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SHORT COMMUNICATION

Mitochondrial pseudo-control region in old world eagles (genus *Aquila*)

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Abstract

The mitochondrial pseudo-control region was analysed in five species of *Aquila* eagles. Indels caused great length differences in the nonrepetitive part of the region, whereas tandem repeat units were highly conserved among the genus. In a reconstructed phylogenetic tree, the clade formed by *A. clanga* and *A. pomarina* showed approximately the same distance to *A. chrysaetos* as did the clade *A. heliaca*/*nipalensis*. In Estonian subpopulations, 12 haplotypes were found among 51 *A. pomarina* individuals and 7 haplotypes among 8 *A. clanga* individuals. Nucleotide diversity in *A. clanga* was greater, and may be caused by decreased breeding density or by gene flow from other subpopulations.

Keywords: bird, endangered species, mtDNA, phylogeny, population diversity, spotted eagle

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Introduction

The control region (CR) has usually been considered to be the most variable part of the mitochondrial DNA (mtDNA) molecule. This noncoding region is located between the genes $tRNA^{Glu}$ and $tRNA^{Phe}$ in birds (Desjardins & Morais 1990). However, in Picidae, Cuculidae, Falconidae and the suboscines group of Passeriformes, a typical CR was discovered between $tRNA^{Thr}$ and $tRNA^{Pro}$, and another noncoding sequence between $tRNA^{Glu}$ and $tRNA^{Phe}$, i.e. at the site corresponding to CR in the standard gene order in birds (Mindell *et al*. 1998). Such rearrangement was later also detected in Accipitridae (*Buteo* species), and the second noncoding sequence was named the pseudo-control region (ΨCR; Haring *et al*. 1999). The ΨCR has been suggested to occur in the other raptors, because the region between $tRNA^{Glu}$ and $tRNA^{Phe}$ that was sequenced in *Aquila chrysaetos japonica* by Masuda *et al*. (1998) contained features resembling the ΨCR — a long unique 5′ region followed by a number of tandem repeats (Haring *et al*. 1999). Indeed, recently, the novel gene order described by Mindell *et al*. (1998) has been found in 20 of 22 *Falconiformes* species tested (Muñoz *et al*. 2001).

Six species of eagles of the genus *Aquila* are distributed across Eurasia: spotted eagle, *Aquila clanga*; lesser spotted eagle, *A. pomarina*; golden eagle, *A. chrysaetos*; steppe eagle, *A. nipalensis*; imperial eagle, *A. heliaca*; and Spanish imperial eagle, *A. adalberti* (Snow & Perrins 1998). The phylogeny of the genus has been investigated by sequencing the cytochrome b gene (*cyt b*) from mtDNA (Seibold *et al*. 1996). Contradictions were found when molecular data were compared with morphological data. For example, using *cyt b*, *A. chrysaetos* is about as divergent from spotted eagles (*A. clanga* and *A. pomarina*) as it is from the group formed by *A. heliaca* and *A. adalberti*, although the plumage characters of adults suggest close relatedness between latter species and *A. chrysaetos*.

According to the *cyt b* sequence data, *A. clanga* and *A. pomarina* diverged ≈ 1 Ma (Seibold *et al*. 1996). These species are morphologically and ecologically similar, and even hybridize in areas where their ranges overlap (Lõhmus & Väli 2001). *A. clanga* is the least studied and one of the most threatened eagle species in Europe (Meyburg *et al*. 2001). The population size of this species is unknown, but it is probably < 1000 pairs in Europe, i.e. at least 20 times fewer than *A. pomarina* (Meyburg *et al*. 2001). Population genetics of both species remain unexplored but investigation of genetic variability is essential for conservation.

In this study, I first describe the sequences of ΨCR in five Eurasian *Aquila* species. Although CR has been studied intensively in recent decades and is described in a large number of animal taxa (Baker & Marshall 1997 and references therein), current knowledge about ΨCR remains very poor. Second, I use ΨCR for phylogeny reconstruction in *Aquila* species and

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compare the results with those derived from the *cyt b* sequence. The *cyt b* is considered to be an appropriate tool for taxonomic studies above the (sub)species level (Moore & DeFilippis 1997), but is too robust for population studies. Although CR has been used extensively as a population marker, studies of ΨCR have usually remained at the species level thus far (but see Irwin *et al*. 2001). Here, I examine the suitability of NR-ΨCR for population studies using two spotted eagle species. I ask whether the genetic structure of the rare *A. clanga* differs from the more common *A. pomarina* in the northern edge of both species' ranges.

