Mitochondrial DNA analysis reveals Holarctic homogeneity and a distinct Mediterranean lineage in the Golden eagle (*Aquila chrysaetos*)

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Received 27 January 2015; revised 9 April 2015; accepted for publication 9 April 2015

The Golden eagle (*Aquila chrysaetos*) is among the most widespread of the birds of prey, covering basically the whole Palaearctic from Europe and North Africa through Asia and Japan, to the North American continent. Only few studies have addressed the species’ genetic structure and the consequences of its demographic history so far, and none of them has covered larger areas of the distribution range. Our present study aims at closing this gap. Based on 283 samples (mostly feathers collected in the field or from museum collections) across the species’ distribution, but with a focus on Europe, we uncover the phylogeography of the Golden eagle. Results imply a phylogeographic split between mainly Northern Europe, Continental Asia, Japan and North America on the one hand and Central–Southern Europe on the other. The observed pattern is likely to be caused by the Last Ice Age, when the population survived in two reproductively isolated glacial refugia. Repopulation of Northern Europe occurred from a presumed Asian refugium, whereas the Alpine range was probably repopulated from a refugium in the Mediterranean region. In Eastern Europe, the Mediterranean and Alpine region we find a co-occurrence of both lineages that heavily influences the local genetic diversity. This pattern is unlike that in most other large raptors in which usually a western and an eastern Eurasian lineage have been recovered.


INTRODUCTION

The contemporary genetic structure of a species contains information on historical events such as population growth, range expansion, dispersal and bottlenecks. In the Northern Hemisphere these processes are heavily influenced by natural climatic oscillations (Hewitt, 1999, 2000), but the more recent anthropogenic factors may also be detected through their impact on the genetic diversity and structure of populations. In Europe, most of these human disturbances occurred from the 18th through the 20th century and consisted in habitat destruction and direct persecution. The latter was particularly aimed at large carnivore and raptor species – Wolves (*Canis lupus*), Brown bears (*Ursus arctos*), Lynx (*Lynx lynx*), Bearded vultures (*Gypaetus barbatus*), Golden eagles (*Aquila chrysaetos*) and White-tailed eagles (*Haliaeetus albicilla*) – because they were regarded as a threat to stock and game animals, or even to humans. Population breakdowns can result in severe bottlenecks, which not only expose animal populations to increased extinction risk through stochastic processes (Caughley, 1994), but also decrease genetic diversity which in turn increases the risk of inbreeding depression and the loss of evolutionary potential.

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Legal protection and conservation programmes have recently helped some of these species to recover and – in case of local extinctions – recolonize parts of their former distribution range, either naturally or through reintroduction programmes.

The Golden eagle (Aquila chrysaetos) belongs to the most abundant and widespread raptor species in the world and is found in a variety of habitats in the Northern Hemisphere in both the Palaearctic and the Nearctic. It depends on open or semi-open landscapes as hunting grounds. Golden eagles are found as far north as the Southern Arctic tundra and south to the highlands of Ethiopia (Clouet & Barrau, 1993; Watson, 2010). Territorial adult birds are sedentary, and dispersal behaviour is usually found in juvenile or older non-breeding individuals. Only in the very northernmost region, where food is not accessible all year round, the Golden eagle shows annual migration (Brodeur et al., 1996). Due to its vast distribution and an overall high population size (estimated at 125 000–300 000 individuals; Rich et al., 2004; Watson, 2010), the species is not listed as threatened (IUCN, 2013: Least Concern, http://www.iucnredlist.org/details/22696060/0). However, its numbers and range in Europe were severely reduced during the period of persecution. In Central Europe, breeding birds have always been present in the Alpine region, but the Golden eagle was extirpated in the more accessible Alpine foothills and lowlands of Germany, Austria, the Czech Republic and Poland by the end of the 19th century or beginning of the 20th century. For example, historical breeding sites were recorded in East and West Prussia, Moravia, the Bohemian and Thuringian Forest (all in Central or Central-Eastern Europe), but they are extinct there today (Dresser, 1871; Naumann, 1899; Glutz, Bauer & Bezzel, 1971). The entire Alpine population today harbours between 800 and 1200 breeding pairs, and the population is reported to be at or near carrying capacity (BirdLife International, 2004; Fasce et al., 2011; Mebs & Schmidt, 2014) but was much lower at the beginning of the 20th century (Corti, 1959; Gamauf, 1991).

