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Genetic Differentiation and Molecular Phylogeny of European *Aquila* Eagles according to Cytochrome b Nucleotide Sequences.

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ABSTRACT

We studied the molecular genetic differentiation and phylogeny of five European eagles of the genus *Aquila*. Special emphasis was placed on the possible species status of the Spanish Imperial Eagle, *A. (heliaca) adalberti*. The mitochondrial cytochrome b gene was amplified by PCR and 1026 bp were sequenced directly. Nucleotide sequences of *A. adalberti* and nominant *heliaca* differed by 1.8 %, the same amount as between the undisputed species *A. clanga* and *A. pomarina*. Although sample sizes are small, we found no evidence for recent gene flow between them. The Golden Eagle, *A. chrysaetos*, morphologically similar to the *A. heliaca* superspecies, is surprisingly divergent and in the phylogenetic tree falls outside the clade formed by the other four taxa.

The molecular data along with substantial differences in morphology and ecology support full species status for the Spanish Imperial Eagle, a finding which has important implications for the conservation and management of this globally threatened taxon.

INTRODUCTION

Within the family of Accipitridae, between 9 and 12 species of eagles are included in the genus *Aquila*, a group of powerful raptors which mainly occur in the Old World (Hartert 1914; Sibley & Monroe 1990; Newton 1990). We studied the molecular genetic differentiation and phylogeny of four closely related *Aquila* species breeding in Europe (*A. chrysaetos, pomarina, clanga, heliaca*) plus one well-marked (sub-?)species that may have reached full species status, *A. (heliaca) adalberti*. Special attention was focussed on the question whether the latter has attained a level of genetic differentiation that corresponds to species status when compared to other eagles.

Traditionally, *Aquila heliaca* has been separated into the nominate subspecies *A. h. heliaca* (Savigny 1809), breeding locally in parts of eastern Europe, across central Asia, and wintering in Africa, India, Indochina and China, and the western form *A. h.*

adalberti, described by Brehm (1861) as a distinct species, which is an endemic resident of the Iberian peninsula (Hartert 1914; Cramp & Simmons 1980). Today the two forms have widely disjunct ranges, but they may have been parapatric as late as the middle of the last century (González *et al.* 1989). Given the lack of evidence for past hybridization and the marked differences in plumage, morphology and ecology (Table 1), the two forms were recently regarded as constituting distinct species *A. heliaca* and *A. adalberti* (Hiraldo *et al.* 1976; González *et al.* 1989; Newton 1990). This view was followed in the most recent taxonomic world list uniting the two in a superspecies (Sibley & Monroe 1990), while most earlier handbooks treated them as subspecies (Hartert 1914; Glutz von Blotzheim *et al.* 1971; Cramp & Simmons 1980).

Table 1: Summary of phenotypic differences between *Aquila heliaca* and *Aquila adalberti* (after Glutz von Blotzheim *et al.* 1971; Hiraldo *et al.* 1976; Cramp & Simmons 1980).

Character	*A.heliaca*	*A.adalberti*
Breeding distribution	Eastern Europe, central Asia	Spain (Portugal)
Migratory status	partial to long-distance migrant	resident
Winter distribution	NE-Africa, Gulf, India, China	Iberian Peninsula (North Africa)
Size: bill length (mm)	M: 42.0; F: 45.3	M: 44.4; F: 48.4
wing length (mm)	M: 587; F: 622	M: 591; F: 630
tail length	M: 274; F: 290	M: 288; F: 303
Adult plumage:		
scapulars	white edges and blotches	white scapulars
leading edge to inner wing	dark	white
Juvenile plumage	mainly yellow-brown, copiously streaked	rusty tone, less streaked
Start breeding season	April	mid-Febuary onwards
Nest location	trees, rarely cliffs	trees only
Clutch	2 (3) eggs	2-3 (rarely 4) eggs
Care of nestlings	mostly female	larger role of male

Possible species status of *A. adalberti* is of particular interest, because of its restricted distribution and the severe reduction in the number of breeding pairs in historic times (Cramp & Simmons 1980). With only about 125 pairs left in the wild it has been classified as endangered (Collar & Andrew 1988), although recent data suggest that the population may again be increasing (Meyburg 1989; Ferrer & Calderón

1990; González 1991). Knowledge of its genetic differentiation compared to eastern *A. heliaca* and other *Aquila* eagles has so far been lacking and will be important in designing conservation and management strategies to prevent its total extinction.

Nucleotide sequences of the mitochondrial cytochrome b gene have been used extensively in studies of avian phylogeny (Edwards & Wilson 1990; Edwards *et al.* 1991; Quinn *et al.* 1991; Richman & Price 1992; Seibold *et al.* 1993; Wink *et al.* 1993; Avise *et al.* 1994) and in some cases population differentiation (Baker 1992; Taberlet *et al.* 1992) and have revealed several previously unrecognized species or semi-species (Smith *et al.* 1991; Helbig *et al.* 1993). We amplified and sequenced 1026 bp of the cytochrome b gene of five *Aquila* taxa in order to analyse the degree of their genetic differentiation and phylogenetic relationships. Outgroups were chosen in order to represent three levels of increasing phylogenetic distance from *Aquila*: 1) representatives of three relatively derived Accipitriform genera (*Accipiter, Buteo, Haliaeetus*), 2) the Honey Buzzard (*Pernis apivorus*) as a presumably primitive Accipitrid (Brown & Amadon 1968) and 3) the White Stork (*Ciconia ciconia*).