Materials and methods

Samples

Blood samples from two captive individuals of *Aquila chrysaetos*, *A. heliaca* and *A. nipalensis* and one individual of *A. clanga* (A.cla2, caught as an adult in Estonia) were obtained from Tallinn Zoo. In addition, blood samples from one wild adult and nestlings of two pairs of *A. clanga*, as well as nestlings of 50 pairs of *A. pomarina* were collected during fieldwork in Estonia (58° – 59° N, 24° – 27° W). Samples of five hybrid nestlings of *A. clanga* and *A. pomarina* were also included in the analyses. Four of these individuals had mtDNA of *A. clanga* and one of *A. pomarina*. Altogether, 8 ΨCR sequences of *A. clanga* and 51 sequences of *A. pomarina* were used in this study.

DNA extraction, amplification and sequencing

One millilitre of blood was collected into a vacuntainer containing EDTA buffer. Blood cells were separated from serum and stored at –20 °C. Total DNA was isolated after incubating blood cells at 56 °C overnight in extraction buffer (10 mM Tris–HCl pH 8.0, 2 mM EDTA, 400 mM NaCl, 10 mM $MgCl_2$, 10 mM KCl, 1.5% SDS, 0.5 mg/mL proteinase K), purification with 5 M NaCl and ethanol precipitation. In the polymerase chain reaction (PCR) 34 cycles of 94 °C for 20 s, 61 °C for 20 s, 40 °C for 60 s with a final extension of 72 °C for 5 min followed the 4 min initial heating at 94 °C. Primers E-ACH and CT2A (Masuda *et al*. 1998), positioned on $tRNA^{Glu}$ and $tRNA^{Phe}$, respectively, were used first to amplify ΨCR in two individuals from each *Aquila* species. To avoid technical problems in amplification generated by tandem repeats (see Masuda *et al*. 1998), a novel downstream primer REV-ACP 5′-TTGTTGGTTRAGTTTATGTGTGG-3′ was developed for the 3′ terminus of NR-ΨCR and, after proving the sequence identity, was used in further amplifications with primer E-ACH at an annealing temperature of 57 °C in *A. clanga* and *A. pomarina*.

PCR product was purified using shrimp alkaline phosphatase and exonuclease I treatment at 37 °C for 15 min and at 80 °C for an additional 15 min; it was sequenced with ABI PRISM 377 automated sequencer using DYEnamic sequencing kit (Amersham) and primers IE-ACH, CT2A (Masuda *et al*. 1998) and REV-ACP. In addition, to sequence the ΨCR region downstream of the 500 bases, primers C-ACP 5′-CCTAATTAGCCCTAYGAYC-3′ and C-AHN 5′-CGAGAAATCARCAACCTTACG-3′ were designed for *A. clanga*/*pomarina* and *A. heliaca*/*nipalensis*, respectively.

All mutations were checked by reamplification and resequencing. In addition, analysis of nestlings sampled in different years on the same breeding territory (12 cases) proved the precision of amplification and sequencing.

Because avian erythrocytes have a nucleus, PCR may favour the amplification of nuclear copies of mitochondrial genes (numts) when blood is used as a source of DNA (Sorenson & Quinn 1998). However, the probability of numt amplification instead of ΨCR is low because: (i) amplification of DNA from muscle cells of *A. pomarina* gave a product with the same sequence as that obtained from blood; (ii) all hybrid individuals obtained ΨCR sequences typical to the female species. This was checked also by restriction analysis; (iii) the results of amplification and sequencing a region of the *cyt b* gene according to Seibold *et al*. (1996) in 2 *A. clanga*, 10 *A. pomarina* and 2 hybrid individuals were identical with those obtained from ΨCR analysis. However, NR-ΨCR of the common buzzard *Buteo buteo* was collected, amplified and analysed using the same methods, it showed absolute identity with the mitochondrial sequence published by Haring *et al*. (2001; AF380305).

Sequence analysis and phylogeny reconstruction

Sequences were aligned using CLUSTAL W (Thompson *et al*. 1994) and were improved manually. Phylogenetic and molecular evolutionary analyses were conducted mainly using MEGA Version 2.1 (Kumar *et al*. 2001). For neighbour-joining tree reconstruction pairwise distances were estimated using the Kimura 2-parameter method (Kimura 1980). To compare results with published *cyt b* data, p-distances were also calculated. In the parsimony analysis, a heuristic search with TBR branch swapping of 1000 bootstrap replicates was performed. FASTDNAML Version 1.0 (Olsen *et al*. 1994) with transition/transversion ratios of 2.0 and 100 bootstrap resamplings was used for maximum likelihood analysis.

