University of Veterinary Medicine
Doctoral School of Veterinary Science

Phylogeography and population genetics of white-tailed eagles *(Haliaeetus albicilla)* in the Carpathian Basin

Ph.D. dissertation

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Abbreviations

AMOVA	analysis of molecular variance
AR	allelic richness
CI	confidence interval
DDT	dichloro diphenyl trichloroethane
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
F_{ST}	fixation index
h	number of haplotypes
hd	haplotype diversity
H_E	expected heterozygosity
H_O	observed heterozygosity
HWE	Hardy-Weinberg equilibrium
LD	linkage disequilibrium
LRr	Lynch-Ritland relatedness
MLr	maximum likelihood relatedness
ms	microsatellite
mt-hvr1	mitochondrial control DNA region hypervariable region 1
NDD	natal dispersal distance
PCBs	polychlorinated biphenyls
P_{ID}	probability of identity
WTE	white-tailed eagle (*Haliaeetus albicilla*)
π	nucleotide diversity

Definitions

Active nest	This term is used here for WTE nests where visual signs of reparation or breeding attempt were observed in the year of sampling (e.g. Oehme 2003). Also referred to as 'occupied nest'.
Breeding dispersal	Movements between an individual's subsequent breeding territories.
Floater	Vagrant individual that owns no territory. Floaters are usually juveniles and have fundamental importance as future members of the breeding population.
Genetic monogamy	A species is assumed as genetically monogamous, if great majority of the offspring are fathered by the social mate of their mother. Note, that this term is different from social monogamy, which refers to persistence of social mating pairs, but does not consider the occurrence of extra-pair paternity.
Haplotype	Here this term refers to a cluster of single nucleotide polymorphisms inherited together.
Hardy-Weinberg equilibrium	A population is at HWE, if mating is random, and neither mutation, nor selection, nor drift, nor migration changes genotype frequencies. Then allele and genotype frequencies remain constant through generations. When concentrating on a given DNA locus, the diploid genotype frequencies can be calculated by the Hardy-Weinberg equation.
Heterozigosity	Refers to the proportion of heterozygotes at a given locus in a population (i.e. diploid individuals carrying two different alleles on their corresponding maternal and paternal chromosomes).
Intruder	Any individual attending at a territory, if it is not a member of the resident pair and is not the pair's offspring from the same year. (Accordingly, an offspring of the breeding pair that hatched earlier and returned to the natal territory is also considered as intruder.)
Juvenile	Here this term refers to any non-mature individual WTE (up to its 5th calendar year). For a more specified terminology see Forsman (1999).

Linkage disequilibrium	Non-random association between alleles at different loci. Loci in LD may be physically linked on the same chromosome, but other factors can influence LD as well (e.g. the mating system or the population structure).
Microsatellite	A DNA locus consisting of 2-9 base pair long sequences, which are repeated several times, adjacent to each other (i.e. tandem repeats). Microsatellites are generally present in non-coding regions of the nuclear DNA, and therefore mutations in these loci are generally neutral (i.e. do not affect the individual's fitness). In microsatellites, the term 'alleles' refer to different-length copies of the same locus.
Null allele	Copy of a DNA locus which cannot be detected due to mutation at the binding site of any of the PCR primers used. Individuals carrying a null allele are mistakenly assumed as homozygotes instead of heterozygotes on the concerned locus.
Natal dispersal	An individual's movement between the territories where it hatched and first attempted to breed.
Occupied nest site	This term is used here for close vicinity (~100 m) of an active WTE nest.
Philopatry	Here this term refers to individual faithfulness to the natal area when establishing a breeding territory. That is, relatively short-distance natal dispersal compared to the extent of previous vagrant movements.
Probability of exclusion	Probability of false judgement in parentage analyses using a given set of DNA loci in the study population. That is, despite being sampled, the actual parent of a randomly chosen individual would be mistakenly excluded from its candidate parents.
Probability of identity	Probability that two individuals share the same genotype across a given set of DNA loci in the study population. This also means the probability that samples from two different individuals would be mistakenly identified as samples from one individual.
Raptor	Avian predator (as a synonym for bird of prey).
Territory	Home area defended by a single resident breeding pair. It contains one or more nests which may be used alternately across years.

Summary

The present dissertation explores the population structure of white-tailed eagles (WTE) in Europe, with a special focus on the Carpathian Basin; and contributes to the discovery of its underlying behavioural background, such as mate choice, dispersal and territoriality. Most of these topics were studied by analysing genetic data obtained from moulted feathers, and the dissertation pays attention to the reliability of this non-invasive sampling method as well.

Although I performed the vast majority of both the laboratory work and the analyses and I participated in the field work as well, several experts of each partial process have contributed to these studies. Therefore, hereafter I will refer each aim and result of the dissertation as 'ours' instead of 'mine'.

In the Carpathian Basin, DNA samples extracted from a total of 247 moulted feathers of adult or juvenile WTEs and small feathers pulled from 167 nestlings were used to investigate several topics related to conservation genetics of the species. Additionally, DNA extracted from different tissue samples of 118 individuals was used to investigate genetic relationships of WTE populations across Europe. Multilocus nuclear microsatellite genotypes allowed us to investigate questions both on population and individual level and sequencing of a 500 bp fragment of the mitochondrial control region provided a better understanding of population history of the Carpathian Basin.

We showed that moulted feathers collected at occupied nest sites of WTEs are reliable DNA sources for studies concentrating on resident individuals of the sampled territories. This non-invasive sampling method can be more suitable for this large raptor species than conventional methods which require capturing of adult individuals. Although feathers shed by intruders and nestlings were found as well at some nest sites, we suggest that the residents can be identified with confidence by analysing a suitably high number of moulted feathers from each nest site.

Analyses of 11 loci microsatellite genotypes across Europe found three genetic clusters and their geographic distribution suggest a division for three major WTE populations: southern (Carpathian Basin countries: Hungary, Croatia, Serbia and Slovakia, south-eastern Czech Republic and north-eastern Austria), central (Poland, Germany, northern Austria and probably autochthonous Czech birds), and northern (Finland, Lithuania and probably Estonia). The northern population could be further divided to a coastal and an inland population.

We found a unique genetic cluster in the Carpathian Basin based on 11 microsatellite loci and found that mitochondrial haplotype B12 is not only unique, but frequent in this population. Our results both confirmed a mainly local recolonization in the Carpathian Basin after a population

bottleneck in the 1970s, and suggest that some WTEs coming from more northern populations contributed to its current genetic structure as well. We also showed that WTEs released in the Czech Republic after a local population extinction in the 20th century could have had a significant impact on the current genetic structure of this population.

Pairwise genetic relatedness of resident individuals was calculated from 12 microsatellite loci to test if actual breeding pairs of south-western Hungary are less related than expected under random mating. Based on our results, WTEs can avoid kin in mate choice.

Using feathers shed by nest site intruders, we investigated the background of intrusion events. Our results confirmed that a significant proportion of nest site intruders were juveniles and consequently can be assumed as floaters. Our observations on nest site intrusion were consistent with the ideas that female floaters might search for an appropriate mate while males search for a good-quality territory.

Besides direct mechanisms, long-distance or sex-biased natal dispersal can also decrease inbreeding in a population. Our results on pairwise breeding distance of closely related same-sex individuals confirmed that natal dispersal is female-biased in the Carpathian Basin WTE population. This pattern may contribute to the generally low intersexual relatedness we found among WTEs breeding close to each other in south-western Hungary.

General introduction

The white-tailed eagle (*Haliaeetus albicilla*, Linneus 1758; hereafter referred to as WTE) is a large raptor species distributed across the Palearctic and Greenland (BirdLife International 2015; for its morphology see Figure 1). It belongs to the subfamily *Haliatinae* ('sea eagles') of the family *Accipitridae* in the order *Falconiformes*. Although they are called 'eagles' or 'sea eagles', the *Haliaeetus* species are more related to *Milvus* kites than to *Aquila* eagles (Schreiber and Weitzel 1995; Lerner and Mindell 2005).

Figure 1. Adult WTE perching in its territory. Note that the tarsi are partly bare and yellow, and the beak is conspicuously large. The yellow beak and iris, the pale neck and head and the white tail are adult characteristics. Females are generally larger than males, but their appearance is otherwise uniform. *(photo: Edina Nemesházi)*

Sampling issues

When studying dispersal, migration, territoriality or other behaviour in wild populations of birds, researchers conventionally capture and mark the individuals with rings or wing tags (e.g. Wheelwright and Mauck 1998; Helander 2003; Alcaide et al. 2009). These individuals can be individually identified when resighted in the wild. However, there are a number of limitations of such methods: direct observation of the individuals is needed, which may be especially difficult if the individual movements cover a large geographical range. Furthermore, identification of individual marks on the field can be challenging (Helander 1985a; Helander 2003), depending on the environmental conditions and the distance between the observed individual and the observer. Modern technology allows to follow individual movements of birds using radio telemetry or GPS data loggers (e.g. Nygård et al. 2003; Krone et al. 2013). When using such methods, researchers equip the individuals of interest with a transmitter after capturing them, and depending on the method, positions of each individual can be followed with high confidence. However, these devices are expensive and therefore, such studies generally collect data with a limited sample size (that is, they follow only a few individuals).

WTEs are sensitive to human disturbance and therefore their investigations should be planned with caution, to minimise negative effects on the individuals' fitness: their disturbance should be avoided, especially during the incubation and hatching period (e.g. Helander 1985). Therefore, conventional methods requiring capture of adults are less suitable for studies of this species. Collection of moulted feathers at breeding territories is an increasingly used non-invasive DNA sampling method in raptor species (Rudnick et al. 2005; Booms et al. 2011; Vili et al. 2013b; Bulut et al. 2016). Nestlings are generally sampled with minimally invasive methods during the annual ringing process (e.g. taking feather or blood samples; Rudnick et al. 2005; Booms et al. 2011; Ponnikas et al. 2013; Treinys et al. 2016). Genetic data obtained from such samples can be used to investigate several questions either on individual or population level. Such as, nest site fidelity (Booms et al. 2011), space use (Bulut et al. 2016), or turn-over rate (Vili et al. 2013b) of resident individuals, parentage of nestlings (Rudnick et al. 2005) and population structure (Ponnikas et al. 2013).