MATERIALS AND METHODS

Collection of blood and tissue samples

Samples consisted of blood (ca. 100 µl) collected from the brachial vein and in a few cases of muscle tissue of dead birds that had been deep-frozen. Samples of the following unrelated individuals were studied: Three *A. adalberti* from Spain (J. J. Negro); five *A. heliaca*, of which three were trapped on migration near Taif, Saudi Arabia (P. Gaucher), one in Kazakhstan (W. Bednarek), and one in Yugoslavia (C. Fentzloff); five *A. pomarina* from eastern Poland (1), northeastern Germany (3) and Hungary (1); six *A. clanga* (4 from eastern Poland; 2 from Turkey); five *A. chrysaetos* (4 from Switzerland, U. Schneppat), one falconer's bird of unknown origin). Blood was stored in EDTA-NaF-Thymol buffer (Arctander 1988) at ambient temperature during field work, transferred to Heidelberg and stored at -20°C until extraction.

DNA isolation

Small amounts of homogenized muscle tissue or 0.3 ml of the blood samples were mixed with 3 ml lysis-buffer (25 mM EDTA, 75 mM NaCl, 10 mM Tris, pH 7.5, 1% SDS) containing 2 mg proteinase K. After overnight incubation at 37°C, proteins and cell fragments were removed by precipitation with 1/3 volume of a saturated NaCl solution. By adding 0.8 volumes of isopropanol to the supernatant the DNA was precipitated and collected with a glass rod. The DNA was washed in 70% ethanol, air-dried and dissolved in TE buffer.

Polymerase chain reaction (PCR) and DNA-sequencing

Primer sequences used for PCR and direct sequencing were modified from Kocher

et al. (1989) in order to most closely approach general consensus sequences across all birds (Table 2). A 1100 bp portion of the cytochrome b gene was amplified using 1 µg of total DNA as target, 50 pmol each of primers A and F, 1.5 mM $MgCl_2$ and 2 units Taq-polymerase (Promega). After initial denaturation (2.5 min at 94°C), 32 cycles of 30 sec at 93°C, 45 sec at 45°C and 90 sec at 72°C were performed on a

Table 2: Primer sequences (5' - 3') used for PCR and direct sequencing (modified from Kocher *et al.* 1989). Positions in the chicken mitochondrial genome corresponding to the 3'-end of each primer are given in parentheses. L = L-strand, H = H-strand.

PCR Primers:

mt-A	(L-14995):	CTCCCAGCCC CATCCAACAT CTCAGCATGA TGAAACTTCG
mt-F	(H-16065):	CTAAGAAGGG TGGAGTCTTC AGTTTTTGGT TTACAAGAC

Sequencing primers:

mt-B	(H-15298):	TTGTGATTAC TGTAGCACCT CAAAATGATA TTTGTCCTCA
mt-C	(L-15320):	TAYGTCCTAC CATGAGGACA AATATCATTC TGAGG
mt-D	(L-15578):	AAAATCCCAT TCCACCCCTA CTACTCCACA AAAGA
mt-G	(L-15180):	CWTCCTTMTT CTTCATCTGC ATCTAC
mt-H	(L-15722):	CCYCCACACA TCAAACCMGA ATGATACTTC CTATT

Biometra thermocycler. PCR products were run on a 1% agarose gel, excised and extracted using the Qiaex gel purification kit (Diagen). After elution, the amplified DNA was precipitated with isopropanol, sodium acetate and glycogen as a carrier. The pellet was redissolved in 6.5 µl H_2O. Direct sequencing of the double-stranded DNA was carried out with the chain termination method (Sambrook *et al.* 1989) at room temperature using ^{35}S-dATP as a radioactive marker and Sequenase 2.0 (USB) according to the distributor's specifications. Primer B, G, C, D and H were used as sequencing primers (Table 2) in such a way that overlapping sequences were obtained. Products of the sequencing reactions were separated on a 6 % polyacrylamide/7 M urea gel by electrophoresis at 65 Watt. After drying, the gel was exposed to X-ray film for 3-4 days. About 300-400 nucleotides were readable per sequencing run.

