eagles (*Aquila clanga, A. pomarina*). The size of *circles* is proportional to their frequency in a sample of 83 birds (*grey*: *pomarina*; *black*: *clanga*). *Small dots* denote haplotypes that were not observed

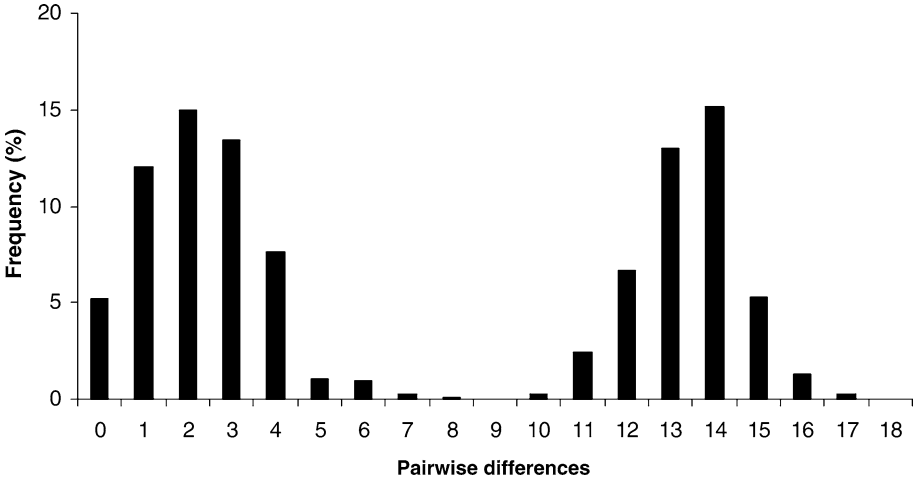

Fig. 2 Frequency distribution of pairwise sequence divergences (mismatch distribution) among all HVR-1 haplotypes found

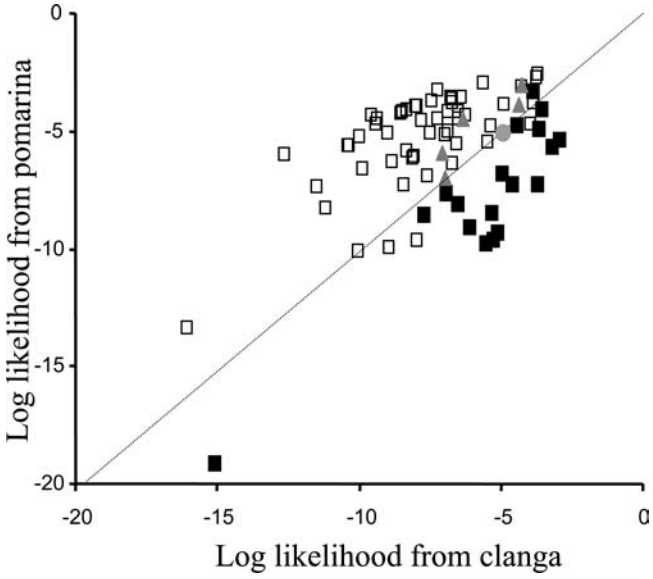

Fig. 3 Result of an assignment test based on 41 polymorphic AFLP fragments in supposedly pure *A. pomarina* (*open squares*) and *A. clanga* (*black squares*). *Grey triangles* denote the five individuals of the *A. pomarina* phenotype with *A. clanga* mtDNA haplotype and the F1 hybrid is denoted with a *grey circle*. On the *dotted line*, the log likelihood of origin is identical for the two species