When using non-invasively collected DNA samples researchers generally cannot observe directly the individuals investigated. Lack of observation data can limit the usability of these samples for certain investigations; for example, the age or breeding status of the individuals sampled may influence the results. In WTEs, juveniles and adults differ considerably in plumage. Therefore, moulted feathers can also be used to estimate the age of the sampled individual (Forsman 1999; Cieślak and Dul 2006). However, while some feathers show different colour pattern before and after maturation, others cannot be used for age estimation.

In territorial raptors, it is generally assumed that moulted feathers collected at occupied nest sites likely belong to the territorial pair (Rudnick et al. 2005; Booms et al. 2011; Vili et al. 2013b; Bulut et al. 2016). Nevertheless, conspecific territorial intrusions do occur, and some intruders can approach the nest sites as well (Rutz 2005; Meyburg et al. 2007; Turrin and Watts 2014). Feathers shed by nest site intruders could potentially influence investigations concentrating on the resident individuals. To our knowledge, no study aimed to assess the reliability of moulted feathers for non-invasive sampling of residents at their nest sites in WTEs so far.

Population trends of white-tailed eagles in Europe

The WTE is a flagship species for the European nature conservation. Due to landscape changes, direct persecution and intensified agriculture, populations of this large raptor decreased dramatically in the early 20th century in Europe: in many countries only a few breeding pairs survived or the species became regionally extinct. With banning the use of DDT in agricultural practice and PCBs in industry, and strict legal protection of the species, its populations started to increase again since the 1970s. The European breeding population is currently estimated at 9,000-12,000 breeding pairs and since 2005 the species is assessed as least concern in the IUCN Red List (BirdLife International 2015). However, individuals are still exposed to a number of threats in several populations (Helander and Stjernberg 2003; Probst and Gaborik 2011), such as electrocution and poisoning in Hungary (Horváth 2009).

The Carpathian Basin is close to the southernmost WTE breeding area in Europe. The species was one of the most frequent large raptors in this area in the 19th century (e.g. Wildburg 1897), but similarly to the rest of Europe, the Carpathian Basin population decreased dramatically in the 20th century. During the 1970s, breeding populations disappeared from Austria and Slovakia, north-eastern Serbia and most parts of Hungary. Only about 10-12 pairs remained in Hungary, all in the southern Transdanubia region (Hám et al. 2009; Horváth 2009; Probst and Gaborik 2011). The former breeding areas have been recolonized by the 21st century, and more than 500 WTE pairs breed in the Carpathian Basin today. This area has provided an important wintering place for WTEs coming from several European areas, even during the population decline (Horváth 2010; Horváth 2012). Recently, extensive investigations have been published on the genetic structure of European WTE populations, but the southernmost breeding area remained poorly studied (Cederberg et al. 2003; Hailer et al. 2006; Literák et al. 2007; Hailer et al. 2007; Honnen et al. 2010; Langguth et al. 2013; Ponnikas et al. 2013; Treinys et al. 2016). It is unknown whether recolonization of the Carpathian Basin occurred exclusively from the local surviving population (as expected from philopatry) or individuals coming from more distant populations played a role as well (e.g. through settlement of wintering individuals).

WTEs were released in some European countries during the 20th century (e.g. Evans et al. 2009; Fentzloff 1984). In the Czech Republic, WTEs were reintroduced after a local extinction, and this reintroduction possibly has affected the genetic structure of the present population: the released individuals originated from two captive breeding pairs found injured in the wild (Claus Fentzloff pers. comm.) and their population of origin remained unknown.

Ecology of the white-tailed eagle

The WTE is dependent on large water bodies and predominantly feeds on fish and waterfowl. However, individuals well adapt to local prey compositions (Horváth 2009; Sándor et al. 2015). As a top predator, it plays an important role in water-related ecosystems. Top predators can facilitate resources essential to other species, initiate a cascade effect in lower trophic levels of ecological communities and may have a positive impact on biodiversity through several ecological processes (Sergio et al. 2008). Notably, raptors have further potential in environmental conservation as tools for monitoring presence of persistent environmental pollutants (Helander et al. 2008; Eulaers et al. 2011), because their eggs and feathers accumulate such chemicals from lower trophic levels.

WTEs generally build their nest on trees, but in some northern populations nesting occurs on cliff ledges and the ground as well (Helander and Stjernberg 2003). They can nest on several tree species (e.g. *Pinus, Fagus, Populus, Fraxinus, Quercus* sp.), but they prefer old and strong specimens (Helander and Stjernberg 2003; Horváth and Pintér 2005; Radović and Mikuska 2009). In the Carpathian Basin, WTEs build their nests on average height around or above 20 m, varying between tree species and location (Horváth and Pintér 2005; Radović and Mikuska 2009). Breeding pairs can produce a single clutch consisting of 1-3 eggs each year (Helander and Stjernberg 2003). The start of egg-laying varies with latitude and climate (Helander and Stjernberg 2003), and it generally starts around late February in the Carpathian Basin. Young WTEs become independent in a few months after fledging and they are vagrant until they settle down and start breeding around their 6th calendar year (Helander and Stjernberg 2003; Bělka and Horal 2009; Horváth 2009). Individual lifespan can exceed 20, or even 30 years in wild WTE populations (Helander 2003; Helander and Stjernberg 2003).

In most European populations adult WTEs stay close to their territories throughout the year (Dementavičius and Treinys 2009; Krone et al. 2013). Juveniles discover large areas during their vagrant movements and can visit populations in several hundred or thousand kilometres from their natal area (e.g. Bělka and Horal 2009; Horváth 2009). In general, juveniles tend to move towards more southern areas for the winter (Nygård et al. 2003; Bělka and Horal 2009; Horváth 2009; Horváth 2010).

Although they disperse large distance from their hatching place as juveniles, WTEs tend to breed relatively close to their natal area; the species is therefore assumed as philopatric (Helander and Stjernberg 2003; Struwe-Juhl and Grünkorn 2007; Whitfield et al. 2009b; Whitfield et al. 2009a). After settling down, breeding individuals are long-term faithful to their territories (Helander 2003; Helander and Stjernberg 2003; Krone et al. 2013).

Similarly to other large raptors, WTEs are socially monogamous (Helander and Stjernberg 2003). Production of extra-pair offspring is generally rare in such raptor species (i.e. they are also genetically monogamous; see also Mougeot 2004 and Rudnick et al. 2005). Inbreeding avoidance may be more crucial in mate choice in these species compared to those birds which have high divorce rate or produce many extra pair offspring. Despite the general philopatry of WTEs, sex-biased or long-distance natal dispersal might decrease inbreeding in this species. However, occurrence of such strategies have been studied only in a few populations so far (Struwe-Juhl and Grünkorn 2007; Whitfield et al. 2009a).

Ability of kin recognition has been shown in some bird species (Sharp et al. 2005; Bonadonna and Sanz-Aguilar 2012) suggesting that individuals could actively avoid kin when choosing a mate. To our knowledge, so far no investigations attempted to reveal whether direct kin avoidance occur in mate choice of WTEs.

Similarly to other territorial raptors, conspecific territorial intrusions occur, even around the breeding season (Krone et al. 2013). Several authors suggested that intruders could gain opportunity to breed or acquire a territory (Garcia and Arroyo 2002; Rutz 2005; Mougeot et al. 2006; Ferrer et al. 2015), but our knowledge on the background of this behaviour is scarce.

Aims of the dissertation

1. We tested the **reliability of a non-invasive DNA sampling method** for breeding WTEs: We assessed whether moulted feathers collected at occupied nest sites belong to the breeding pairs, or feathers lost by intruders can potentially bias studies using such samples. [SECTION I]
2. With a comprehensive sampling of WTEs across Europe we studied two main topics on population level:
    - We investigated the **genetic structure of several European breeding populations** (from the northern to the southernmost areas) to reveal the history of the population recovery in the Carpathian Basin: did it occur exclusively through local expansion or did gene flow from other populations substantially contribute to it? [SECTION II]
    - We inferred the **origin of the captive birds released** between 1978 and 1989 in the Czech Republic to compare this population and its history with the naturally recovered neighbouring populations. [SECTION II]
3. Occurrence of two strategies related to **inbreeding avoidance** was tested in the Carpathian Basin population:
    - We tested the hypothesis that WTEs consider relatedness when **choosing a mate,** by comparing mean pairwise genetic relatedness of actual breeding pairs to mean values predicted under random mating. [SECTION III]
    - We addressed whether **natal dispersal** is sex-biased in the Carpathian Basin, inferring from genetic and spatial data on male and female WTEs breeding in the area. [SECTION IV]
4. Using moulted feathers collected at occupied WTE nest sites, we investigated whether **nest site intrusions** can be explained by three non-exclusive hypotheses. Accordingly, intruders may visit their natal area, seek opportunity to breed, or to occupy a suitable territory. [SECTION III]

I. Assessing reliability of a non-invasive DNA sampling method for resident white-tailed eagles

I.1. Introduction

Investigation of large raptors is challenging: conventional methods requiring capture of adults are generally not suitable in these species. Recently, increasing number of researchers have used non-invasively collected moulted feathers as DNA sources to study several questions requiring individual identification; e.g. annual turnover and mate fidelity of breeding individuals (Rudnick et al. 2005; Vili et al. 2013b) or nest site fidelity and dispersal of adults and juveniles (Booms et al. 2011; Bulut et al. 2016). These studies generally assumed that moulted feathers collected at nest sites of a territorial raptor belong to the resident individuals with a high probability. Nevertheless, feathers lost by conspecific intruders could possibly influence the results of such investigations; observations suggest that some intruders can approach occupied nests as well (Rutz 2005; Meyburg et al. 2007; Turrin and Watts 2014).

To our knowledge, reliability of moulted feathers collected at nest sites for sampling resident individuals was not tested in white-tailed eagles (*Haliaeetus albicilla*; hereafter WTE) so far. Breeding pairs of this species are territorial, but conspecific intrusions occur (Krone et al. 2013). Using moulted feathers collected at WTE nest sites Bulut et al. (2016) found that in some territories moulted feathers belonged to more than one individual from the same sex. This observation suggests that some of those feathers were lost by nest site intruders (or the pair's offspring) and not residents.