Holarctic or Palaearctic species for which phylogeographic studies have been conducted show varying genetic patterns. In Eurasia there were either several genetic lineages, mostly geographically separated with an area of overlap (e.g. Saker falcon Falco cherrug, Nittinger et al., 2007; White-tailed eagle Haliaeetus albicilla, Hailer et al., 2007; Ponnikas et al., 2013; Cinereous vulture Aegypius monachus, Poulakakis et al., 2008; Bearded vultures Gypaetus barbatus, Godoy et al., 2004; Red fox Vulpes vulpes, Statham et al., 2014; Brown bear Ursus arctos, Davison et al., 2011) or a single haplogroup and a lack of genetic structuring (e.g. Raven Corvus corax, Omland et al., 2000; Haring, Gamauf & Kryukov, 2007). For Holarctic taxa, there are usually two alternative patterns: (1) a distinct American lineage that occurs in sympatry with a Eurasian haplogroup in either North America (e.g. Raven, Red fox and Brown bear, references see above) or Asia (Moose Alces alces, Hundertmark et al., 2002), suggesting recent genetic gene flow across Beringia or the Bering Strait; and (2) lack of gene flow across the Bering Strait and subsequent speciation into a Palaearctic species (e.g. White-tailed eagle, Eurasian three-toed woodpecker Picoides tridactylus, Grey heron Ardea cinerea and Eurasian beaver Castor fiber) and a Nearctic sister taxon (Bald eagle Haliaeetus leucocephala, American three-toed woodpecker Picoides dorsalis, Great blue heron Ardea herodias and North American beaver Castor canadensis). The Golden eagles of North America (A. chrysaetos canadensis) and Siberia (A. chrysaetos kamtschatica) are, apart from body size, phenotypically very similar, suggesting no deep split between the continents in this species.

Previous population genetic results on Golden eagles are only available on a local or regional scale for Japan (Masuda et al., 1998), the Swiss Alps (Suchentrunk, Haller & Ratti, 1999), Scotland (Bourke et al., 2010), Slovakia (Bielikova et al., 2010) and California (Sonsthagen et al., 2012). These studies analysed genetic diversity of populations that either suffered from a bottleneck due to human persecution (Scotland and Switzerland) or have recently been declining (Japan), investigated the founder effect of recently populated islands (Channel Islands, California) or provided the basis for paternity testing in captive Golden eagles (Slovakia). In Scotland and Switzerland there was no indication of a recent population breakdown that could be linked to human persecution (Suchentrunk et al., 1999; Bourke et al., 2010). These results suggest that genetic diversity in Golden eagles was largely retained, possibly due to the species’ long generation time (similar to the case of the White-tailed eagle, Hailer et al., 2006) or because the population breakdown was not as severe or widespread as believed.

Although the Golden eagle is an iconic bird, comprehensive phylogeographic information is still lacking as previous genetic studies used different genetic markers and were restricted geographically. We close this gap and aim to uncover the Holarctic genetic structuring of the species. The geographic focus is on Europe, especially the Alpine and Mediterranean regions, but we also include samples from Fennoscandia, the British Isles, continental Asia, Japan and North America, making this study the first to comprise Golden eagles from throughout the distribution range. Taking advantage of this spatially
more inclusive sampling, we address the following research questions:
1 Are there distinct phylogeographic lineages as a result of different glacial refugia as found in other raptors, or is there no genetic structuring, indicating a more continuous distribution south of the uninhabitable area during the Last Ice Age?
2 What is the level and distribution of genetic diversity in this large predator, and what does it tell us about its demographic history with respect to signatures of bottlenecks and/or expansion events?

MATERIAL AND METHODS

SAMPLING AND DNA EXTRACTION

In total samples of 534 Golden eagles (feathers \( n = 362 \), and footpad or muscle \( n = 172 \)) were obtained from museum collections and private collectors (Table 1, for a total list see Supporting Information, Table S1), covering the period from 1817 to 2013. Some of the Japanese specimens came from a breeding programme; their pedigree was carefully checked and specimens excluded if they came from the same maternal lineage. For almost all of our samples, information on collection site and year was available.

As a high percentage of our samples (47%) were collected before 1950, we took particular care in avoiding contamination. This included both handling in the laboratory and data interpretation. Extractions and polymerase chain reactions (PCRs) were done in a clean room, and all laboratory equipment was DNA-free (DNA-free laboratory ware; other tools: flame treatment or UV-irradiated).

As described in the literature (Horváth et al., 2005), we used either the umbilicus superior or interior or both for DNA extraction. Extraction was conducted using the Gen-ial First-DNA all tissue kit (Gen-ial GmbH, Trossdorf, Germany). DNA was extracted following the manufacturers’ standard protocols. To optimize extraction, 5 µL DTT (dithiothreitol) were added to each sample that was extracted with the Gen-ial kit. Lysis time was prolonged to 24 h for both feathers and tissue.