Sequence analysis

Nucleotide sequences were aligned with the cytochrome b sequence of *Gallus gallus* (Desjardins & Morais 1990). Phylogenetic trees were reconstructed using the maximum parsimony method with the phylogeny program PAUP 3.1.1 (Swofford 1993) and the neighbour-joining method (Saitou & Nei 1987) using the program package MEGA (Kumar *et al.* 1993). In the neighbour-joining analyses genetic distances were calculated based on the Kimura 2-parameter model (Kimura 1980),

Table 3. Pairwise genetic distances between *Aquila* eagles and representatives of four other Accipitriform genera used as outgroups (compare Fig. 1). Data are based on 1026 nucleotides of the cytochrome b gene (uncorrected for multiple substitutions). Below diagonal: numbers of observed nucleotide differences (transitions/transversions); above diagonal: proportion of nucleotide positions differing between taxa (p-distance).

	Species No.	1	2	3	4	5	6	7	8	9	10
1	*Buteo buteo*	-	0.102	0.125	0.127	0.125	0.121	0.122	0.123	0.111	0.115
2	*Accipiter gentililis*	74/30	-	0.135	0.134	0.131	0.127	0.137	0.138	0.132	0.133
3	*Aquila pomarina*	92/36	95/43	-	0.018	0.072	0.072	0.072	0.076	0.147	0.121
4	*Aquila clanga*	96/34	96/41	16/2	-	0.075	0.075	0.070	0.074	0.146	0.120
5	*Aquila chrysaetos* 1	93/35	96/38	65/9	70/7	-	0.008	0.076	0.076	0.135	0.114
6	*Aquila chrysaetos* 2	91/33	92/38	65/9	70/7	8/0	-	0.072	0.074	0.133	0.114
7	*Aquila heliaca*	90/35	98/42	67/7	67/5	70/8	66/8	-	0.018	0.133	0.126
8	*Aquila adalberti*	91/35	99/42	70/8	70/6	69/9	67/9	17/1	-	0.137	0.127
9	*Haliaeetus albicilla*	94/20	101/34	121/30	120/30	105/33	103/33	109/27	113/27	-	0.121
10	*Pernis apivorus*	76/42	87/49	80/44	81/42	74/43	74/43	86/43	87/43	80/44	-

which takes into account the strong transition-transversion bias found in our data. With PAUP, both heuristic and exact algorithms ("Branch & Bound") were employed and always yielded identical results. Bootstrap analysis (heuristic search with PAUP) was performed to obtain confidence estimates for each clade in both the maximum parsimony and the neighbour-joining searches.

RESULTS

We first sequenced 1026 bp of two individuals each of *A. heliaca, A. adalberti* and *A. chrysaetos* and one individual of all other species (Appendix). In order to obtain some measure of intrataxon variation, a smaller portion of 300 bp was sequenced (with Primer B) in all remaining *Aquila* individuals yielding partial cytochrome b sequences for 3-6 birds per taxon . The number of cyt b haplotypes found thus represents a minimum estimate. Intrataxon sequence variation was restricted to single nucleotide substitutions in *A. heliaca, A. adalberti* and *A. pomarina* (2 haplotypes each) or was absent as in *A. clanga*, all six individuals of which carried the same haplotype. In *A. chrysaetos*, two haplotypes were found, one in four birds from the Swiss Alps (type 1 in Fig. 1 & Appendix) and the other in a falconer's bird of unknown origin (type 2) that differed by 8 substitutions among 1026 nucleotides (0.8% sequence divergence).

Table 3 shows absolute numbers of nucleotide differences and percent values of sequence divergence (p-distance, uncorrected for multiple substitutions). *A. heliaca* and *A. adalberti* differed by 17-18 nucleotide substitutions (1.7 - 1.8 %; Table. 3). The other most closely related species pair were *A. pomarina* and *A. clanga*, differing by 18 substitutions (1.8 %). The Golden Eagle, *A. chrysaetos*, which is morphologically very similar to the *A. heliaca* superspecies and generally regarded to be its sister species, differs from both *heliaca* and *adalberti* by a much larger number of 74-78 substitutions (7.2 - 7.6 %). Sequence divergence between these and other eagles such as *A. nipalensis* and *verreauxii* was 2.9 - 7.8 % as has been reported in more detail elsewhere (Seibold 1994). Therefore genetic distances between established *Aquila* species span a range of 1.8 - 7.8 %, within which the pairs *heliaca - adalberti* and *pomarina - clanga* occupy equal levels of differentiation at the lower end of the scale. The ratio of transitions (A/G and C/T changes) to transversions (A/G versus C or T), which in mitochondrial genes is well known to be inversely related to p-distance (von Haeseler *et al.* 1993), ranged from 17:1 between the most closely related taxa down to 2:1 for comparisons involving *Pernis apivorus* (Table 3).

Among the five *Aquila* species, there were 132 variable nucleotide positions (out of 1026 = 12.9 %), of which 65 were phylogenetically informative between species. As expected from earlier studies of cytochrome b sequence evolution, substitutions occurred only in first (18) and third (114) codon positions. For eleven out of 128 variable codons we inferred amino acid substitutions (Appendix), which included three between *heliaca* and *adalberti* and two between *clanga* and *pomarina*.