WTEs generally start breeding around their 6th calendar year (Helander and Stjernberg 2003). Appearance of the individuals transform significantly during the first years of their life. Nestlings have dark beak and iris, and the colour of those gradually changes to yellow by the adulthood (adult characteristics are shown in Figure 1 in the 'General introduction'). An adult WTE can also be told from a juvenile based on plumage (Figure I.1; older subadults show mixed characteristics, see Forsman (1999). Moulted feathers can be used for age estimation (i.e. telling adult from juvenile), without actually observing the sampled individual. However, not all feathers are reliable for age estimation: some feathers show discriminatory pattern in juveniles or adults, while others look similar before and after maturation (Forsman 1999; Cieślak and Dul 2006).

Figure I.1. Juvenile (left) and adult (right) WTEs. Note colour differences of the tail feathers and the underbody plumage: the juvenile has mottled tail feathers and tawny-mottled plumage with dark head, while the adult has white tail, uniform underwings and pale head and neck. *(photos: Edina Nemesházi)*

Our goal was to assess whether moulted feathers collected at occupied WTE nest sites are reliable for sampling the resident individuals. Furthermore, when we found feathers with unusual colouration, we recorded if such feathers belonged to individuals with known age, to assess the potential of the colouration for age estimation.

I.2. Materials and methods

DNA samples were collected from WTEs across the Carpathian Basin between 2010 and 2016 during the breeding season. We searched for moulted feathers within about 100 m from occupied nests, and nestlings were sampled by pulling one growing body feather during the annual ringing process.

DNA was extracted with Quiagen DNEasy Blood and Tissue Kit or Thermo GeneJet genomic kit by the manufacturers' instructions, but additional 10 µl of 1M dithiotreitol was used during the digestion step. Molceular sexing of each DNA sample was performed with the 2550F/2718R (Fridolfsson and Ellegren 1999) or the GEfUp/GErUp and GEfLow/GErLow

primer pairs (Ogden et al. 2015). PCR reactions were performed in a 16 µl volume. PCR reactions of 2550F/2718R contained 1.6 µl PCR buffer (10 µl Dream Taq™, Fermentas), 16.25 mM $MgCl_2$ (Promega), 1.30 mM dNTP-mix (Fermentas), 0.33 units of DNA-polymerase (Dream Taq™, Fermentas), 6.43 pmol of each primer and 10-70 ng of template DNA. Amplification of the GEfUp/GErUp and GEfLow/GErLow primer pairs was performed in a multiplex reaction, with 3.75 pmol of each primer (composition of the reaction mixture was otherwise the same). Sex-specific bands were visualized in UV light following electrophoresis on 2% agarose gel containing ECO Safe (Pacific Image Electronics Co., Ltd.). PCR profiles followed the original articles for each sex marker (Fridolfsson and Ellegren 1999; Ogden et al. 2015), but the profile described by Ogden et al. (2015) was completed with an initial touch-down section where annealing temperature decreased from 65 to 60°C in 7 cycles.

A total of 12 microsatellite loci were used for genotyping: Hal01, Hal03, Hal04, Hal09, Hal13 (Hailer et al. 2005), Aa27, Aa35, Aa49 (Martínez-Cruz et al. 2002), IEAAAG04, IEAAAG05, IEAAAG12 and IEAAAG14 (Busch et al. 2005). Forward primers were 5' labelled with a fluorescent dye (VIC™, FAM6™, PET ™, NED™, or HEX). PCR reactions were singleplex or multiplex, depending on the used primer pairs (see below), performed in 16 µl volume similarly to molecular sexing described above. For IEAAAG and Hal loci, a modified PCR profile following Hailer et al. (2006) was used: reactions were repeated in 37 cycles and both annealing and amplifications steps lasted for 45 seconds. Aa loci were amplified as described by Martínez-Cruz et al. (2002). Annealing temperatures for Aa and Hal loci were set as described in the original papers (Martínez-Cruz et al. 2002; Hailer et al. 2005). Following Hailer et al. (2006), we used 56°C for loci IEAAAG04, IEAAAG05 and IEAAAG14, while annealing was performed on 60°C for IEAAAG12.

Microsatellite fragment analysis of the 12 loci was optimised as follows: two pools of PCR products were used, where in the first pool primers IEAAAG05 and IEAAAG14 were amplified separately, while IEAAAG04 (4-4 pmol forward and reverse) and Hal04 (6-6 pmol), and IEAAAG12 (4.26-4.26 pmol) and Hal01 (5.75-5-75 pmol) were amplified in mupltiplex reactions. The second pool contained products of three singleplex (Aa35, Hal03 and Hal09) and two multiplex reactions, where Hal10 (5.5-5.5 pmol) and Hal13 (4.5-4.5 pmol), and Aa27 (3-3 pmol) and Aa49 (5.25-5.25 pmol) were amplified together. Fragment length analyses were performed on an ABI3130 sequencer (Applied Biosystems, using Gene Scan™ -500LIZ™ Size Standard). To minimise genotyping errors, trace files were scored by two experts independently in Peak Scanner 1.0 (Applied Biosystems), and PCRs were repeated when they yielded uncertain results (e.g. low or ambiguous peaks).

Based on analyses of several subset of DNA samples overlapping with samples used here, we assumed that our 12 loci are reliable for individual identification of WTEs in the Carpathian

Basin, and even 7 loci genotypes would be sufficient (see results of SECTION III and SECTION IV). Using genotypes of feathers collected at the same nest site, consensus individual genotypes were prepared manually. Two moulted feathers collected at the same nest site were assumed to belong to the same individual if they were successfully genotyped on at least 8 loci and showed maximum 2 homozygote/heterozygote mismatches; meaning that on the same locus, two alleles were amplified from one moulted feather, but only one of those was amplified from the other feather. In moulted feathers, such mismatches can occur due to poor DNA quality.

Addressing residents

We tested the reliability of moulted feathers collected at nest sites for addressing the residents of the sampled territories. In this study, we used moulted feathers only from those nest sites which met all the following criteria: (i) breeding was successful in the year of sampling, (ii) at least 4 moulted feathers were collected and yielded sufficient DNA for further analyses, and (iii) DNA samples from nestlings were available as well (either from the same year or the previous one).

An individual was addressed as resident breeding bird in a territory, if its consensus genotype matched with the nestlings' genotypes from the same nest site where its moulted feathers were collected (i.e. the putative parent shared an allele with the nestling on each locus).

Fisher's exact test was used to compare the ratio of residents to non-residents among the individuals identified from at least three feathers to the ratio of residents to non-residents among the individuals identified from less than three feathers in a sampling event. A total of 25 territories were investigated, out of which two were sampled in two different years each, resulting in a total of 27 sampling events (hereafter 'territory-years'). Notably, these sampling events were considered to be independent in Fisher's exact test.

We similarly tested whether the odds of finding the resident females differed from the odds of finding the resident males: we recorded whether the resident females and males were sampled or not at each nest site in each year. As the breeding was successful in each territory in each year investigated, and 'territory-years' were considered to be independent, the total numbers of both the resident females and the resident males present in the study population were considered to be equal to the number of 'territory-years'. Two-sided p-values were calculated in both Fisher's exact tests.

Age estimation from moulted feathers

When moulted feathers allowed age estimation based on the literature (Forsman 1999; Cieślak and Dul 2006), the assumed age of the sampled individual was recorded (i.e. juvenile or adult).

Some feathers showed unusual colour pattern compared to most feathers from the same type (appendix Table AI.1). Sampling individuals with multiple moulted feathers gave us opportunity to note whether certain feather types showing some extent of mottling or unusual colouration belonged to juveniles or adults. We assessed whether these feathers could potentially be used for age estimation of the individuals.

I.3. Results

Addressing residents

Genotypes of a total of 152 randomly chosen moulted feathers collected in overall 25 WTE territories were used to test the reliability of moulted feathers for sampling resident individuals (Figure I.2). Two territories were represented by two sampling years each in the analyses (B-T1 and B-T6; see appendix Table AI.2**.**). Percentages of feathers shed by certain types of individuals (e.g. resident or intruder) for these two territories were calculated as averages across two sampling years each.

The number of analysed moulted feathers ranged between 4 and 12 per territory per year (median=6; appendix Table AI.2**.**). Out of the total of 152 shed feathers, 115 belonged to resident females, 31 to resident males, and 6 feathers belonged to other individuals (4 intruders and 2 nestlings).

While we were able to sample the resident female of each territory, no feathers were found from the resident males at 8 of the 25 territories investigated. Proportion of moulted feathers belonging to resident females ranged from 50 to 100% (on average 79%) at different territories; and 0 to 40% (on average 18%) of the moulted feathers belonged to resident males.

Some feathers were lost by non-resident individuals: these were intruders or nestlings. Feathers shed by intruders were collected in 4 territories, and only 1 feather was found from these individuals each. Across the 25 territories, on average 2% of the feathers belonged to intruders. In two territories, single feathers were lost by the nestlings (on average 1% of all feathers collected across the 25 territories). However, in one of these two cases the nest fell down, and the remains of the nestlings were still present at the nest site. This nestling feather presumed as moulted feather was found further from the remains of the nestling, but it was probably lost an extraordinary way (i.e. during or after falling down with the nest).

A total of 41 resident and 6 non-resident (intruder or nestling) individuals were identified, out of which 29 residents and 0 non-residents were sampled by a minimum of three feathers, while 12 residents and all 6 non-residents were sampled by less than three feathers. Fisher's exact test showed that the odds that the sampled individual was a resident was significantly higher

if it had been identified from at least three moulted feathers than if it had been identified from less than three feathers (p=0.0017, CI: 2.336 - Inf.).