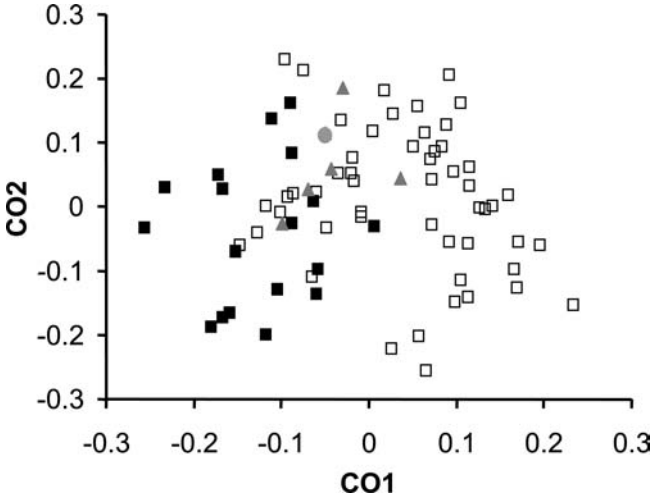

Fig. 4 The relationship between the first two principal coordinate axes (CO1 and CO2) derived from the 41 polymorphic AFLP fragments. Symbols as in Fig. 3

umented F1 hybrid from the analysis, the mean absolute difference in log-likelihood scores to *clanga* was significantly larger ($t = 2.45$, $p = 0.018$) for the pure *pomarina* (2.68 ± 1.57) than for the mismatched birds (0.94 ± 0.71).

Using principal coordinate analysis to delineate the differences between the species showed significant differences along three axes. The spread of data points along two axes is illustrated in Fig. 4. Along the first axis (CO1), the mismatched birds were clearly intermediate and significantly different from the pure *pomarina* ($t_{60} = 2.35$, $p = 0.022$). Along CO2 and CO3, they were more similar and not significantly different from pure *pomarina*.

Based on the AFLP data, there was a significant genetic structure between the two species ($F_{st} = 0.179$; $p < 0.0001$). Assuming equilibrium between mutation/drift and migration, this estimate corresponds to a gene flow of 1.1 migrants per generation (N_m).

Discussion

Our results show that greater and lesser spotted eagle populations in east-central Europe are significantly differentiated for mitochondrial markers. However, these markers are not diagnostic, i.e. there is no perfect segregation of mitochondrial haplotypes with phenotypes. The two species are thus not represented by mutually monophyletic clades in the gene tree. Initially, we hypothesized that this lack of reciprocal monophyly was due to ancestral polymorphism or to hybridization in the distant past. In both of these cases we would expect nuclear genomes of "mismatch" birds to conform to their phenotype. However, the AFLP analysis showed that this is not the case: birds with a mismatch between phenotype and mitochondrial haplotype were intermediate between typical *clanga* and *pomarina* AFLP genotypes. Although all five of these birds (excluding the two documented hybrids) had the typical *pomarina* phenotype, their AFLP scores differed significantly from all other *pomarina* and they were more similar to *clanga* than *pomarina* individuals that did not show a mismatched mtDNA. This result strongly suggests that "mismatched" lesser spotted eagles were actually recent back-cross hybrids, i.e. offspring of hybrid females breeding with *pomarina* males. If this interpretation is correct, it would mean that female hybrids are at least partially fertile.

Based on mtDNA analyses, back-crosses resulting from matings between male F1 hybrids and *pomarina* females will go undetected because they will have *pomarina* haplotypes. The AFLP method, on the other hand, generates information from multiple independent loci, which can be used in unbiased assignments of the genetic composition of individuals. Such assignments, e.g. as implemented in the program AFLPOP (Duchesne and Bernatchez 2002), require that reference data are available from pure populations not affected by introgression. This prerequisite is not fulfilled in our data for either of the two species. Although it might be possible to find non-introgressed *clanga* populations further east, this will be less likely for *pomarina* as we have already found appreciable mtDNA introgression up to the western edge of its rather restricted range. Hence, without the unbiased reference populations, we cannot estimate how many of the birds with matching phenotype and mtDNA genotype are of back-cross origin.