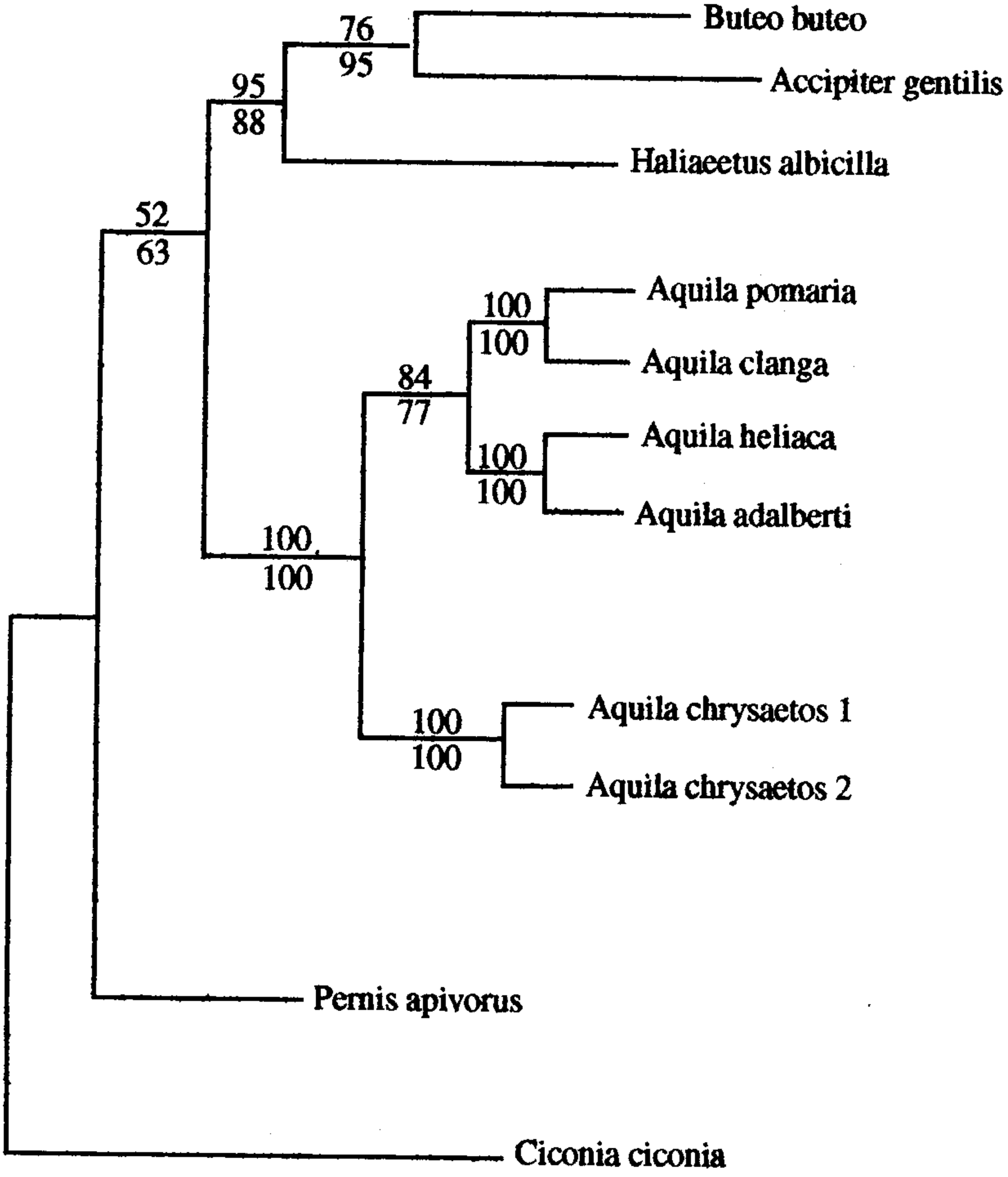
A hypothetical phylogenetic tree was reconstructed with one sequence per taxon, i. e. neglecting the small amount of intrataxon variation described above, except in *A. chrysaetos*, of which the two haplotypes were distinct enough to both warrant inclusion. One most parsimonious tree (556 steps long) was found, which grouped all *Aquila* species in a monophyletic clade (Fig. 1). Among the raptor species studied, *Pernis apivorus* appeared as the most basal taxon and the genera *Buteo*, *Accipiter* and *Haliaeetus* together formed the sister group to *Aquila*, suggesting that *Aquila* represents a fairly old genus within the Accipitriform raptors. There was an indication of *heliaca/ adalberti* and *pomarina/clanga* being sister groups (84 % bootstrap frequency). On morphological grounds one would expect *heliaca*, *adalberti* and *chrysaetos* to form a monophyletic clade.