Table 1. The distribution of samples in our data set, number of samples \((n)\) from which PCR products (402 bp or 350 bp) could be obtained, origin, subspecies and assigned genetic lineage

<table>
<thead>
<tr>
<th>Region</th>
<th>Country</th>
<th>Time range</th>
<th>PCR product ((n))</th>
<th>Lineages ((M/H))</th>
<th>Taxonomic assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mediterranean</td>
<td>Morocco, Spain, Sardinia, Apennine (Italy), Bulgaria, Greece, Turkey</td>
<td>1898–2011</td>
<td>27</td>
<td>10</td>
<td>M (35) + H (2)</td>
</tr>
<tr>
<td>Scotland (UK)</td>
<td>Scotland (UK)</td>
<td>1904–1997</td>
<td>13</td>
<td>0</td>
<td>H</td>
</tr>
<tr>
<td>Fennoscandia</td>
<td>Norway, Finland</td>
<td>1884–2012</td>
<td>38</td>
<td>4</td>
<td>H</td>
</tr>
<tr>
<td>Alps</td>
<td>Austria, Germany, Switzerland, South Tyrol (Italy), France</td>
<td>1850–2013</td>
<td>137</td>
<td>0</td>
<td>M (115) + H (22)</td>
</tr>
<tr>
<td>East Europe</td>
<td>Ukraine, European Russia, Romania, Georgia</td>
<td>1857–1935</td>
<td>8</td>
<td>9</td>
<td>M (6) + H (11)</td>
</tr>
<tr>
<td>Continental Asia</td>
<td>Central Asia, Asian Russia, Kashmir (India), China</td>
<td>1919–2012</td>
<td>8</td>
<td>6</td>
<td>H</td>
</tr>
<tr>
<td>Japan</td>
<td>Japan</td>
<td>1977–2013</td>
<td>12</td>
<td>0</td>
<td>H</td>
</tr>
<tr>
<td>North America</td>
<td>Canada, USA</td>
<td>1919–2012</td>
<td>5</td>
<td>2</td>
<td>H</td>
</tr>
</tbody>
</table>

Subspecies were inferred by collection sites. M, Mediterranean lineage; H, Holarctic lineage. Not included are three samples of unknown origin (Mediterranean lineage) and a single sample from Normandy (A. c. chrysaetos, Holarctic lineage). Taxonomic assignment based on Watson (2010). If both lineages occur sympatrically, numbers of individuals for each lineage are given in parentheses.
taxa. Primers for this marker amplifying a 402-bp fragment were established by Sonsthagen et al. (2012). The short length is advantageous for the amplification of old or degraded DNA (ancient DNA, aDNA) (Gilbert et al., 2005) as the PCR success for short fragments is higher in such samples. Furthermore, the utilization of already established primers allowed us to include the Californian haplotypes obtained by Sonsthagen et al. (2012) (accession numbers: JQ246417–JQ246421). The forward primer was modified by using the software Amplify 3 and the online tool Primer-BLAST. The final primers for PCR and sequencing were modGOEA_CR1L 5′-CCC CCGTATGTATTATTGTA-3′ and GOEA_CR595H 5′-GCAAGGTCGTAGGACTAACC-3′ (reverse primer introduced by Sonsthagen et al., 2012). A second reverse primer was designed (GOEA_CR350 5′-GG TTAGCTCATCGACCTTG-3′) to amplify smaller mtDNA fragments in case of PCR failure that, in combination with the forward primer modGOEA_CR1L, amplified a 350-bp fragment of the mtDNA control region. These shorter sequences were not included in most subsequent analyses and were only used for lineage identification (see below). Taq polymerase was used for PCR amplification (40 cycles of 30 s at 95 °C, 30 s at annealing temperature and 40 s at 72 °C; with an initial 15 min at 95 °C and a final extension step at 72 °C for 5 min). Annealing temperatures for the 402- and 350-bp fragment PCRs were 48 and 51 °C, respectively. No gaps were discovered in the sequences, alignment was done manually in BioEdit 7.2.3 (Hall, 1999).

**PHYLogeographic STRUCTURE AND DIFFERENTIATION**

Relationships among mtDNA haplotypes including frequency information were visualised by means of a median-joining network using Network 4.6.1.2 (Bandelt, Forster & Röhl, 1999). A maximum-likelihood tree with 1000 bootstrap replicates (Felsenstein, 1981) was constructed using MEGA 5.2 (Tamura et al., 2011) to test for reciprocal monophyly of the two major haplogroups (see Results). As outgroups we chose *Clanga pomarina* (Genbank AJ875219, AJ875200), *C. clanga* (AJ875198, AJ875199) (formerly classified as *Aquila pomarina* and *A. clanga*) and the more distantly related *Nisaetus nipalensis* (AP008238). We used the online tool FindModel, (http://www.hiv.lanl.gov/) to determine which substitution model fit our data set best. Accordingly, Hasegawa–Kishino–Yano (HKY) + Γ (α = 0.04) was used for ML tree reconstruction in MEGA 5.2 and the Bayesian Skyline analysis (see below). However, as this model is not implemented in Arlequin, we used the related Tamura–Nei model (Tamura & Nei, 1993) in that programme.