Mitochondrial gene flow appeared to be asymmetrical with "mismatched" mtDNA being found only in the *pomarina* and not in the *clanga* populations. Although

sample size was much smaller for *clanga*, this result may be real. First, due to the "reversed" sexual size dimorphism in these eagles (females being larger than males; Cramp et al. 1980; Bergmanis 1996), mixed matings are more likely to occur between the larger *clanga* females and the smaller *pomarina* males than the other way round (in which case the size difference between the sexes would be minimal). This is exactly the pattern that Väli et al. (2004) observed in Estonia. Second, for the same reason of reversed sexual size dimorphism, back-crossing would be expected to be biased in favour of female hybrids. A hybrid female is more likely to accept as mate a *pomarina* male, which would always be smaller than herself, rather than a *clanga* male, which might be the same size, if not larger, than herself.

We conclude that reproductive isolation between these two species of eagles is incomplete and that F1 hybrids are fertile, at least to some extent, which leads to asymmetrical gene flow between them. The estimated number of migrants per generation was an order of magnitude higher for the nuclear (AFLP markers) than for the mitochondrial genome. This may indicate that female hybrids survive or reproduce less well than male hybrids, a pattern that conforms to Haldane's (1922) rule. Due to the body size relationships between the species and sexes, male hybrids are expected to back-cross preferentially with *clanga* females, which are much rarer than *pomarina* females in the entire zone of sympatry. Gene flow may, in addition, be restricted by hybrid disadvantages also in males and/or by positive assortative mating (by taxon) in the sympatry zone, but sufficient data are not available to document this.

A surprising finding was that apparent hybrid genotypes occurred at an appreciable frequency (> 10%) in the lesser spotted eagle population even at the western edge of the species' range, in Germany. Greater spotted eagles, until recently, were known to breed no further west than eastern Poland, i.e. 650–750 km east of where the apparent hybrid genotypes were found in Germany. Although dispersal distances of these eagles are not well known, gene flow over such distances is unlikely to occur within one or two generations. Our findings therefore suggest that some greater spotted eagles (occasionally?) breed further west than is currently recognized, far within the range of lesser spotted eagle. Here, they may have greater difficulties to find conspecific mates, making hybridization more likely. Support for this hypothesis comes from the recent discovery of a mixed *clanga* (female) × *pomarina* (male) breeding pair near Greifswald, NE Germany, in the years 2003 and 2004. In 2003, the pair reared one offspring which indeed carried a *clanga*-type (cluster B, Fig. 1) mitochondrial haplotype. The fact that such cases have not been detected earlier in Germany or the western half of Poland is not surprising in the light of the great phenotypic similarity between the two species and their secretive way of life during the breeding season.

Many cases of incomplete reproductive isolation, with or without mitochondrial gene flow, have been documented between bird species that are phenotypically quite distinct (Grant and Grant 1992). Greater and lesser spotted eagles represent one of the few documented cases of a "zone of overlap and hybridization " (sensu Short 1969) among Accipitriform raptors, i.e. a broad zone where two species breed sympatrically, but hybridize regularly at low frequency. Such zones clearly indicate secondary contact after prolonged, or repeated, periods of allopatric divergence. In Europe, secondary contact zones between taxa re-expanding from separate glacial refugia and representing all stages of the genetic divergence process have been found in diverse kinds of organisms (Hewitt 2000). The hybridization frequency resulting in one migrant per generation, as estimated from the AFLP markers, for spotted eagles may be high enough to erode the existing genetic differentiation between the two species in the long run. The fact that the two species are still significantly differentiated phenotypically and genetically might indicate that contact between them is fairly recent. During recent glaciations, the two species are likely to have been isolated on different continents as indicated by their present winter quarters: *pomarina* probably in Africa and perhaps in some refugial area in the eastern Mediterranean basin, *clanga* somewhere in southern Asia. Given the postglacial vegetation history of the Western Palearctic, contact between these two inhabitants of mature deciduous forest probably occurred no earlier than about 5,000–6,000 years ago. This is the time when a contiguous deciduous forest belt was reestablished between Europe and Asia (Huntley 1990). Given the fairly long generation time of these eagles (first breeding occurs at 3–4 years of age), contact between them probably dates back less than 1,500 generations.