Unweighted parsimony and neighbour-joining reconstructions yielded the same tree topology and similar bootstrap frequencies (Fig. 1). The topology was not influenced by the distance measure chosen in the neighbour-joining analysis (Jukes-Cantor, Kimura 2-parameter, Tamura-Nei distance, synonymous substitution rate calculated according to Li 1993). When in the parsimony searches transversions were weighted 20:1 over transitions a) in third codon positions only or b) in all positions, *Haliaeetus* and *Accipiter* changed places in the reconstructed tree and the bootstrap value excluding *Pernis* from the other raptors jumped from 52 % to 96 and 97 %, respectively. The improved resolution of the phylogenetically oldest branch is due to the fact that transversions, due to their relative rarity in mitochondrial protein coding sequences (Edwards *et al* 1991), do not become saturated by multiple substitutions even over long time spans and therefore retain phylogenetic information much longer than transitions.

DISCUSSION

The amount of mitochondrial genetic differentiation between *A. heliaca* and *A. adalberti* was found to be the same as in at least one other pair of undisputed species in the same genus, namely *A. pomarina* and *A. clanga*. A separation between the mitochondrial lineages of each of these pairs could have occurred slightly less than 1 million years ago, if we assume a substitution rate of 2% per million years for mitochondrial genes (Shields & Helm-Bychowski 1988). Since this rate applies to the whole mitochondrial genome but cyt b might evolve more conservatively, the age of *ca.* 1 Mio years should be regarded as a minimum estimate. Although our sample sizes are too small to allow any meaningful probability of historical mitochondrial gene flow to be calculated, there is no evidence that gene flow has occurred between *adalberti* and *heliaca* since their ranges first became separated in the Pleistocene. Today the range disjunction spans 1700-1800 km and *A. heliaca* is so rarely observed in western Europe that any gene flow would be extremely unlikely on geographical grounds alone. Note, however, that the two forms may have been parapatric as recently as 150 years ago without evidence of hybridization having ever

Figure 1. Hypothetical phylogenetic tree of *Aquila* eagles plus outgroups. Tree topology was identical using parsimony (equal character weights) and neighbour-joining methods. Tree length: 556 steps; consistency index: 0.730 (0.625 excluding uninformative characters). Percent bootstrap frequencies (500 replicates) are indicated for parsimony (heuristic search, random sequence addition) above and for neighbour-joining search (Kimura 2-parameter distance) below each branch. The full sequences of *Aquila* taxa are given in the Appendix.



been found (González *et al.* 1989; González 1991). Taking into account the distinct differences in adult and especially immature plumage, morphology, migratory status and many details of behaviour and ecology (see Table 1; Hiraldo *et al.* 1976; González 1991), all available evidence supports the idea that these taxa should be considered allospecies of a superspecies (*sensu* Mayr 1963; Amadon 1966).

Among other diurnal birds of prey studied so far, minimum genetic distances between closely related species are also as low as 1.8%, e.g. *Falc concolor* vs. *F. subbuteo* (1.7%, Seibold *et al.* 1993) and *Milvus milvus* vs. *M. migrans* (1.7%, Seibold *et al.* unpublished). On the other hand, within-species sequence divergence values of 1.5% in *Parus caeruleus* (Taberlet *et al.* 1992), 2.5% in *Branta canadensis* (Van Wagner & Baker 1990) and even 10% in *Falco cherrug* (Seibold *et al.* 1993) have also been reported. The latter is certainly exceptional and most likely due to past interspecific hybridization. Clearly, such distance values do not *per se* indicate limits between species. They are nonetheless a useful measure of differentiation, especially in cases of disjunct distributions (as with Imperial Eagles), where it is impossible to apply the most decisive criterion for biological species status, i.e. the lack of fertile, natural interbreeding.

The data presented here are important in assessing conservation priorties for *A. adalberti*. It is a taxon genetically as distinct from its closest relatives as some other eagle species are from each other. The highest priority should therefore be given to preventing its extinction on the Iberian Peninsula, where it represents one of only three endemic bird species. The other two are *Sturnus unicolor* and *Phylloscopus brehmii,* both of which have much larger populations. Introduction of eastern Imperial Eagles to Iberia, which might be proposed as a last resort to prevent its extinction there, should be rejected, because it would either swamp the genetic distinctiveness of *A. adalberti* or no fertile interbreeding would occur. A recent study of allozyme variation in the remaining subpopulations of Spanish Imperial Eagles failed to find any genetic variation at 22 loci that were resolved (Negro & Hiraldo 1994). Interestingly, mitochondrial genetic diversity seems to be greater given that we identified two haplotypes among only three individuals sampled at random. Few comparable data exist on allozyme versus mitochondrial genetic diversity in birds and possible discrepancies between them certainly warrant further study.