To assess genetic structuring, we carried out analyses of molecular variance (AMOVAs) in Arlequin 3.5 (Excoffier & Lischer, 2010). We calculated ΦST as a measure of FST accounting also for haplotype divergence and not only frequencies. We partitioned our total data set into the two major lineages (see Results). ΦST is a measure of how much of the total variance can be accounted for by differences among populations as opposed to variability within them.

**GENETIC DIVERSITY AND DEMOGRAPHIC HISTORY**

Haplotype (h) and nucleotide (π) diversities were calculated in Arlequin 3.5. The combination of these measures contains information on demographic history, e.g. high haplotype diversity combined with low nucleotide diversity implies a low historic effective population size with a subsequent population expansion, whereas both high haplotype and nucleotide diversities suggest large historic and present population sizes (Grant & Bowen, 1998).

We also addressed demographic history in a more quantitative manner by means of mismatch analyses, neutrality tests (Tajima’s D and Fu’s Fs) and Bayesian Skyline Plots (BSPs). The mismatch analysis is based on the pairwise differences for all sequences. The shape of the distribution of these mismatches depends on demographic history. A unimodal distribution is typical of a recent population expansion, whereas a bimodal distribution is usually found in populations with admixed origin (e.g. through secondary contact of distinct lineages); populations that are in demographic equilibrium show a ragged or multimodal distribution (Rogers & Harpending, 1992). Mismatch analyses were carried out with Arlequin 3.5, and we tested for deviations from the sudden expansion model. When an expansion event was inferred by the mismatch analysis we also calculated the time t since expansion, based on the equation $t = \frac{\tau}{2u}$, where τ is a unit of mutational time and the mode of the unimodal mismatch distribution and $u$ is the cumulative probability of substitutions across the sequence. These calculations have been shown to be error-prone due to misconceptions of $u$ (Schneekar & Weiss, 2011) and we therefore used the spreadsheet tool of Schneekar & Weiss (2011) for our calculations.

Tajima’s D (Tajima, 1989) and Fu’s Fs (Fu, 1997) are originally tests of selective neutrality, but they also contain demographic information and are often used in this way for non-coding sequences such as the control region. Statistically significant negative values suggest a recent population expansion, while
significantly positive values are indicative of bottleneck.

The BSP analysis was carried out with the software package BEAST 1.8 (Drummond & Rambaut, 2007) and Tracer 1.5 (Rambaut et al., 2014). We applied a strict clock rate using a normally distributed range (mean = 7.23% per Myr, SD = 4.5E-9). This clock rate was estimated in an analysis of control region data in grouse (Tetraoninae), and our results should therefore be interpreted with caution (Drovetski, 2003). We ran BSPs for the complete data set and meaningful partitions (Mediterranean lineage, Holarctic lineage, Alpine data set with and without Holarctic individuals; see Results). For each run, different lengths of MCMC steps were used, sampling every 1000 generations with a 10% burn-in to obtain large enough effective sample size (ESS) values (>200). The HKY + Γ substitution model with an alpha of 0.04 was used.

RESULTS

In total we obtained 283 mtDNA control region sequences (252 for the 402-bp fragment, 31 for the 350-bp fragment; for details see Supporting Information, Table S1). Fifty-three percent of all samples yielded reliable sequences (Supporting Information, Table S2, Fig. S1), dating from 1817 to 2013. The data set comprised 26 haplotypes, defined by 22 polymorphic sites. The origin of the samples for which DNA extraction and sequencing was successful covers major parts of the Golden eagle’s distribution range (Table 1, Fig. 2 and Supporting Information, Table S1).

The median-joining network (Fig. 1) shows a split into two lineages or haplogroups with the most common haplotypes of the two lineages (M1 and H1) differing at 10 positions. The two lineages are geographically parapatric: lineage M is largely restricted to the Mediterranean region, but also occurs in the Alps and Eastern Europe, whereas lineage H (for Holarctic) is found in Scotland, Fennoscandia, Eastern Europe, continental Asia, Japan and North America. In the Alps, Eastern and Southern Europe, there is some overlap between both lineages (Fig. 2). The extent of co-occurrence was found to decrease from the East (60% H haplotypes in Eastern Europe) to the West (6% in Spain) (Fig. 2). The two lineages M and H proved to be reciprocally monophyletic in the ML tree, but node support was overall moderate to low (82% and 66% bootstrap support for lineages M and H, respectively, Fig. 3). Two haplotypes (H2 and H4) showed a very wide distribution, occurring in Europe and Asia including Japan. Intraspecific sequence divergence in the Golden eagle was comparable with interspecific values between the sister species of Spotted eagles (Clanga clanga and C. pomarina; Fig. 3 and Helbig et al., 2005).