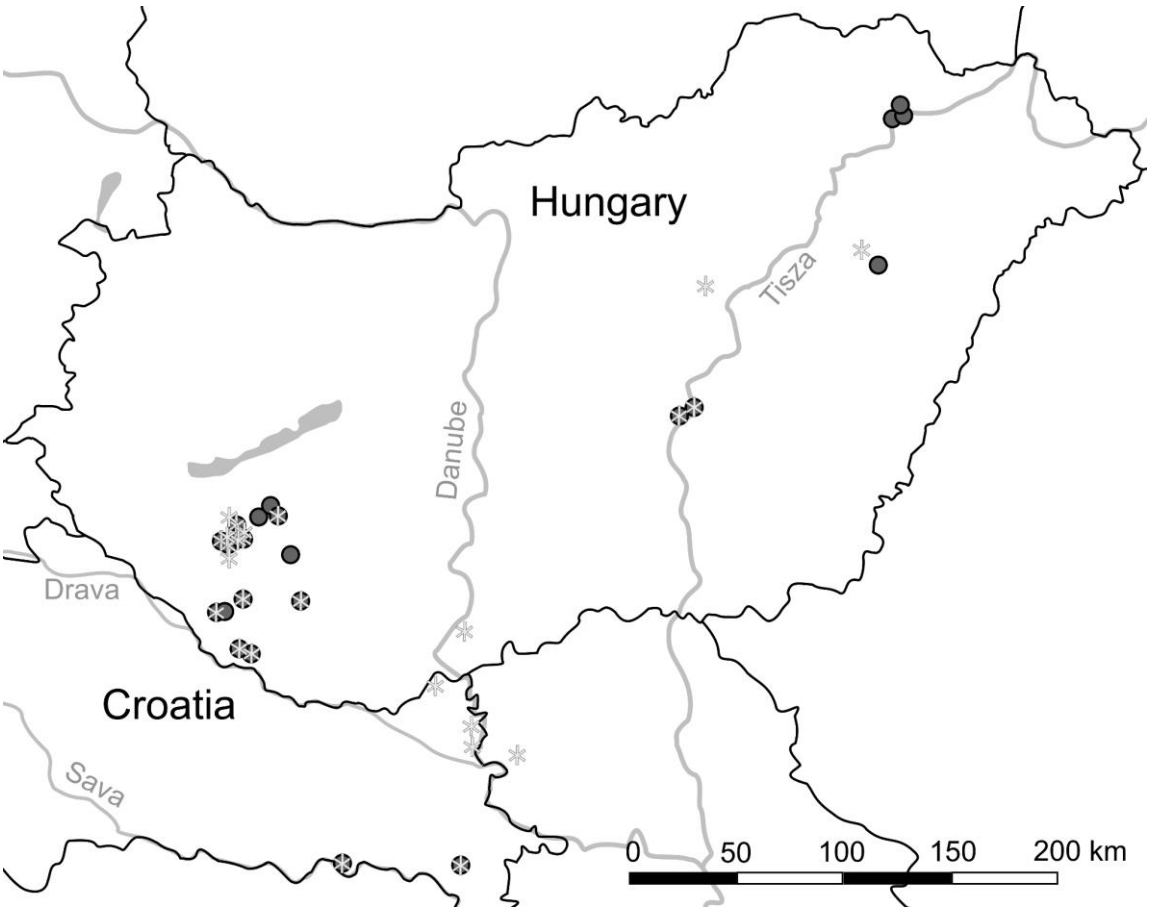

Figure I.2. WTE territories investigated across the Carpathian Basin. Dark circles refer to territories where the reliability of moulted feathers for sampling residents was tested. ***Grey asterisks*** refer to those territories where moulted feathers with age-discriminatory or unusual colour patterns were investigated.

Resident females were sampled in all 27 'territory-years', while resident males were sampled in 18 and not sampled in 9 'territory-years'. According to Fisher's exact test, the odds for finding a resident female significantly differ from the odds for finding a resident male (p<0.0018, CI: 2.506 - Inf.). Note that the actual odds ratios could not be calculated in any of the two Fisher's exact tests, because the number of intruders being sampled by at least three moulted feathers was zero in the first test, and the number of 'territory-years' with failed sampling of resident females was also zero in the second test; therefore, the odds for such cases was calculated to be zero.

Age estimation from moulted feathers

We analysed genotypes from a total of 67 moulted feathers allowing age estimation or having unusual colour pattern compared to similar WTE feathers (for sampling locations see Figure I.2). Out of these, 26 were discriminatory for adults (all large tail feathers) and 10 for juveniles (2 large tail feathers, 6 body coverts and 2 large flight feathers) based on the literature (Forsman 1999; Cieślak and Dul 2006). These feathers allowed us to assess the age of 32 individuals (22 adults and 10 juveniles). All but two of the adults were known to be residents at the sampling territory (status of the remaining two remained unknown). One out of 10 feathers with discriminatory characteristics for juveniles was lost by a nestling, the remaining 9 feathers belonged to intruders (N=8) and an individual with unknown status (N=1). For feathers allowing age estimation see Figure I.3 and appendix Table AI.1.

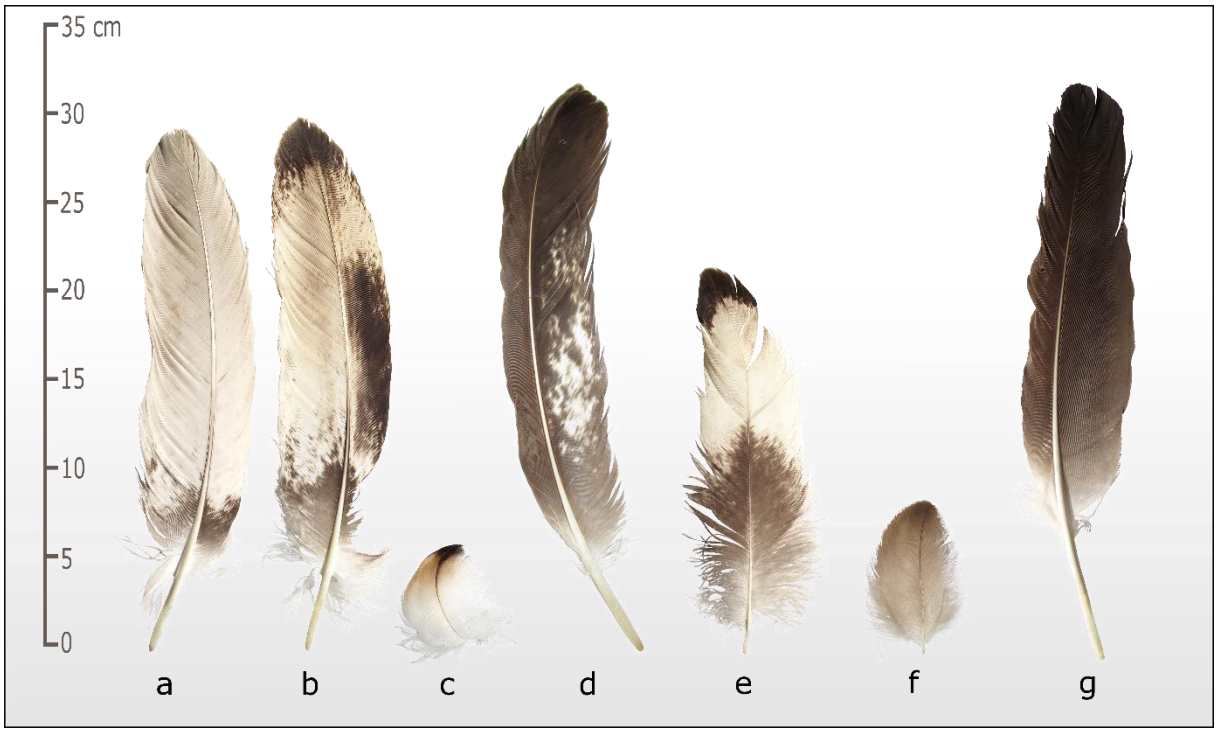

Figure I.3. Aging based on moulted feathers. We assumed an individual as adult if it was identified from a large tail feather with the upper half of the vane being completely white (**a**). A bird was assumed as juvenile if it moulted a large tail feather with extensive brown mottling (**b**), a secondary flight feather with extensive white mottling (**d**), or a body covert with the vane being pale but its top being dark (**c**). As colouration of the uppertail coverts (**e**) can show considerable individual variance, these feathers were excluded from the age estimation procedure. Body coverts (**c, f**) and flight feathers (**d, g**) did not allow us to identify adults, because immature birds can also moult such feathers similar to those of adults (see Forsman 1999). Notably, we observed that white mottling can also occur on body coverts moulted by adults.

As extensive white mottling on primary or secondary flight feathers refers to juveniles (Forsman 1999; Cieślak and Dul 2006), we suspected that appearance of mottling on body coverts or small flight feathers (e.g. alula or great coverts) may also be a juvenile feature. This presumption proved to be wrong: such feathers were shed by adults as well (appendix Table AI.1).

Large tail feathers are the most reliable type of feathers for aging WTEs. The upper half of these feathers is white in adults and shows extensive brown mottling in juveniles (but some adults retain moderately dark tips to their tail feathers; Forsman 1999; Cieślak and Dul 2006). The uppertail coverts show similar colouration, and one could easily come to the assumption that uppertail coverts having dark tip and considerable extent of brown mottling belong to juveniles. Note, that these feathers are not suitable for aging, as adults show significant individual variability in the colouration of their uppertail coverts. Accordingly, we found such feathers with dark tip and variable extent of brown mottling shed by adults (Figure I.3, appendix Table AI.1).

I.4. Discussion

We collected moulted WTE feathers at occupied nest sites to assess whether this method is reliable for sampling resident individuals. We used nestling DNA samples to identify the breeding pair in each territory. Extra-pair paternity is overall rare among large raptors (e.g. Mougeot 2004; Rudnick et al. 2005). For example, Rudnick at al. (2005) found only three out of 166 eastern imperial eagle nestlings which had mismatching genotype with one of their putative parents, and they assumed that two of those nestlings showed mismatching genotypes due to presence of null alleles rather than extra-pair paternity. Accordingly, Booms et al. (2011) assumed that an individual gyrfalcon is resident in the territory where its moulted feathers were found, if it had matching genotype with the nestlings hatched in the same territory. In our study, we assumed the same.

Majority of the moulted feathers collected at WTE nest sites belonged to breeding individuals: on average 79% to the females and 18% to the males. Although feathers from conspecific territorial intruders were found as well, the overall proportion of these was only 2%, with no such feathers found in most cases. Fisher's exact tests found significant difference between 1) the odds of identifying residents and that of identifying intruders or nestlings when sampling an individual by at least three moulted feathers, and 2) the odds of sampling resident females and that of sampling resident males. These results are consistent with the idea that the more time an individual spends at an area, the more feathers it moults there. We collected moulted feathers during the breeding season in territories with successful broods. Similarly to most

raptors, sex roles are partitioned in parental care of WTEs (Andersson and Norberg 1981). During the breeding season, the female spends considerable time on or near the nest: incubates the eggs and later protects and feeds the nestlings. The male spends considerable time hunting further from the nest site, but regularly brings prey for the female and the nestlings and perches nearby the nest. Although WTEs are territorial, conspecific intruders can visit occupied territories from time to time (Krone et al. 2013), and can approach the nest site as well (for reports on similar raptors see Rutz 2005, Meyburg et al. 2007 and Turrin and Watts 2014). These intruders spend only a short period at the nest site compared to the residents; therefore, only a small proportion of the moulted feathers present at occupied nest sites belong to them. Accordingly, collecting moulted feathers at raptor territories several authors reported that they generally found larger number of feathers belonging to resident birds while mostly single feathers belonged to intruders (Meyburg et al. 2007; Bulut et al. 2016).