It was surprising that on the mitochondrial DNA level *A. chrysaetos* is about as divergent from the *A. heliaca - adalberti* superspecies as it is from *A. clanga - pomarina*, confirming Kleinschmidt's (1934) view that Golden and Imperial Eagles are not closely related. This contradicts the fact that the adult plumage of *chrysaetos* is much more similar to *heliaca* (making field identification of adults sometimes difficult) than it is to *clanga* or *pomarina.* Interestingly, immature (especially juvenile) plumages are more highly distinct than adult plumages in all *Aquila* species, but do not provide a clue as to whether *chrysaetos* is closer to *heliaca-adalberti* or to *pomarina-clanga.*

One can only speculate about reasons for this discrepancy: The adult plumage

pattern retained by *chrysaetos* and *heliaca* may represent a plesiomorphic condition, from which *clanga* and *pomarina* have diverged. This could in some way be related to their largely forest-dwelling habits during the breeding season, whereas *chrysaetos*, *heliaca* and *adalberti* live in open habitats year-round. A second possibility is that nuclear gene flow between *chrysaetos* and *heliaca* was interrupted much later than was mitochondrial gene flow, leading to closer similarity in nuclear encoded characters such as adult plumage pattern than in mitochondrial characters. Similar cases have been reported among flycatchers (Tegelström & Gelter 1990) and leaf warblers (Helbig *et al.* 1993) and may be rather widespread in birds. Hybridization between Golden Eagle and either *heliaca* or *adalberti* is - to our knowledge - unrecorded in the wild, but has been achieved with *adalberti* in captivity (Cugnasse *et al.* 1993). In the future it will be desirable to sequence nuclear genes of *Aquila* eagles and compare levels of nuclear versus mitochondrial differentiation.

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APPENDIX

Appendix. Cytochrome b nucleotide sequences of the five *Aquila* species studied including two haplotypes of Golden Eagle (1026 bp). The data correspond to positions 14995 - 16020 of the chicken mitochondrial genome (Desjardins & Morais 1990). Underlined letters (R = A/G; Y = C/T) show positions that varied intraspecifically. Codons involving amino acid changes are framed.

A.heliaca	GGC	TCC	CTG	CTA	GGA	ATC	TGC	CTA	CTA	ACA	CAA	ATT	CTA	ACC	GGC	CTC	CTA	CTA	GCC	ATA	CAT	TAC	ACT	GCA
A.adalberti	...	...	...	...	...	...	...	...	...	...	...	..Y	...	...	...	...	...	...	...	...	...	...	...	...
A.clanga	..T	...	T.A	...	...	...	...	...	...	...	...	..C	...	...	...	...	...	...	...	..G	..C	...	..C	...
A.pomarina	..T	...	T.A	...	...	...	...	...	...	...	...	..C	...	...	...	...	...	...	...	..G	..C	...	..C	...
*A.chrysaet.*1	..	...	..C	...	...	...	...	...	A..	...	...	..C	...	...	...	...	...	T..	...	...	..C	...	..C	...
*A.chrysaet.*2	...	...	..C	...	...	...	...	...	A..	...	...	..C	...	...	...	...	...	T..	...	...	..C	...	..C	...

A.heliaca	GAC	ACC	ACT	CTA	GCC	TTC	TCG	TCC	GTC	GCC	CAC	CCA	TGC	CGG	AAC	GTA	CAG	TAT	GGC	TGA	CTA	ATC	CGC	AAC
A.adalberti	...	..T	...	...	...	...	...	...	...	...	...	...	...	..A	...	...	...	...	...	...	...	...	...	...
A.clanga	...	...	...	...	...	...	...	..T	...	...	...	...	...	..A	...	...	..A	...	...	...	...	...	..T	...
A.pomarina	...	...	...	...	...	...	...	..Y	...	...	...	...	...	..A	...	...	..A	...	...	...	...	...	..T	...
*A.chrysaet.*1	...	...	..C	...	...	...	..A	...	...	...	...	...	.T	...	...	...	...	..C	...	...	...	...	...	...
*A.chrysaet.*2	...	...	..C	...	...	...	..A	...	...	...	...	...	..T	...	...	...	...	..C	...	...	...	...	...	...

A.heliaca	CTA	CAT	GCC	AAC	GGA	GCA	TCC	TTC	TTC	TTC	ATC	TGT	ATC	TAC	CTA	CAC	ATC	GGC	CGA	GGA	CTT	TAC	TAC	GGC
A.adalberti	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	A..	...	...	..T
A.clanga	...	...	..T	...	...	...	...	...	...	...	...	..C	...	...	...	...	...	...	...	...	...	...	...	...
A.pomarina	...	...	..T	...	...	...	...	...	...	...	...	..C	...	...	...	...	...	...	...	...	...	...	...	...
*A.chrysaet.*1	...	...	...	...	...	...	...	...	...	...	...	..C	.T	...	.T	...	...	...	...	..G	..C	...	...	...
*A.chrysaet.*2	...	...	...	...	...	...	...	...	...	...	...	..C	.T	...	.T	...	...	...	...	...	..C	...	...	...

A.heliaca	TCA	TAC	CTA	TAC	AAA	GAA	ACC	TGA	AAC	ACA	GGA	ATC	ATT	CTC	CTA	CTT	ACA	CTC	ATA	GCA	ACC	GCC	TTC	GTA
A.adalberti	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
A.clanga	...	...	T..	...	...	..G	...	...	...	...	...	...	...	...	...	..C	..C	...	...	...	..T	...	...	...
A.pomarina	...	...	T..	...	...	..G	...	...	...	...	...	...	..C	...	...	..C	..C	..T	...	...	..T	...	...	...
*A.chrysaet.*1	...	...	..T	...	...	..G	...	...	...	...	...	...	...	...	...	...	..C	..T	...	...	...	...	..T	...
*A.chrysaet.*2	...	...	..T	..T	...	..G	...	...	...	...	...	...	...	...	...	...	..C	..T	...	...	...	...	..T	...