For the global data set haplotype and nucleotide diversities were 80% and 1.4%. The respective values for the two haplogroups separately were 58% and 0.2% (lineage M, nine haplotypes) and 75% and 0.41% (lineage H, 17 haplotypes). The four additional H haplotypes found by Sonsthagen et al. (2012) were not included in diversity calculations because their frequencies were not published. Diversity values for single populations are given in Table 2. The Alps show high values because of the co-occurrence of both lineages; Fennoscandian eagles are lowest in diversity. Surprisingly, the island samples from Japan displayed very high diversity, higher even than the Alpine region despite the presence of only the Holarctic lineage.

The Mediterranean region was characterized by one frequent central haplotype (M1) occurring in the West (Spain), Central (Italy) and East Mediterranean (Bulgaria, Anatolia). A private haplotype was found in Sardinia (M8), but whether it is really unique to this island can only be answered if more Southern French and Italian samples are analysed.

POPULATION STRUCTURE AND DEMOGRAPHIC HISTORY

The two distinct haplogroups and the star-shaped pattern with a central dominant haplotype in each of them suggest recent expansion events in at least two geographically separated populations during the Last Glacial Maximum (Fig. 1). Significant negative values for Tajima’s D and Fu’s Fs, indicative of expansion events, were only found for the Holarctic haplogroup. The results of the mismatch analyses (with the exception of Fennoscandia for which results were ambiguous) and the low nucleotide diversity with intermediate haplotype diversity for the two haplogroups is indicative of bottleneck events followed by population expansion (Table 2). For the global data set, the mismatch analysis was in accordance with an expansion event, but neither Tajima’s D nor Fu’s Fs were significantly different from zero. To test whether the long temporal range affected our results we recalculated all demographic tests for our largest data set (Alps) excluding all samples older than 1950. Results only differed slightly (significant deviation from expansion based on sum of squared deviations – but not raggedness – in the mismatch analysis for the data set including both M and H lineages; Mediterranean data set and all tests of Fu’s Fs and Tajima’s D did not change). Demographic conclusions were thus not affected.

The τ-based expansion times derived from the mismatch analyses are given in Supporting Information,
Table S4. They have to be viewed with caution as possible mutation rates cover a large range. It is interesting, though, that both the Mediterranean and the Holarctic lineage show similar results that are in accordance (at least based on the minimum values) with an expansion after the Last Glacial Maximum.

ΦST between the two haplogroups was 0.918 (P < 0.01) meaning that 91.8% of the total genetic variance was due to differences between the two groups.

In the Bayesian Skyline plot analyses the median trend was not supported by the upper and lower highest posterior density intervals in all but one data set: only the results of the partial Alpine population (individuals of the Mediterranean lineage only) showed a consistent signal of a recent increase in female effective population size (Fig. 4).

**DISCUSSION**

We analysed more than 250 Golden eagle sequences from throughout their distribution range and found two distinct mtDNA lineages (Mediterranean and Holarctic). Genetic diversity was comparable with that of other large raptors. Mismatch analyses were mostly in accordance with recent population expansion, but other demographic tests (Tajima’s D, Fu’s Fs and BSPs) were more ambiguous.

The occurrence of two (main) haplogroups in large raptor species is not uncommon. White-tailed eagles (*Haliaeetus albicilla*, Honnen et al., 2010; Langguth et al., 2013), Cinereous vultures (*Aegypius monachus*, Poulakakis et al., 2008), Bearded vultures (*Gypaetus barbatus*, Godoy et al., 2004) as well as the Saker falcon (*Falco cherrug*, Nittinger et al., 2007) show a partitioning into two mtDNA lineages across their Eurasian distribution (none of them occurs in the Nearctic). What is unusual, however, is the geographic distribution of the two lineages in Golden eagles. While all the afore-mentioned species show a western and an eastern lineage with or without a zone of overlap or a clinal pattern, the Golden eagle has a geographically confined Mediterranean lineage and a single second lineage covering the whole Northern Hemisphere from Western Europe through Eurasia and Japan to North America. It is also remarkable that single haplotypes were found in regions as far apart as Japan, Central Asia and the Alps (H2) or Japan and Fennoscandia (H4). This is
indicative of at least occasionally successful long-distance dispersal, but we cannot completely rule out that it is an ancestral shared polymorphism. Although we cannot define the eastern border of the Mediterranean lineage due to a lack of extensive sampling in West Asia, its distribution is roughly in accordance with that of the subspecies *A. chrysaetos homeyeri*, while our data do not recognise any of the other five subspecies (see Table 1). However, since our data set is a single non-coding mitochondrial marker, it is neither appropriate nor sufficient to settle taxonomic issues of this kind.