In two cases, single feathers which were presumed to be moulted turned out to be lost by nestlings; one of these was possibly lost in conjunction with the fact that the nestling fell down with the nest and died. Nestlings do not moult coverts or larger feathers (for moulting stages see Forsman 1999). Therefore, losing such feathers may be casual in their case. However, they lose their hatchling down feathers in the nest. Down feathers are poor DNA sources, and are generally excluded from non-invasive sampling of adult raptors. Overall, nestling feathers can less likely influence the investigations of breeding individuals using moulted feathers. Still, our results show that occurrence of such error is also possible. Such errors can be eliminated by obtaining nestling genotypes as well, using pulled body feathers or blood samples.

Following Forsman (1999) and Cieślak and Dul (2006), we were able to estimate the age of overall 32 individuals based on moulted feathers collected across the Carpathian Basin. WTEs generally establish a territory and start breeding around their 6th calendar year (Helander and Stjernberg 2003), when their plumage shows all the adult characteristics. Accordingly, none of the resident individuals were found to be juveniles, and among 22 individuals assumed as adults, all but 2 were known to be residents at the sampling territory. Analysing multiple feathers shed by the same individuals, we found that some extent of (generally white) mottling can occur on body or neck coverts and small flight feathers shed not only by juvenile, but adult WTEs as well. Besides those described by Forsman (1999) and Cieślak and Dul (2006), we did not find any colouration or pattern which could potentially be used for age estimation of WTEs; therefore in SECTION III we performed age estimation based on the literature data exclusively.

Although moulted feathers are increasingly used as non-invasively collected DNA samples in territorial raptors, there is no consistency in the literature for the criteria of addressing an individual as resident at the sampled territory. Some authors assumed that any moulted feather

found under nests or perching trees belong to resident individuals, given their territorial behaviour (e.g. in eastern imperial eagle *Aquila heliaca*: Rudnick et al. 2005; Vili et al. 2013). In other studies, an individual was assumed as resident if it was found from the most feathers among same-sex individuals sampled in the same territory (Booms et al. 2011; Bulut et al. 2016).

Our results confirmed that resident WTEs can be identified with high confidence using moulted feathers collected at occupied nest sites during the breeding season. However, investigations based on such samples should be performed with caution. Some feathers laying near occupied nests can originate from intruders or occasionally nestlings as well. Therefore, an individual identified from a single moulted feather should not be automatically addressed as resident. We suggest rather conservative criteria: an individual WTE should be accepted as resident if majority of the feathers moulted by same-sex individuals at a nest site belonged to it, and it was sampled with at least three moulted feathers. If there is no sufficient number of moulted feathers available, non-invasive sampling of adults should be completed by minimally invasive sampling of nestlings. If genotypes of the putative residents match with genotypes of the nestlings, than they can be accepted as residents of the territory.

II. Natural and anthropogenic influences on the population structure of white-tailed eagles in the Carpathian Basin and central Europe

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II.1. Introduction

The white-tailed eagle (*Haliaeetus albicilla* LINNAEUS, 1758; hereafter referred to as WTE) is a large raptor species distributed across the Palearctic and Greenland. Its European populations suffered drastic declines in the early 20th century. In many countries, only few breeding pairs survived, or the species became regionally extinct. With the prohibition of DDT and PCBs and strict legal protection of the species, populations started to increase again in several countries since the 1970s. At the beginning of the 21st century, the European population was estimated at a minimum of 5000-5600 breeding pairs, increasing continually. Therefore, the species is now assessed as Least Concern in the IUCN Red List (BirdLife International 2015).

The WTE is dependent on large water bodies as it predominantly feeds on fish and waterfowl. Breeding individuals are sedentary in most populations, but in some northern regions they are forced to migrate during the winter season (e.g. Helander and Stjernberg 2003). It is well known that juveniles are vagrant, travelling even hundreds of kilometres until settling down as adult breeders (e.g. more than 2000 km from Finland to the Czech Republic (Bělka and Horal 2009) or to Hungary (Horváth 2009) with even larger distances; WTEs reached even Hawaii (Hailer et al. 2015) multiple times). Still, according to observation data the species is overall considered as philopatric (e.g. Hám et al. 1990; Mizera 1999; Helander and Stjernberg 2003; Struwe-Juhl and Grünkorn 2007; Bělka and Horal 2009; Whitfield et al. 2009b). However, there are also data on considerable natal dispersal distances (e.g. birds settled as far from their natal area as 450 km (Struwe-Juhl and Grünkorn 2007) from Germany to Poland). The common limitation of these studies is that they can only use direct observational information on dispersal. Individuals that leave their natal area and are not sighted again cannot be evaluated, but could well breed in distant or simply overlooked places. In this respect, examination of

genetic relationships amongst populations can be effective to complement the data derived from direct observation based on ringing data or telemetry research.

The Carpathian Basin, located in east-central Europe, is close to the southernmost European breeding area of the species, containing four major rivers (Danube, Tisza, Drava and Sava) and further wetlands. Hungary is located in its centre, but it also contains parts of Romania, Slovakia, Croatia, Serbia, Slovenia, Austria and Ukraine. During the 19th century, the WTE was one of the most frequent large raptor species in the region, with substantial numbers of breeding pairs and wintering individuals. Due to landscape changes, direct persecution and intensified agriculture the population decreased dramatically in the middle of the 20th century. During the 1970s, breeding populations completely disappeared from Austria, Slovakia, and the Czech Republic, north-eastern Serbia and from most parts of Hungary (except for the southern Transdanubia region, where 10-12 pairs remained) (Hám et al. 2009; Horváth 2009; Probst and Gaborik 2011). By now, all these areas have been recolonized and the Carpathian Basin harbours more than 500 breeding pairs (for size estimates of the sampled breeding populations see Table II.1). Breeding density at the Kopački rit wetland area (located in Croatia and Serbia, where the river Drava joins the Danube) is one of the highest known worldwide, with up to 15 pairs in 10x10 km grid cells (Mikuska 2009; Probst and Gaborik 2011).

The Carpathian Basin provided important wintering places for eagles originating from several regions of Europe even during the period of population decline. Observations of colour-ringed birds have revealed that wintering WTEs in Hungary come from Poland, the Baltic states, Finland, Sweden and Russia, as well as from other Carpathian Basin countries (Serbia, Croatia and Slovakia) (Horváth 2010; Horváth 2012). Birds ringed as nestlings in Hungary have been observed in Slovakia, Austria, Serbia, Croatia, Poland, Romania and Lithuania (Horváth 2009 and data from the Hungarian Bird Ringing Centre). Yet, also in the Carpathian Basin, individuals tend to breed close to their natal area: all of 7 individuals with known natal and breeding place from Hungary stayed in the country (dispersal distance varied between 25 and 280 km; unpublished data from the Hungarian Bird Ringing Centre), and Hám et al. (1990) reported that even the young WTEs may stay relatively close: 85% were resighted within 100 km from their natal area. It was previously found that eagles in the Danube and the Tisza region of Hungary can be regarded as a single (genetic) population, but the potential contribution of wintering migrants from other populations to the local gene pool could not be assessed due to the lack of samples from other regions (Nemesházi et al. 2013).

Due to the drastically decreased population sizes, WTEs were released in some European countries during the 20th century (e.g. western Scotland: Evans et al. 2009; Schleswig-Holstein in northern Germany: Fentzloff 1984). In the Czech Republic, population recovery started with a reintroduction program which might have affected the present genetic structure of the

population. Eleven young birds were released between 1978 and 1989, all of them being offspring of two captive breeding pairs (Claus Fentzloff pers. comm.). The parents were found injured in the wild and their origin (i.e. to which population they belonged) remained unknown.

Recently a number of publications have aimed to reveal the phylogeography and genetic structure of the European WTE populations (Cederberg et al. 2003, Hailer et al. 2006, 2007, Literák et al. 2007, Honnen et al. 2010, Nemesházi et al. 2013, Langguth et al. 2013, Ponnikas et al. 2013, Treinys et al. 2016). Sequencing WTEs for a mitochondrial hypervariable region across the distribution range, Hailer et al. (2007) found two major genetic lineages (A and B), with a West-East clinal distribution (showing admixture across a wide range in Eurasia), suggesting that the species survived the Last Glacial Maximum in two main (a more eastern and a more western) refugia. A third lineage (C) with only one haplotype occurred at the Fennsocandian Baltic coast.

A similar genetic pattern with two major eastern and western lineages was found in several large raptors distributed widely across the Palearctic, (e.g. bearded vulture *Gypaetus barbatus*: Godoy et al. 2004, Eurasian black vulture *Aegypius monachus*: Poulakakis et al. 2008); but see Nebel et al. (2015) for a different pattern in golden eagles *Aquila chrysaetos*).

Despite extensive investigations across the WTE's distribution range (Hailer et al. 2006; Hailer et al. 2007; Honnen et al. 2010; Langguth et al. 2013), the southernmost parts of the European breeding area are still poorly studied, especially along its highly important river system, the Danube.

Here we use a comprehensive sampling of WTEs from across Europe to study the following main topics: 1) We investigated the genetic structure of several European breeding populations (from the northern to the southernmost areas) to reveal the cause for the recovery of the WTE population in the Carpathian Basin: did it occur exclusively through local expansion or did gene flow from other populations substantially contribute to it? 2) We inferred the origin of the captive birds released between 1978 and 1989 in the Czech Republic to compare this population and its history with the naturally recovered neighbouring populations.