A.heliaca	GGC	TAT	GTC	CTT	CCA	TGA	GGA	CAA	ATA	TCC	TTC	TGA	GGG	GCC	ACA	GTC	ATC	ACC	AAC	CTA	TTC	TCA	GCA	GTC
A.adalberti	..T	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
A.clanga	..T	...	...	..C	...	...	..G	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	A..
A.pomarina	..T	...	...	..C	...	...	...	...	...	...	...	...	...	..T	...	...	...	...	...	...	...	...	...	A..
*A.chrysaet.*1	..T	...	...	..C	..C	...	...	..G	...	...	...	...	..A	...	...	...	...	...	...	...	...	...	...	A..
*A.chrysaet.*2	...	...	...	..C	...	...	...	..G	...	...	...	...	..A	...	...	...	...	...	...	...	...	...	...	A..

A.heliaca	CCA	TAC	ATC	GGA	CAG	ACC	CTT	GTA	GAA	TGA	GCC	TGA	GGC	GGA	TTC	TCC	GTA	GAC	AAC	CCT	ACC	CTT	ACT	CGC
A.adalberti	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
A.clanga	..G	...	...	..T	..A	...	..C	...	...	..G	..T	...	...	...	...	...	...	..T	...	..C	...	..C	...	..T
A.pomarina	..G	...	...	..T	..A	...	..C	...	...	..G	..T	...	...	...	...	...	...	..T	...	..C	...	..C	...	..T
*A.chrysaet.*1	...	...	...	...	..A	...	..C	...	..G	..G	...	...	..T	...	...	..T	...	..T	...	..C	...	..C	...	...
*A.chrysaet.*2	...	...	...	...	..A	...	..C	...	..G	..G	...	...	..T	...	...	..T	...	..T	...	..C	...	..C	...	...

A.heliaca	TTC	TTC	GCC	CTA	CAC	TTC	CTA	CTT	CCA	TTC	CTC	ATC	GCA	AGT	CTT	ACC	CTA	ATC	CAC	CTT	ACC	TTC	TTA	CAC
A.adalberti	...	..T	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	C..	..T
A.clanga	...	...	..T	...	..T	..T	T..	..C	...	...	...	...	...	..C	...	...	...	..T	...	..C	...	...	C..	...
A.pomarina	...	...	...	...	..T	..T	T..	..C	...	...	...	...	...	..C	...	...	...	..T	...	..C	...	...	C..	...
*A.chrysaet.*1	...	...	...	...	..T	..T	T..	...	...	...	..T	...	...	..C	...	...	...	...	...	...	...	...	C..	...
*A.chrysaet.*2	...	...	...	...	..T	..T	T..	...	...	...	..T	...	...	..C	...	...	...	...	...	...	...	...	C..	...

A.heliaca	GAG	TCT	GGA	TCA	AAC	AAT	CCT	CTA	GGA	ATT	ATC	TCA	AAC	TGT	GAC	AAA	ATC	CCA	TTC	CAT	CCA	TAC	TTC	TCC
A.adalberti	...	...	...	...	...	...	...	...	...	..C	...	...	...	...	...	...	...	...	...	...	...	...	...	...
A.clanga	...	..C	...	...	...	..C	..C	...	...	...	...	...	G..	..C	...	...	...	...	...	..C	...	...	...	...
A.pomarina	...	..C	...	...	...	..?	..?	...	...	...	...	...	...	...	...	...	...	...	...	..C	...	...	...	...
*A.chrysaet.*1	...	..C	...	...	...	...	..C	...	...	...	...	...	...	...	...	...	...	...	...	..C	...	...	...	...
*A.chrysaet.*2	...	..C	...	...	...	...	..C	...	...	...	...	...	...	...	...	...	...	...	...	..C	..C	...	...	...

A.heliaca	TTA	AAA	GAC	ATT	CTA	GGA	TTT	GTA	CTA	ATA	CTA	CTC	CCA	CTA	ACA	ACT	CTA	GCC	CTA	TTC	TCA	CCT	AAT	CTG
A.adalberti	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..C	...
A.clanga	C..	...	...	...	...	...	..C	...	...	...	...	...	...	...	...	G..	...	...	...	...	...	...	..	..A
A.pomarina	C..	...	...	...	...	...	..C	...	...	...	...	...	..G	...	...	G.C	...	...	...	...	...	...	...	..A
*A.chrysaet.*1	C..	...	...	..C	...	...	..C	...	...	..G	...	..T	...	...	...	...	...	..T	...	...	...	...	..C	...
*A.chrysaet.*2	C..	...	...	..C	...	...	..C	...	...	...	...	...	...	...	...	...	...	..T	...	...	...	...	..C	...