The present distribution of the two haplogroups suggests that during the last ice age – when major parts of Eurasia and North America were covered by a permanent ice sheet – the species survived the cold in at least two refugia: one around the Mediterranean region and a second refugium somewhere else in the Holarctic, possibly in Asia (for North America, see below). As our sampling outside Europe is limited and no structuring within the Holarctic lineage was found, the exact location of this refugium remains unknown. After the Last Glacial Maximum, the species colonised the Alps mainly from the Mediterranean region whereas the present temperate and boreal regions of Eurasia were recolonized from the second refugium. Whether the presence of both lineages in the Alps (and also further South) is due to initial colonisation also from the north or east or whether it is due to long-distance dispersal in more recent times, is impossible to clarify. Particularly immature Golden eagles from northern populations are known to cross large distances, for example Finnish Golden eagles have been recorded to travel up to 2000 km from their breeding site. They primarily move south and reach Southern Europe, although ringing surveys or radio-tracking have yet to confirm them reaching the Alpine breeding population (Fremming, 1980; Watson, 2010; Mebs & Schmidt, 2014). For breeding, Golden and other eagles are on the whole philopatric and mostly return close to the areas where they hatched (Ferrer, 1993; Watson, 2010), so, in the absence of nuclear genetic data, their collection in the Alps cannot definitively be taken as evidence for gene flow. Still, two individuals with a Holarctic haplotype were sampled in nests in the Alps and the Northern Apennines, which implies at least occasional gene flow from the Holarctic to the populations of the Mediterranean lineage.

In North America, our small sample only yielded the haplotype that was also the most common one

Figure 2. Map of Eurasia; dots represent successfully sequenced (both 402- and 350-bp) individuals. The percentages of Holarctic (black) and Mediterranean (grey) individuals in Central, East and South Europe are shown in pie charts. Sample numbers for every pie chart (from east to west): Ukraine, Romania, Poland, Serbia and Hungary = 6 (H) and 4 (M); Austrian, German and Swiss Alps = 19 (H) and 91 (M); Italian Alps and Apennines = 3 (H) and 10 (M); French Alps = 0 (H) and 22 (M); Spain = 1 (H) and 15 (M). A map including North America can be found in the Supplement (Supporting Information, Fig. S2).
found in California (Sonsthagen et al., 2012; H5 in Fig. 1). It is remarkable that all North American haplotypes found so far fall within the Eurasian diversity (Holarctic lineage) and that both continents even share H5/CR1. Golden eagle fossils have been recorded continuously in the Californian Rancho La Brea tar pits from 35 000 to 10 000 years (Molina & Prothero, 2011) so that a post-glacial recolonization of North America from Asia after an extinction can be ruled out. Whether there was intercontinental gene flow during the Late Pleistocene or more recently (which would explain the striking genetic homogeneity across the Bering Strait) is unknown. Similar genetic patterns, i.e. a genetic lineage that covers both Asia and North America, were also found in other species, e.g. Brown bear (Davison et al., 2011), Raven (Omland et al., 2000) or Red fox (Statham et al., 2014). However, these species also showed further genetic lineages restricted to either Eurasia or North America. Clearly, more extensive sampling across the North American continent is needed to shed further light on this issue and to find or rule out the existence of a third, North American lineage. In Europe, the presence of two genetically different lineages strongly contributes to the observed haplotype and nucleotide diversity, a constellation also found in White-tailed eagles (Langguth et al., 2013). When excluding the rarer Holarctic lineage in the Alpine population, its genetic diversity matches the lowest observed diversity in the Golden eagle found in California (Sonsthagen et al., 2012). Genetic diversity in Fennoscandia and Scotland (the latter has been suggested to have been colonised from Scandinavia (Bourke et al., 2010) and may show a founder effect) is rather low (see Table 1), as would be expected in formerly glaciated regions of the distribution range. Still, overall the Golden eagle harbours considerable genetic variability. This may be due to its large distribution range and consequently potentially high effective population sizes, but the long generation times in large raptors seem to generally function as a buffer against the loss of genetic diversity (Hailer et al., 2006). Low genetic diversity in northern Scandinavia was also found in White-tailed eagles, particularly in Europe’s largest breeding population in Norway (Hailer et al., 2007; Langguth et al., 2013). Norway is also a stronghold for the Golden eagle (860-1040 breeding pairs; BirdLife International, 2004). These regions were recolonized after the Last Glacial Maximum and thus probably show a founder effect in the wake of a leading edge expansion from the South.

The high genetic diversity in the endangered (Howard & Moore, 1991; Red Data Book of Japan 2013) Japanese subspecies A. chrysaetos japonica is surprising given that Japan is an island archipelago (albeit a big one). These findings seem to be at odds with those of another study on Japanese Golden eagles (Masuda et al., 1998) that reported low diversity values. The molecular marker sequence used by Masuda et al. (1998) and thought to be the control region (ψCR) (Haring et al., 1999), a derivative of the functional CR; a direct comparison between Masuda et al. (1998) and the present study is therefore not possible. Yet, the discrepancies between the two studies are interesting as the ψCR
was shown to be even more variable than the true
CR in several raptor species (Kruckenhauser et al.,
2004; Cadahia et al., 2009). The high diversity found
in our study may reflect a generally high genetic
diversity in all of Asia or could have been retained
due to the following (mutually non-exclusive) rea-
sions: (1) Japan was inhabited by Golden eagles con-
tinuously and genetic exchange between Japan and
continental Asia could have reduced the effects of a
genetic bottleneck; (2) genetic exchange could still be
contributing to the high genetic diversity observed
today; and (3) Japan was recolonized from geneti-
cally different Asian glacial refugia (Japanese haplo-
types are the most divergent within the Holarctic
lineage). More extensive sampling from continental
Asia that so far exhibits similarly high genetic div-
ersity is needed to settle this question.