It is, of course, possible that hybridization has also occurred upon contact in previous interglacials, and our initial hypothesis regarding "mismatched" birds was that they represent a footprint of such ancient hybridization. In that case, however, we would expect them to show a nuclear genotype indistinguishable from pure *pomarina*, due to back-crossing over many generations. The fact that this is not what we found shows that mismatches between phenotype and mtDNA in most, if not all, cases are due to recent hybridization.

Zusammenfassung

Genetische Differenzierung und Hybridisation zwischen Schelladler (*Aquila clanga*) und Schreiadler (*Aquila pomarina*)

Schell- und Schreiadler sind zwei nah verwandte Adlerarten, deren Brutgebiete sich in Mittel- und Osteuropa großflächig überlappen. Das Vorkommen von Hybriden zwischen beiden wurde lange vermutet, doch erst in den letzten 15 Jahren wurden Bruten von Mischpaaren beschrieben, die in einigen Fällen erfolgreich verliefen. Um die genetische Differenzierung und das Ausmaß mögli-

chen Genflusses zwischen diesen Arten zu bestimmen, untersuchten wir DNA-Sequenzen der mitochondrialen Kontrollregion und nukleare AFLP-Marker bei 83 Individuen (61 *pomarina*, 20 *clanga*, 2 F1-Hybriden). Die dabei gefundenen 30 mitochondriale Haplotypen bildeten im phylogenetischen Netzwerk zwei Cluster mit einer mittleren Sequenzdivergenz von 3,0%. Die beiden Arten waren sowohl auf mitochondrialem als auch auf nuklearem (AFLP-) Niveau signifikant differenziert. Jedoch fanden sich fünf Individuen (in Lettland, Ostpolen und Deutschland) mit *pomarina*-Phänotyp, die einen mitochondrialen *clanga*-Haplotyp besaßen (''Mismatch''-Vögel), was auf gelegentlichen Genfluss hindeutet. Die AFLP-Marker zeigten, dass diese fünf Vögel auch hinsichtlich ihres nuklearen Genoms intermediär zwischen solchen *clanga*- und *pomarina*-Individuen waren, bei denen Phänotyp und mtDNA übereinstimmten. Dies zeigt, dass Mismatch-Vögel entweder F1- oder frühe Rückkreuzungshybriden mit Schreiadlern waren. Die mitochondriale Introgression verläuft offenbar asymmetrisch, denn bei Schelladlern wurden bisher keine *pomarina*-Haplotypen gefunden. Schätzungen für den nuklearen Genfluss lagen etwa zehnmal höher als für mitochondrialen Genfluss. Dies deutet auf eine geringere Fertilität (oder Vitalität) von weiblichen verglichen mit männlichen Hybriden hin und stimmt mit der Erwartung nach Haldane's Regel überein. Insgesamt deuten unsere Ergebnisse darauf hin, dass Hybridisation zwischen den beiden Adlerarten häufiger ist als bisher angenommen und viel weiter nach Westen in das Areal des Schreiadlers hineinreicht, als bisher bekannt. Dies wird durch die Entdeckung eines Schrei- x Schelladler-Mischpaares in Vorpommern bestätigt, das im Jahr 2003 einen Jungvogel erfolgreich aufzog (und 2004 erfolglos brütete).

Acknowledgements We thank G. Maciorowski, T. Mizera and H. Shirihai for help with the sampling and J.-P. Schwanbeck for sharing information about a mixed pair he discovered in NE Germany. The study was financially supported by grants of Bundesministerium für Forschung und Technologie (to A.J.H.) and the Swedish Research Council (to S.B.).

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