II.2. Materials and methods

Sampling and DNA extraction

The present study is based on a total of 282 samples, collected between 1987 and 2013 in several areas of Europe, of which eventually 249 were successfully analysed. For simplicity we will use country names to denote the sampling areas, even if the samples were collected only from a small part of certain countries (e.g. northeast Austria). For the present study,

sampling was performed by fellow workers of the assigned national parks during the ringing process in most of the Carpathian Basin (Hungary, Croatia, Serbia). Growing feathers were plucked from nestlings and moulted feathers were collected under active nests. To exclude non-independent samples, we used only one nestling from every sampled nest or, if we had no nestling samples, moulted feathers from breeding adults. Samples from additional areas of the Carpathian Basin and further European regions were available from earlier studies (Germany and Austria: Honnen et al. 2010, Czech Republic and Slovakia: Literák et al. 2007, Poland: Langguth et al. 2013, Finland: Ponnikas et al. 2013). These DNA samples were extracted from different types of tissue, such as nestling feathers or blood, moulted feathers around the nests and internal organs or muscle tissue from birds found dead. To minimise the chance of using samples from birds that were incorrectly assigned to a given breeding population (that is, to avoid samples from wintering or juvenile vagrant eagles), only carcasses found between March and August and with an estimated age of less than six months or more than five years were included. A total of five birds found dead were ringed in a country different from the one they were found in. In these cases we classified them as belonging to the population where they were ringed: one young bird hatched in Lithuania and one in Slovakia were found dead in Hungary, two birds originating from Finland were found dead in Austria and the Czech Republic, and one bird hatched in Estonia was found dead in Austria. We also analysed two individuals born in captivity in the Czech Republic whose parents originated from Lithuania (and classified them as Lithuanian WTEs).

For samples collected in Hungary, Croatia and Serbia we used a DNA purification kit (Quiagen - DNEasy Blood & Tissue Kit or Thermo - GeneJet genomic DNA purification kit) following the manufacturer's instructions, but using an additional 10 µl of dithiotreitol (1M) during the digestion step. DNA was extracted from the tip of the calamus in nestling feathers and the superior umbilicus in moulted feathers (Horváth et al. 2005). For samples from the other countries we already had extracted DNA (see original publications for methods).

Microsatellite analyses of the European populations

We used 11 nuclear microsatellite loci to study the genetic structure of the sampled populations. Some of the primer pairs were optimized for *Haliaeetus albicilla* (Hal01,04,09,10,13: Hailer et al. 2005), others for *Aquila* spp. (Aa27,35: Martínez-Cruz et al. 2002; IEAAAG04, 05, 12, 14: Busch et al. 2005). Forward primers were 5'-labeled with fluorescent dyes (FAM6™, PET™, NED™, VIC™ or HEX).

PCR reactions were performed in a 16µl volume, containing 10-70 ng of template DNA, 1.6 µl PCR buffer (10x Dream Taq™, Fermentas), 1.30 mM dNTP-mix (Fermentas), 16.25 mM $MgCl_2$ (Promega), 6.43 pmol of each primer and 0.33 units of DNA-polymerase (Dream Taq™,

Fermentas) for singleplex reactions. Aa27 and 35, Hal10 and Hal13, IEAAAG04 and Hal04, and IEAAAG12 and Hal01 were amplifiable as multiplexes as well (for amplification details see appendix Table AII.12).

For the Hal and IEAAAG loci, we used the PCR profile described by Hailer et al. (2006), with some modifications (37 cycles, 45 seconds for both annealing and amplification). The Aa loci were amplified following Martínez-Cruz et al. (2002). We used the annealing temperatures from the original publications for the Aa and Hal loci, and the temperatures used by Hailer et al. (2006) for IEAAAG04, 05 and 14. For IEAAAG12, annealing temperature was 60°C.

PCR products were run on an ABI3130 sequencer (Applied Biosystems, using Gene Scan™-500LIZ™ Size Standard), alleles were scored with Peak Scanner 1.0 (Applied Biosystems).

To check for genotyping errors, we randomly repeated 150 PCRs and sequence runs for 81 samples. This comparison resulted in an overall accuracy of 95%. In the case of uncertain results (e.g. low or ambiguous peaks in the trace files), we also repeated the PCR reactions. All runs were scored independently by at least two persons. Only genotypes with unambiguous results were included in the final data set (N=249, for final sample sizes per country, see Table II.1).

The final dataset was checked for null alleles and scoring errors caused by stutter bands or large allele dropouts with Micro-Checker 2.2.3. (Van Oosterhout et al. 2004) and for linkage disequilibrium (LD) between pairs of loci with Genepop 4.3. (Rousset 2008). P-values of multiple tests for LD were adjusted with Bonferroni correction, using the “bonferroni” method in the function “p.adjust” in R 3.1.2. (R Core Team 2015).

Genetic structure was investigated using two Bayesian clustering methods. Structure 2.3.4. (Pritchard et al. 2000) was used to infer the most probable number of genetic clusters without information on geographical distances between the individuals. In the Geneland 4.0.4. software (Guillot et al. 2008), spatial coordinates of the samples were included, allowing for a better definition of spatial genetic units.

Structure settings were the following: admixture model with correlated allele frequencies, burn-in: 200 000, MCMC: 400 000, K = 1-7, 10 iterations per K. The most probable number of clusters was inferred using both the ΔK method (Evanno et al. 2005) and the highest LnP(D) value. Average individual membership values for each cluster across 10 replicate runs were calculated with CLUMPP (Jakobsson and Rosenberg 2007).

In Geneland, information on the sampling localities was given with different accuracy according to the available information (exact WGS84 coordinates, name of the nearest town, region of the country or just the country was available for a given sample). Ten independent runs were

performed using the correlated allele frequency model (spatial model, K=1-7, number of iterations: 200 000, thinning: 200, using the first 200 saved steps as burn-in).

Table II.1. Population census for the sampled countries at the period of minimum numbers and during recent years, as well as sample sizes for microsatellite (*ms*) and mitochondrial control region (*mt-hvr1*) data.

Country	Number of breeding pairs		Sample size	
	1970s minimum [1]	around 2010 [1]	ms	mt-hvr1
Hungary	10-12	226	76	44
Croatia	30 [11] *	150	14	9
Serbia	10 [7] *	90-92	41	30 **
Slovakia	0	8	7	6 **
Austria	0	13-15	3	13 **
Czech Republic	0	25-30	29	9 **
Poland	80-90 [9]	1000+ [10]	29	55 **
Germany	110-120 [8]	630-660	13	85 **
Estonia	10-15 [6]	150-170 [4]	1	24 **
Lithuania	0 [5]	120 [5]	3	45 **
Finland	30-40 [2]	430 [3]	32	86 **

[1] Probst and Gaborik 2011, [2] Stjernberg et al. 2006, [3] Stjernberg et al. 2011, [4] Elts et al. 2009, [5] Treinys et al. 2016, [6] Randla and Tammur 1996, [7] Hám et al. 2009, [8] Hauff 1998, [9] Mizera and Szymkiewicz 1991, [10] Tadeusz Mizera unpublished data, [11] István Hám unpublished data

* 11 pairs at the Kopački rit in 1976 (Mikuska 2009)

** data from the literature (Hailer et al. 2007; Honnen et al. 2010; Langguth et al. 2013; Ponnikas et al. 2013; Treinys et al. 2016), for Serbia six new samples were added to the previously sequenced 24 (details described in the Methods section). Countries at least partly located within the Carpathian Basin are highlighted in bold.

As a third approach of visualising genetic clusters we carried out a factorial correspondence analysis (FCA) with Genetix 4.05.2. (Belkhir et al. 2004).

Analysis of molecular variance (AMOVA) based on F- statistics, Nei's genetic distances, expected and observed heterozygosities, allele frequencies and the number of private alleles for each cluster were calculated using Genalex 6.5. (Peakall and Smouse 2012). Deviations from Hardy-Weinberg equilibrium (HWE) for geographical regions (East European Plain, Great European Plain and Carpathian Basin) were calculated in Genalex 6.5. We chose geographical regions rather than Structure clusters because these are inferred such that deviations from

HWE are minimal, and HWE calculations therefore would be circular. P-values for multiple tests were adjusted with Bonferroni correction in R 3.1.2. (R Core Team 2015).

To measure allelic diversity, we calculated allelic richness (AR) in Fstat 2.9.3.2. (Goudet 1995). The AR calculations were based on the lowest number of completely genotyped individuals in any of the compared populations. The samples were chosen randomly in each population, across ten repeats.

Mitochondrial analyses in the Carpathian Basin population

We compared the Carpathian Basin with all previously investigated populations using a 499 bp fragment of the mitochondrial control region hypervariable region 1 (mt-hvr1) using the same primers as described by Hailer et al. (2006). There is no evidence of nuclear copies of the target region (Hailer et al. 2007; Honnen et al. 2010; Langguth et al. 2013; Ponnikas et al. 2013).

Amplification of the mitochondrial control region was performed following Hailer et al. (2006). Sequencing was conducted on an ABI3130 sequencer (Applied Biosystems). Trace files were analysed using the Pregap4 and Gap4 softwares implemented in the Staden package (Staden et al. 2000). Alignment with previously described haplotypes (Hailer et al. 2006; Honnen et al. 2010; Langguth et al. 2013) was done with the ClustalW software (Larkin et al. 2007) implemented in Mega5 (Tamura et al. 2011).

A median-joining network was constructed with Network 4.6.1.3. (Bandelt et al. 1999).

Number of haplotypes and haplotype and nucleotide diversities per population (as suggested by the microsatellite data) were calculated with DnaSP 5.10.01 (Librado and Rozas 2009).

Origin of the recolonized populations

To identify source populations for the recolonized areas of the Carpathian Basin and the Czech Republic and to investigate the level of current gene flow, we carried out assignment tests on our microsatellite dataset with GeneClass2 (Piry et al. 2004), using both frequency-based (Paetkau et al. 1995) and Bayesian (Rannala and Mountain 1997) approaches. Both tests were performed after simulation of 1000 individuals with the algorithm proposed by Cornuet et al. (1999). In these analyses, we assigned individuals sampled in the recolonized areas to reference populations (i.e. major geographical units where the species survived the 1970s population decline).

As the results of individual assignment analyses can differ according to the approach chosen, we ultimately summarized the suggested origin of each individual over all analyses that used genetic information alone (i.e. Structure and two approaches in GeneClass2). Assumed origin

of an individual was accepted if all three methods agreed in its assignment (note that only samples from the Czech population and the recolonized region of the Carpathian Basin were included here).

II.3. Results

Microsatellite analyses of the European populations

When searching for genotyping errors, we divided the samples into 3 populations (N=218, corresponding to the genetic clusters suggested by Structure, see details below) to avoid false implications caused by deficiency of heterozygotes due to population substructuring (Wahlund-effect) across the large sampling area. Micro-Checker did not indicate presence of null alleles, large-allele dropout or stutter bands.