Although the network shows a star-like pattern
with a central dominant haplotype and many less
frequent satellite haplotypes for both haplogroups,
recent expansion signals were equivocal from the
mismatch analyses (usually indicative of an expan-
sion) vs. Tajima’s D and Fu’s Fs (almost no signifi-
cant signal). It does seem plausible that effective
population sizes on the whole remained large enough
to veil an unambiguous expansion signal. Analysis of
demographic history based on coalescence theory as
performed by means of BSPs did not yield reliable
support for changes in (female) effective population
sizes for most analysed data sets. Only for the par-
tial Alpine data (including only individuals of the
Mediterranean lineage) was there evidence of an
expansion in the past 2000 years. The reason for this
is unclear, but generally, as with the other
approaches, there is a lot of contingency here as inferences are based on a single marker. What can
be said, though, is that population expansion is much
less evident in Golden eagles than for example the
White-tailed eagle (Langguth et al., 2013) that over-
laps with the former in a huge area in Eurasia. Fur-
ther studies including a more comprehensive
sampling from non-European parts of the distribu-
tion range and adding nuclear markers are indis-
ispensable to a deeper understanding of the
biogeographic history of this iconic raptor.

ACKNOWLEDGEMENTS

We thank everyone who has sent us Golden eagle
samples. Their generosity made this study possible.
They are R. Sutcliffe (Glasgow Museums Resource
Centre; GB), R. McGowan & Z. Floody (National
Museums Scotland; GB), R. Barrett (Tromsø University
Museum; NO), I. Byrkjedal (University Museum
of Bergen; NO), M. Hildén and T. Stjernberg (Finn-
ish Museum of Natural History; FI), N. Morel
(Musée Le Mans; FR), L. Besson (Bourges Musée
d’Histoire Naturelle; FR), C. Gauthier and P.
Can-
degabe (Musée de Grenoble; FR), D. Freychet

<table>
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<tr>
<th>Population/lineage</th>
<th>Sample N</th>
<th>Lineages</th>
<th>Diversity parameters (SD)</th>
<th>Neutrality tests</th>
<th>Mismatch expansion signal</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>h, π (%)</td>
<td>n</td>
<td>Tajima’s D</td>
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<tr>
<td>Holarctic (Lineage H)</td>
<td>107</td>
<td>–</td>
<td>0.75 (0.04) 0.41 (0.27)</td>
<td>17</td>
<td>–0.74**</td>
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<tr>
<td>Mediterranean (Lineage M)</td>
<td>145</td>
<td>–</td>
<td>0.58 (0.04) 0.20 (0.1)</td>
<td>9</td>
<td>–1.80</td>
</tr>
<tr>
<td>Total</td>
<td>252</td>
<td>–</td>
<td>0.80 (0.02) 1.40 (0.7)</td>
<td>26</td>
<td>1.68</td>
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<tr>
<td>Alps (both lineages)</td>
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<td>M + H</td>
<td>0.67 (0.04) 0.79 (0.46)</td>
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<tr>
<td>Alps (only Mediterranean lineage)</td>
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<td>M</td>
<td>0.55 (0.04) 0.2 (0.1)</td>
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<td>–0.55</td>
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<tr>
<td>East Europe</td>
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<td>M + H</td>
<td>0.93 (0.08) 0.9 (0.6)</td>
<td>6</td>
<td>–0.80</td>
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<tr>
<td>Scotland</td>
<td>13</td>
<td>H</td>
<td>0.60 (0.09) 0.2 (0.1)</td>
<td>3</td>
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<td>Fennoscandia</td>
<td>38</td>
<td>H</td>
<td>0.41 (0.09) 0.1 (0.15)</td>
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<td>Continental Asia</td>
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<td>Japan</td>
<td>12</td>
<td>H</td>
<td>0.81 (0.08) 0.85 (0.5)</td>
<td>5</td>
<td>0.96</td>
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Lineages were defined by the topology of the haplotype network: M, Mediterranean, H, Holarctic. Values for geographi-
cally meaningful populations are also given (Alps, Fennoscandia, Japan, Continental Asia and Scotland). Total calcula-
tions do not include samples from Sonsthagen et al. (2012). Mismatch analysis: + and – denote expansion signal and
lack thereof (signal present if data do not deviate significantly from sudden expansion model as assessed by tests of sum
of squared deviations SSD and raggedness). * and ** denote statistical significance of neutrality tests at the 0.05 and 0.01 level, respectively.
Figure 4. Bayesian skyline plots (BSP) for the Golden eagle control region (402 bp). BSPs depict the median population size through time for the total data set, total Alpine data set, the partial Alpine data set (Mediterranean lineage only), the Mediterranean lineage as well as the Holarctic lineage. The light grey lines show the upper and lower bounds of the 95% highest posterior density interval. Please note the differently scaled x and y axes. Zero indicates the present. The graphs go back in time to the most recent common ancestor (MRCA). The vertical grey bar indicates the Last Glacial Maximum (LGM) between 20,000 and 26,000 years ago (Clark et al., 2009).