All sequences are deposited in GenBank under Accession nos AF435093–AF435099 (with alignment) and AF487438–AF487453.

Results

ΨCR in the genus Aquila

The amplified product of *Aquila* species had a nonrepetitive region in the 5′ part (NR-ΨCR) and a cluster of tandem repeats in the 3′ repetitive part (R-ΨCR).

	Aquila clanga	*A. pomarina*	*A. nipalensis*	*A. heliaca*	*A. chrysaetos*
A. clanga		0.0450	0.2725	0.3227	0. 1865
A. pomarina	16/1		0.2817	0.3331	0. 1978
A. nipalensis	69/17	72/16		0.1035	0.2237
A. heliaca	82/15	85/14	35/2		0.2209
A. chrysaetos	51/12	55/11	55/19	56/17	

Table 1 Kimura 2-parameter distances (above diagonal) and transitions/transversions ratio (below diagonal) of the 413-bp sequence that occurred in all species

Large indels (up to 218 bp) caused great differences in the length of NR-ΨCR, from 491 bp (*A. chrysaetos*) to 962 bp (*A. clanga*). In *A. heliaca* the NR-ΨCR had a length of 885 bp, in *A. nipalensis* a length of 927 bp and in *A. pomarina* a length of 945 bp. However, *A. clanga* had only one (17 bp) and *A. nipalensis* two (17 and 19 bp) short insertions compared with *A. pomarina* and *A. heliaca*, respectively.

Because of the large indels in NR-ΨCR a total of only 413 bp from the three fragments found in all species was chosen for phylogenetic analysis (positions 8–100, 596–695 and 840–1060 in the alignment). The similarity of the 413 bp fragment varied from 72 to 95% among species. There were 137 available sites and 78 were phylogenetically informative. Kimura 2-parameter distances of sequences, and the numbers of transversions and transitions are shown in Table 1. The average ratio of transitions/transversions was 6.9; transitions outnumbered transversions greatly in all species pairs. No saturation effect was found. Comparing the entire NR-ΨCR, the Kimura 2-parameter distance between *A. clanga* and *A. pomarina* was 19% greater than the divergence by the 413 bp fragment.

The average p-distance for the 413 bp sequence differed by 4.3% from the Kimura 2-parameter distance, and was 2.60 ± 0.41 (SD; $n = 6$) times greater than Seibold *et al*. (1996) estimated for the *cyt b* gene. According to the entire NR-ΨCR divergence between *A. clanga* and *A. pomarina* sequences, the evolutionary rate of NR-ΨCR is 3 times greater than in *cyt b*.

It was not possible to obtain any adequate alignment between the 413 bp fragment from *Aquila* and NR-ΨCR from other raptor species. Therefore, no outgroup was chosen for phylogeny reconstruction and an unrooted tree was generated. Three methods, neighbour joining (NJ), maximum parsimony (MP) and maximum likelihood (ML), yielded trees of identical topology and maximum bootstrap values (100). All branches in the ML tree were significantly positive at $P < 0.01$. *A. clanga* and *A. pomarina* formed a clade with the shortest branches. A second clade was formed by *A. heliaca* and *A. nipalensis*. *A. chrysaetos* was situated between these two groups and it had approximately the same distance to both (Fig. 1).

Repeat units in R-ΨCR were 48–49 bases long and highly conserved: the similarity between species was 88–100%, reflecting 0–5 single point mutation differences. *Aquila* tandem repeats showed 61–67% similarity with both

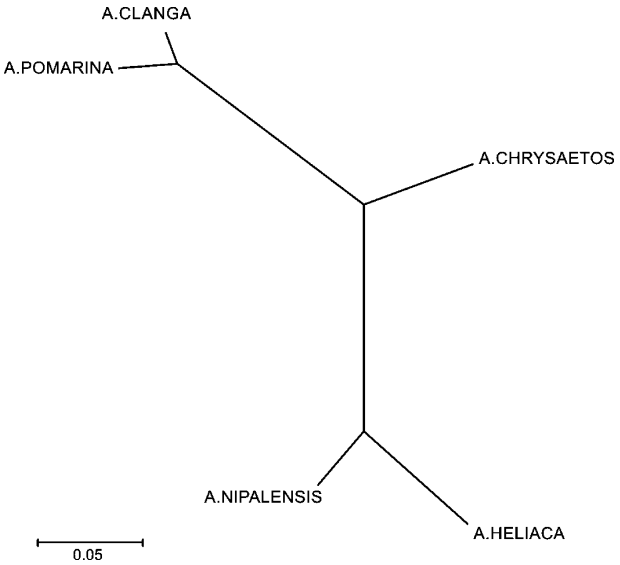

Fig. 1 Unrooted neighbour-joining tree of five *Aquila* species based on Kimura 2-parameter distance of 413 bp sequence from NR-ΨCR.