No signs of linkage disequilibrium (LD) were found by Genepop after Bonferroni correction when partitioning the samples according to geographical regions (East European Plain, Great European Plain and Carpathian Basin). Notably, from overall 165 comparisons, some p-values for LD between certain locus pairs were below the criterion value 0.05 (but above 0.01, except for one but see below) before Bonferroni correction in one of the three regions: Hal01-Hal09 and IEAAAG04-Hal13 in the Great European Plain, and Aa27-Aa35, Aa35-Hal04, Hal01-IEAAAG05 (p=0.001), Hal09-IEAAAG14, Hal10-IEAAAG14 and Hal09-Hal13 in the Carpathian Basin. P-values for the Aa27-Hal04 and Hal09-IEAAAG05 locus pairs were below 0.05 (but above 0.01) in both the Carpathian Basin and the East European Plain, but not in the Great European Plain.

For the overall dataset, altogether 75 alleles were found at the 11 loci. Number of alleles per locus ranged between three (Aa35) and 17 (IEAAAG05), with an average of 6.8. For heterozygosity and allelic richness see Table II.2.

Structure suggested three genetic clusters (Figure II.1 and appendix Table AII.1) for our samples with predominantly northern, central and southern European distribution. For further analyses based on these results, we used 50, 60 and 70% cut-off criteria (that is, an individual is assigned to a cluster if its membership value is at least 0.5, 0.6 or 0.7, respectively). Populations of neighbouring countries were genetically similar, but differed considerably from the others with each cut-off criteria. While individuals assigned to the northern cluster were found across the whole sampled area, the central cluster was largely confined to Germany and Poland, with only few exceptions. Eagles belonging to the southern cluster were found exclusively in the Carpathian Basin (Figure II.1). Accordingly, three geographical groups can be discerned: the Carpathian Basin (Hungary, Serbia, Croatia and Slovakia), most of central Europe (Germany, Poland and Austria) and northern Europe (Finland, Lithuania, and probably

Estonia) (see also appendix Table AII.2). The population of the Czech Republic differed from those of the surrounding countries, with a high number of birds that were assigned to the northern cluster.

Differentiation among the three Structure clusters was supported by the factorial correspondence analysis (appendix Figure AII.1.).

Geneland separated five clusters in 9 out of 10 runs. In one single run, three samples from Central Poland and two from northern Germany formed a distinct sixth cluster. The confirmed five clusters are: (1) Carpathian Basin (Hungary, Serbia, Croatia, Slovakia, eastern Austria and single samples from southern Poland and the south-eastern Czech Republic), (2) Germany and Poland together with north-western Czech Republic, (3) Czech cluster (rest of the Czech Republic together with the northernmost Austrian sample), (4) Finnish Lapland and Lithuania and (5) Finnish Baltic coast. Assignment of the single Estonian sample was different amongst the independent runs: (4), (5) or (2). Moreover, one northern and one north-eastern Czech sample were assigned to cluster (2) instead of (3) one and three times (out of nine), respectively. Geographical distributions of Structure and Geneland clusters are shown in Figure II.1.

For the proportion of individuals assigned to each Structure cluster within the Geneland clusters, see appendix Table AII.2.

AMOVA analyses found most of the variation within the individuals (85% for the Geneland and 83-85% for Structure clusters, decreasing with stricter cut-off criteria). Variation among populations was 6% for Geneland clusters and 7-10% for Structure clusters (increasing with stricter cut-off criteria). Pairwise F_{ST} values for the Geneland and Structure clusters showed moderate, but significant differentiation ($p<0.001$ for all comparisons of the Structure, and $p<0.003$ for the Geneland clusters). Values for the Structure clusters varied between 0.041 and 0.099 with the 50%, 0.053 and 0.118 with the 60%, and 0.059 and 0.135 with the 70% cut-off criterion. For the Geneland clusters, pairwise F_{ST} values varied between 0.018 and 0.088. Values of pairwise F_{ST} and unbiased Nei's genetic distances are summarized in appendix Tables AII.4-AII.7. Deviations from HWE after Bonferroni correction were found in each geographical region (East European Plain, Great European Plain and Carpathian Basin), but only at one or two loci and the presence of deviation was inconsistent among loci: Hal04 ($p=0.026$), Hal09 ($p=0.002$) and Hal10 in the Carpathian Basin, Aa27 and IEAAAG04 in the Great European Plain, and IEAAAG05 in the East European Plain ($p<0.001$ in all but the two cases highlighted above). P-values of each other locus-region combination were above the criterion value 0.05 (before Bonferroni correction). We assume that deviation from HWE was caused by the mixture of genotypes from different origins within the geographical populations.

Table II.2. Measures of heterozygosity and diversity within the Structure and Geneland clusters based on 11 microsatellites. ***N mt*** and ***N ms***: sample sizes for the mitochondrial and microsatellite data respectively, ***h***: number of haplotypes, ***hd***: haplotype diversity, ***π***: nucleotide diversity, H_O: observed heterozygosity, H_E: expected heterozygosity, uH_E: unbiased expected heterozygosity ([2N / (2N-1)] * H_E; where N is the sample size), ***AR***: allelic richness.

Cluster type		Cluster	N mt	h	hd	π	N ms	H_O	H_E	uH_E	AR*
Geneland	FIN Baltic coast		63	5**	0.665	0.00659	23	0.617	0.597	0.611	4.564
	FIN Lap and Lithuania***		92	9**	0.748	0.00678	12	0.689	0.627	0.654	5.182
	Germany and Poland		140	16	0.590	0.00388	43	0.588	0.563	0.570	3.727
	Carpathian Basin		99	10	0.735	0.00675	143	0.520	0.542	0.544	3.809
	Czech		12	8	0.894	0.00905	28	0.605	0.600	0.612	4.109
Structure	50%	northern	-	-	-	-	66	0.623	0.633	0.638	6.282
		central	-	-	-	-	62	0.577	0.556	0.560	4.600
		southern	-	-	-	-	90	0.500	0.498	0.501	4.455
	60%	northern	-	-	-	-	54	0.614	0.630	0.636	6.064
		central	-	-	-	-	52	0.573	0.545	0.551	4.227
		southern	-	-	-	-	78	0.497	0.489	0.493	3.909
	70%	northern	-	-	-	-	41	0.617	0.638	0.646	5.945
		central	-	-	-	-	40	0.574	0.544	0.551	3.773
		southern	-	-	-	-	61	0.483	0.482	0.486	3.818

*For AR analyses, sample sizes (minimum number of diploid individuals completely genotyped) were 12 for the Geneland and 36, 28 or 23 for the Structure clusters according to the 50%, 60% or 70% cut-off criterion, respectively. Values are means across ten repeats.

**Haplotype and nucleotide data for the Finnish samples (Baltic coast and partly Lapland) are minimum estimations. Some of the 473 bp sequences did not allow discrimination for some haplotypes (for details see Ponnikas et al. 2013 and appendix Table AII.9). To make them comparable with the other populations, we considered these data to be the most frequent possible haplotypes from the neighbouring countries and compared the full (499 bp) sequences.

*** Estonian samples included (for details see appendix Table AII.9)

Number of private alleles did not show any clear pattern across the Structure and Geneland clusters (appendix Table AII.11). For a list of alleles and their frequencies within each cluster see appendix Table AII.10.