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Hecker (AT), R. Osterkorn (OAW, Linz; AT), W. Glawischnig (AGES Innsbruck; AT), M. Päckert (Senckenberg Naturhistorische Sammlungen Dresden; DE), H.-J. Fünfstück (Bayrisches Landesamt für Umwelt; DE), B. Kraft (Landesfür für Vogelschutz in Bayern e.V.; DE), G. Mayr (Senckenberg Forschungsinstitut und Naturmuseum Frankfurt/Main; DE), M. Haase (Universität Greifswald; DE), T. Töpfer (Zoológisches Forschungsinstitut Museum Alexander Koenig Bonn; DE), R. Riegler (Vienna; AT), E. Borgo (Museo Civico di Storia Naturale di Milano; IT), R. Trabucco and M. Bon (Natural History Museum Venice; IT), L. Lubet, R. Salmaso (Museo Verona; IT), K. Bliem (Forstinspektorat Schlanders; IT), T. Chassovnikarova (Soﬁa Museum and Plodiv University; BG), S. Xirouchakis (GR), J. Terraube (University of Turku; FI), J.P. Pompidor (F), A. Gavrilov (Wolgograd; RU), Toru Yamazaki (Asian Raptor Research and Conservation Network, JP) and T. Ozawa (The Society for Research of Golden Eagle, JP). Additionally we thank S. Ho, whose help was crucial for the interpretation of the BSPs, and G. Anderson for discussions on the statistics of time series. C.N. is grateful to H.-M. Berg and P. Sumasgutner for receptive questions. C.N. thanks P. Sumasgutner for receiving all the species inhabiting the Western Palaearctic region. London: Published by the Author.


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Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Figure S1.** Sequencing success rate of different types of material for the 402-bp segment of the control region. The graph shows the success rate for the different types of material including small feathers, the umbilicus superior and inferior and tissue (muscle or footpad) in percentages. For the total number of samples, see Supporting Information, Table S2.

**Figure S2.** Map of Eurasia and North America; small dots represent successfully sequenced (both 402 and 350 bp) individuals. The Californian Golden eagles from Sonsthagen et al. (2012) are represented as one large black circle. The percentages of Holarctic (black) and Mediterranean (grey) individuals in Central, Eastern and Southern Europe are shown in pie charts. Sample numbers for every pie chart (from east to west): Ukraine, Romania, Poland, Serbia and Hungary: 6 (H) and 4 (M); Austrian, German and Swiss Alps: 19 (H) and 91 (M); Italian Alps and Apennines: 3 (H) and 10 (M); French Alps: 0 (H) and 22 (M); Spain: 1 (H) and 15 (M).

**Figure S3.** Cumulative curves that show the increase of individuals and haplotypes in our Alpine data set over time. (for a total list see Table 1 or Supporting Information, Table S1).

**Table S1.** Complete list of the 283 successfully sequenced Golden eagles according to region and country. M = Mediterranean Lineage, H = Holarctic Lineage. Haplotype numbers are the same as those in the network (Fig. 1) and ML tree (Fig. 3). The 350-bp fragment was excluded from all statistics and only used for lineage identification. Taxonomy (subspecies) was assigned based on collection site.

**Table S2.** The complete data set (‘total sample’) and the successfully sequenced data set (N and %) by time and quality. ‘Other’ refers to material that was sampled at an unknown time, could not be classified as a small or large feather or comprised mixed types of materials (e.g. tissue and feather).

**Table S3.** Golden eagle haplotypes discovered in the present study, including information on time range, geographic occurrence and frequencies. The prefix of the haplotypes (M or H) depicts the genetic lineage they were assigned to.

**Table S4.** Estimated expansion times for Golden eagle populations. Times were estimated using the online spreadsheet tool of Schenekar & Weiss (2011). Generation time (u) was assumed to be 12.5 years, the mutation rate was set to range from 4.54% (min) to 12.54% (max). The same mutation range was used for the Bayesian Skyline estimations. Fennoscandia showed significant deviation from an expansion event; therefore, no expansion time was calculated.

**SHARED DATA**

All haplotypes obtained in this study were submitted to GenBank (accession numbers KR259251-KR259276 for the 402-bp fragment and KR336764-KR336790 for 350-bp fragment).