B. buteo (AF380305) and *Haliaeetus albicilla* (AY034150). The number of repeats in *Aquila* remained unclear because direct sequencing allowed us to read only a partial sequence of the repeat region, but it was possible to detect up to 10 repeat units in all species.

Intraspecific NR-ΨCR variation in A. clanga *and* A. pomarina

Intraspecific variability of the two spotted eagle species was analysed by 895 consistently readable bases of NR-ΨCR (bases 4–991 in the alignment). Altogether 25 segregating sites were found, 11 among *A. clanga* and 14 among *A. pomarina*, whereas position 280 was variable in both species (Fig. 2). In addition to the 17 bp indel (bases 523–539), 43 species-specific point mutations were found. Thus, *A. clanga* and *A. pomarina* are two clearly separate groups [mean interpopulation nucleotide diversity 0.0216 ± 0.003 (SE)].

Among 51 individuals of *A. pomarina*, 12 haplotypes occurred, the most common A.pom1 accounted for 79% of the samples (Fig. 2). The others were just splits based on single-point substitutions (13 transitions and 1 transversion).

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                12344444445688                    22233556778
                88201144595599                    08946594689
                80825805810103                    70156577281

A.pom 1 (40)  CAGTCTTATTGCTT     A.cla 1 (2)   -AAGTTGCCA-
A.pom 2 (1)   T.............      A.cla 2 (1)   .G........
A.pom 3 (1)   ...C..........      A.cla 3 (1)   ....C.....
A.pom 4 (1)   ....T.........      A.cla 4 (1)   ....C..A...
A.pom 5 (1)   .....C........      A.cla 5 (1)   ....C.....T
A.pom 6 (1)   ......C.......      A.cla 6 (1)   ......A.T..
A.pom 7 (1)   .......G......      A.cla 7 (1)   C.GA.C...G.
A.pom 8 (1)   ..........A...
A.pom 9 (1)   ............-.
A.pom 10(1)   ..A........A..
A.pom 11(1)   ......C.C.....
A.pom 12(1)   .G.......C...C
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Fig. 2 Variable sites of 12 *Aquila pomarina* and 7 *A. clanga* NR-ΨCR haplotypes, based on the analysis of 895 bp of NR-ΨCR. Site numbers correspond to positions in the reference sequence. The number of individuals within a haplotype is given in brackets. The 26-bp deletion in Acla6 (bases 468–493) is not shown.

Eight individuals of *A. clanga* were divided into seven haplotypes based on a total of eight transitions, one transversion and two single-point insertions/deletions. In addition, one hybrid individual (A.cla6) had a 26-bp deletion (bases 468–493). Nucleotide diversity was greater for *A. clanga* than for *A. pomarina* [0.0029 ± 0.001 (SE) and 0.0006 ± 0.0002 (SE), respectively].

Discussion

Interspecific variation

In the five species of *Aquila*, the mitochondrial region between RNA^{Glu} and RNA^{Phe} had a nonrepetitive 5′ region followed by a number of tandem repeats. This is a typical structure of ΨCR in raptors (Haring *et al*. 2001). ΨCR has been found also in Piciformes, Cuculiformes and Passeriformes (Mindell *et al*. 1998; Bensch & Härlid 2000), but these groups lack tandem repeats.

Nonrepetitive regions of raptor ΨCR sequenced to date have consisted of 169 bases in *Falco peregrinus* (Mindell *et al*. 1998), 338 bp in *Buteo buteo* (Haring *et al*. 1999) and 360 bp in *Haliaeetus albicilla* (AY034150; Haring *et al*. 2001). The length of a similar region in *Aquila chrysaetos* was 472 bp (Masuda *et al*. 1998). In a comparison of five *Buteo* species, no large indels were found in the NR-ΨCR (Haring *et al*. 1999). Therefore it was surprising to find a large interspecific variation in the length of NR-ΨCR between *Aquila* species. However, also within the genus *Phylloscopus*, the corresponding region showed high length variation (from 171 to 308 bp) and only two conserved parts were found in six species, the first had a length of 56 and other of 80 bp (Bensch & Härlid 2000).