A.heliaca	CTA	GGC	GAC	CCA	GAA	AAC	TTC	ACC	CCA	GCA	AAC	CCT	CTA	GTT	ACA	CCC	CCT	CAT	ATT	AAA	CCA	GAA	TGA	TAC
A.adalberti	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
A.clanga	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..G	...	...
A.pomarina	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..A	..C	...	...	...	...	...	...	...
*A.chrysaet.*1	...	...	...	...	...	...	...	...	...	...	...	..C	...	...	...	...	...	...	..C	...	...	...	...	...
*A.chrysaet.*2	...	...	...	...	...	...	...	...	...	...	...	..C	...	...	...	...	...	...	..C	...	...	...	...	...

A.heliaca	TTC	CTA	TTT	GCA	TAC	GCC	ATT	CTA	CGC	TCA	ATC	CCA	AAC	AAA	CTG	GGA	GGT	GTA	CTA	GCC	CTA	GCC	GCC	TCA
A.adalberti	...	...	...	...	...	...	..	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
A.clanga	...	...	...	...	...	...	..C	..C	...	...	...	...	...	...	T..	...	..A	...	...	...	...	...	...	...
A.pomarina	...	...	...	...	...	...	..C	..C	...	...	...	...	...	...	T..	...	..A	..G	...	..T	...	...	...	...
*A.chrysaet.*1	...	...	...	...	...	...	..C	...	..T	...	...	...	...	..G	...	...	..A	..G	...	..T	...	..T	...	...
*A.chrysaet.*2	...	...	...	...	...	...	..C	...	..T	...	...	...	...	..G	...	...	..A	..G	...	..T	...	..T	...	...

A.heliaca	GTA	CTR	ATC	CTA	TTC	CTC	ATC	CCT	CTC	CTC	CAT	AAA	TCT	AAA	CAA	CGT	ACA	ATA	ACC	TTC	CGA	CCT	TTC	TCC
A.adalberti	...	..A	...	...	.....	...	...	...	...	...	...	...	...	...	...	...	...	...	..	...	...	..C	C..	...
A.clanga	...	..G	...	...	.....	...	...	...	...	...	..C	...	..C	..G	...	..C	...	...	..	...	..G	..C	C..	..T
A.pomarina	...	..G	...	...	...	...	...	...	...	...	..C	...	..C	...	...	..C	T..	...	..	...	..G	..C	C..	..T
*A.chrysaet.*1	...	..G	...	...	...	..T	...	..C	T..	...	...	..G	..C	...	...	..C	...	...	..	..T	...	..C	C..	...
*A.chrysaet.*2	...	..G	...	...	...	..T	...	..C	T..	...	...	..G	..C	...	...	..C	...	...	..	..T	...	..C	C..	...

A.heliaca	CAA	CTC	CTA	TTC	TGA	ACC	CTA	GTC	GCC	AAC	CTC	CTC	ATC	CTC	ACA	TGA	ATC	GGC	AGC	CAA	CCA	GTA	GAA	CAT
A.adalberti	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..G	...	...	...	...	...	...	...	...
A.clanga	...	...	...	...	...	..T	...	A..	...	...	...	...	...	..T	...	...	...	...	...	...	...	...	...	..C
A.pomarina	...	...	...	...	...	..T	...	A..	...	...	...	...	...	..T	...	...	...	...	...	...	...	...	...	..C
*A.chrysaet.*1	...	...	...	...	..G	...	...	A..	...	...	...	...	...	...	...	..G	..T	...	...	...	...	...	...	..C
*A.chrysaet.*2	...	...	...	...	..G	...	...	A..	...	...	...	...	...	...	...	..G	..T	...	...	...	...	...	...	..C

A.heliaca	CCG	TTC	ATC	ATC	ATT	GGC	CAA	ATC	GCC	TCC	CTC	ACC	TAC	TTC	TCC	ACC	CTC	CTA	ATC	CTC	TTC	CCT	CTA	ATT
A.adalberti	..	...	..T	...	..C	...	...	...	...	...	...	...	...	...	...	...	...	...	...	T..	...	...	...	...
A.clanga	..A	...	..	...	..C	...	...	C..	...	...	...	...	...	...	...	...	...	...	...	...	...	..C	...	...
A.pomarina	..A	...	...	...	..C	...	...	C..	...	...	...	...	...	...	...	...	...	...	..T	...	...	..C	...	...
*A.chrysaet.*1	..A	...	..T	...	..C	...	...	C..	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
*A.chrysaet.*2	...	...	..T	...	..C	...	...	C..	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...

A.heliaca	GGA	GCC	CTC	GAA	AAT	AAA	ATG	CTC
A.adalberti	...	...	...	...	...	...	...	...
A.clanga	...	...	...	...	...	...	...	...
A.pomarina	...	...	...	...	...	...	...	...
*A.chrysaet.*1	...	...	...	...	...	...	...	...
*A.chrysaet.*2	...	...	...	...	...	...	...	...