Also in CR, remarkable differences in length have been registered both in birds and in mammals. These occur between genera (Baker & Marshall 1997), species (Edwards & Wilson 1990) and subspecies (Edwards & Wilson 1990; Stewart & Baker 1994). Although the difference in length is usually caused by a variable number of tandem repeats, several findings report large indels that regulate the length of the CR (Baker & Marshall 1997 and references therein).

The number of tandem repeats in the 3′ region of ΨCR varies among raptor genera. Twenty-five repeats were found in *F. peregrinus* (Mindell *et al*. 1998), 23 in *B. buteo* and 11 in *A. chrysaetos* (AY034151; Haring *et al*. 2001). According to our study, the other *Aquila* species also have at least 10 repeat units. Haring *et al*. (2001) concluded that there is no apparent relatedness between *Aquila*, *Buteo* and *Haliaeetus* tandem repeats, but I succeeded in finding 61–67% similarity between aligned sequences of single repeat units. However, tandem repeat unit sequences are very conserved within genera according to the studies of *Buteo* (Haring *et al*. 1999) and *Aquila* (this study).

Phylogenetic analysis of NR-ΨCR showed that *A. clanga* and *A. pomarina* were most closely related, as were *A. heliaca* and *A. nipalensis*. *A. chrysaetos* had similar genetic distance to both. A phylogenetic tree based on the *cyt b* sequence showed the same features (Seibold *et al*. 1996). Mitochondrial genes have a substitution rate of 2%/Myr (Shields & Helm-Bychowski 1988), but it may be even slower in *cyt b* (Seibold *et al*. 1996). According to the results of this study, the evolutionary rate of NR-ΨCR is 2.2–3 times greater than in *cyt b*, thus the divergence rate for NR-ΨCR should be at least 4.4–6%/Myr. This calculation may be biased, because it relies on only an independent estimation of divergence time for *A. clanga* and *A. pomarina*. However, some of the divergences in coding regions and ΨCR between *Buteo* species (Haring *et al*. 1999) showed values similar to those obtained in this study.

Intraspecific variation

Twelve haplotypes were found among 51 *A. pomarina* individuals and 7 among 8 *A. clanga* individuals. The relative number in *A. pomarina* otherwise resembles the 5 haplotypes in 23 individuals of *A. chrysaetos* (Masuda *et al*. 1998), but the length of NR-ΨCR in spotted eagles is twice that in *A. chrysaetos*, giving more opportunity for mutation. In contrast, spotted eagles were sampled in an area of 30 000 km², whereas *A. chrysaetos* was studied in different parts of Japan, China and Korea.

The 26 bp deletion in NR-ΨCR of one *A. clanga* individual was surprising, but within *A. chrysaetos* a 4 bp deletion was found (Masuda *et al*. 1998) and in Turnstones (*Arenaria interpres*) one exceptional individual may differ much more in CR than other within a geographical region (Wenink *et al*. 1994).

In *A. clanga*, one should expect similar genetic variety as in *A. pomarina*, or it may be even lower as is usual in small populations of rare species (Haig & Avise 1996). But the rare *A. clanga* had greater nucleotide diversity than *A. pomarina* (see, e.g. Haig & Avise 1996; Pestano *et al*. 2000;

for similar results). This may indicate a recent crash in numbers of *A. clanga* and / or gene flow from other subpopulations. *A. clanga* numbers have decreased over all of its European range during the 20th century (Meyburg *et al*. 2001). In Estonia, for example, *A. pomarina* is ≈ 20 times as numerous as *A. clanga* (Lõhmus 1998). Hence, competition for mates or breeding territories may be much weaker in *A. clanga*, and the integration of foreign individuals into the local population is easier. Another explanation is that the Estonian *A. clanga* population originates from several glacial refugia. This is supported by the fact that *A. clanga* winters in several parts of southern Europe, the Middle East and northern Africa, whereas *A. pomarina*'s wintering grounds are in central and southern Africa linked with breeding grounds via only one migration route over Bosporus (Cramp & Simmons 1980).

Although NR-σCR showed great diversity in *A. clanga* in this study, no sequence variation was found in *cyt b* from six *A. clanga* individuals sampled in Poland and Turkey (Seibold *et al*. 1996). However, *cyt b* is considered to be most suitable for studies above the (sub)species level (Moore & DeFilippis 1997), and only part of this gene was sequenced by Seibold *et al*. (1996). Similarly, no variability was found in the allozymes of *A. adalberti* (Negro & Hiraldo 1994), but a more sensitive random amplified polymorphic DNA method revealed a high level of heterozygosity (Padilla *et al*. 2000). It is essential to explore the genetic diversity of spotted eagles in other regions of their ranges and NR-ΨCR seems to be an appropriate marker for it.

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