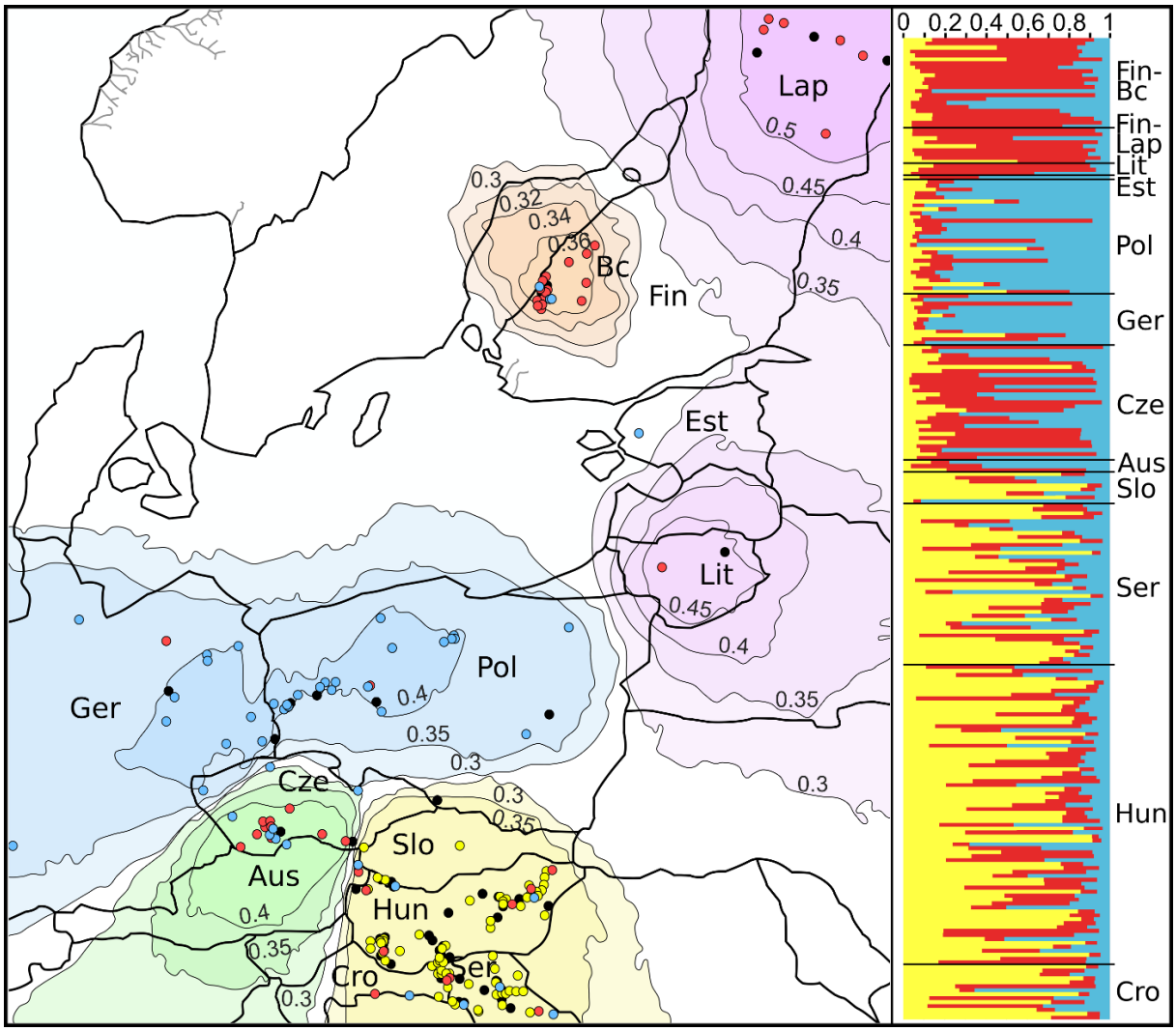

Figure II.1. Genetic clusters in Europe. Left: distribution of Structure (K=3) and Geneland (K=5) clusters across the sampled countries in Europe (N=249). Lines with numbers indicate posterior probabilities of belonging to the given Geneland cluster for samples within the circumscribed area. Coloured dots refer to individuals assigned to a given Structure cluster according to the 60% cut-off criterion (***red***: northern, ***blue***: central, ***yellow***: southern, ***black***: not classified individual). Colour marking for the Geneland clusters is as follows: Finnish Baltic coast (***orange***), Finnish Lapland and Lithuania (***purple***), Germany and Poland (***blue***), Czech (***green***) and Carpathian Basin (***yellow***). (Detailed data on the Structure clusters within each Geneland cluster for all cut-off criteria are shown in appendix Table AII.2) **Right**: barplot of the three genetic clusters suggested by Structure. Each individual is represented by a bar, which is partitioned into three coloured segments according to the percentage of assignment to the genetic clusters (colour code for each cluster is identical with that on the map). Abbreviations refer to country names; Finland (***Fin***) is subdivided to Lapland (***Lap***) and Baltic coast (***Bc***).

Mitochondrial analyses in the Carpathian Basin population

We successfully sequenced mt-hvr1 in 59 Carpathian Basin samples, and together with 40 previously published samples, we had altogether 99 Carpathian Basin haplotypes for comparison with data published from throughout the distribution range of the species.

The median-joining network yielded the three lineages known to occur in WTEs (appendix Figure AII.2.). Thirty-six percent of these 99 eagles belonged to the A (western) and 64% to the B (eastern) haplogroup. Earlier studies (Honnen et al. 2010; Langguth et al. 2013) found ten haplotypes in the region, four of them frequent and six singletons, found in single birds from Austria, Serbia and Slovakia (see also Figure II.2). The newly sequenced 59 samples only yielded the remaining four common haplotypes. Proportions of these within the Carpathian Basin (N=99) are as follows: A01 (34%), B01 (34%), B06 (8%), B12 (17%).

Haplotype distributions within the Geneland clusters are shown in the appendix Table AII.9. There is a clinal variation in the frequencies of the main lineages across the distribution area of the species (with only A in the West and almost exclusively B in the Far East; Hailer et al. 2007). However, A01 and B01 have been found in most populations studied so far. Haplotype B06 is rare in several countries across Eurasia.

Among the six singleton haplotypes in the Carpathian Basin, five (A09, A10, B10, B11 and B16) were not found anywhere else (but see also Figure II.2). The bird carrying B03 in the Carpathian Basin originated from Estonia, and was found dead in Austria in its 6th calendar year (Honnen et al. 2010) (for the microsatellite analyses, we classified it as Estonian, but it is possible that this bird settled in Austria before its death).

In earlier studies, B12 was found only in Austria (N=1) and Serbia (N=2). According to our compiled dataset, the distribution of this haplotype indicates a subdivision within the Carpathian Basin: it is relatively frequent in Hungary (27%) but rare in the southern (10% in Croatia and Serbia together) and rare or missing in the northern areas (10% in southeast Austria, absent from Slovakia).

We did not find a clear pattern in the presence of haplotypes across the different Structure clusters (see also appendix Table AII.8).

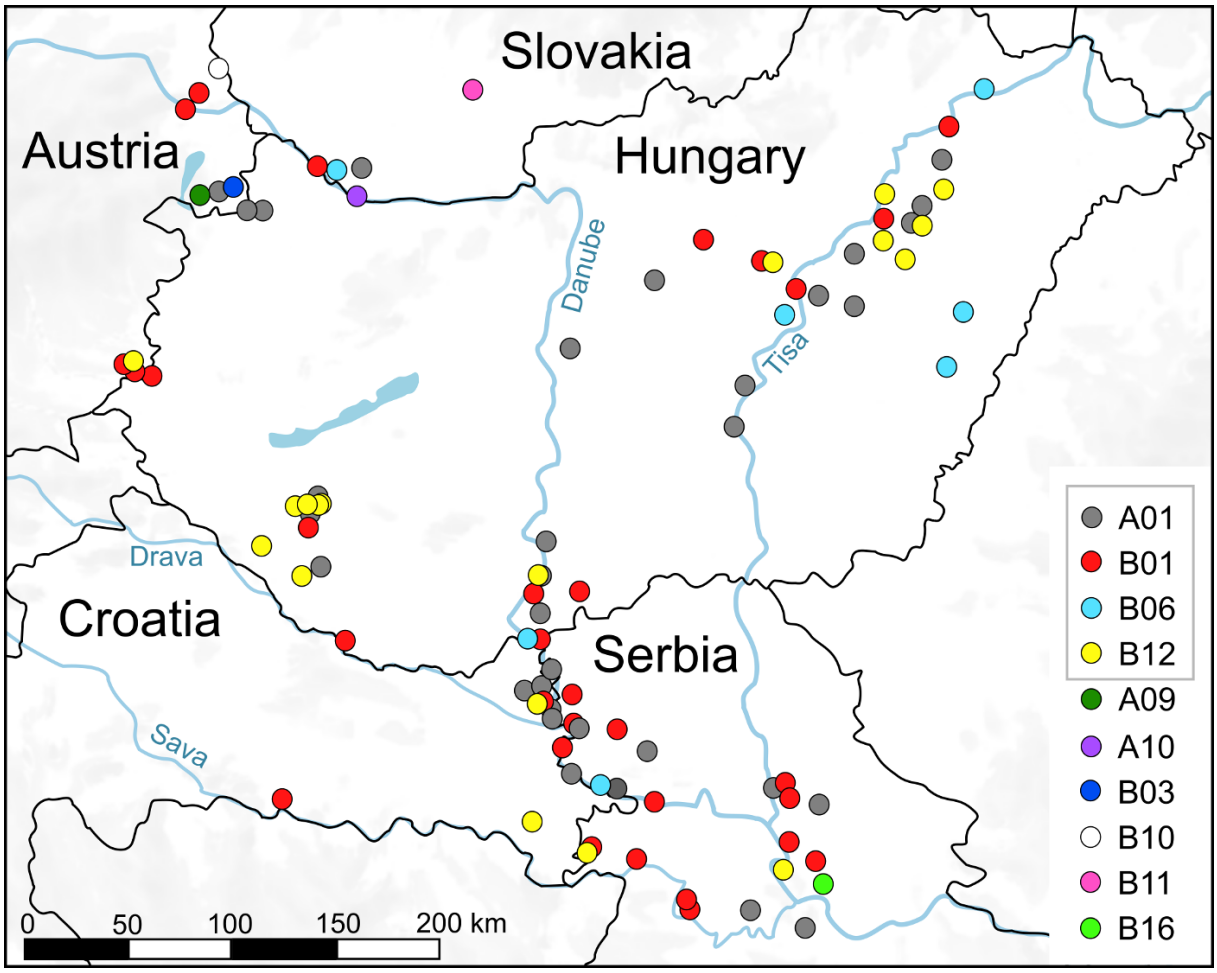

Figure II.2. Distribution of the mt-hvr1 haplotypes found within the Carpathian Basin. Haplotypes found in more than one individual are framed.

To compare the Carpathian Basin population with other European populations investigated so far, individual sequences sampled in different countries were grouped by the Geneland clusters. Haplotype and nucleotide diversity values varied between 0.590 and 0.894 and 0.00388 and 0.00905, respectively, with the lowest value in the cluster of Germany and Poland and the highest in the Czech cluster (Table II.2). The values for the Carpathian Basin were 0.735 (hd) and 0.00675 (π).

Origin of the recolonized populations

To assess the possible origin of birds in the recolonized regions (Slovakia, Austria and the Czech Republic, and northern, central and eastern regions of Hungary, see also Figure II.3), we performed assignment tests in GeneClass2. In these analyses, we assigned individuals sampled in the recolonized areas to “reference populations”. As “reference” (i.e. possible population of origin for the given individuals), we used genetic data from those regions where populations survived the 1970s. These reference populations were defined according to the

Geneland clusters, with an exception for the populations of the Finnish Baltic coast, Lapland and Lithuanian populations: as these were not separable using microsatellite markers without prior spatial information, we did not distinguish them for these analyses. Therefore, “reference populations” were as follows: northern Europe, Germany and Poland (together as central European population) and those regions of the Carpathian Basin where the species survived the bottleneck of the 1970s.

Results of the frequency-based and Bayesian methods used in GeneClass2 were compared with the individual assignments suggested by Structure. Out of 79 samples from the recolonized regions, 69 birds were classified to one of the three Structure clusters (50% cut-off criterion) and 36 were assigned consistently to a northern/central/southern cluster by all three methods. According to this subdivision, birds of presumably northern origin were found in every recolonized country except for Slovakia. The proportion of these northern birds was highest in the Czech Republic (N=12, 41.4% of the sampled birds). Birds of presumably *central* European origin were found in Slovakia (N=1, 12.5%) and the Czech Republic (N=2, 6.9%), while *southern* birds were restricted to Hungary (N=13, 33.3%) and Slovakia (N=3, 37.5%). (See also Figure II.3; numbers of assigned individuals for all cut-off criteria are summarized in appendix Table AII.3.)

Since birds can be assigned to different clusters by Geneland and Structure, it is not surprising that the different assignment approaches performed here proposed different classifications in some cases (i.e. assignment in a full dataset without spatial information in Structure vs. geographically distinct populations in GeneClass2). The high number of non-consistently assigned individuals can be caused by high rates of admixture of genetic clusters of different origin within an individual. However, individuals with non-informative genotypes (i.e. carrying alleles that are common in more than one genetic cluster) can result in controversial assignments as well.

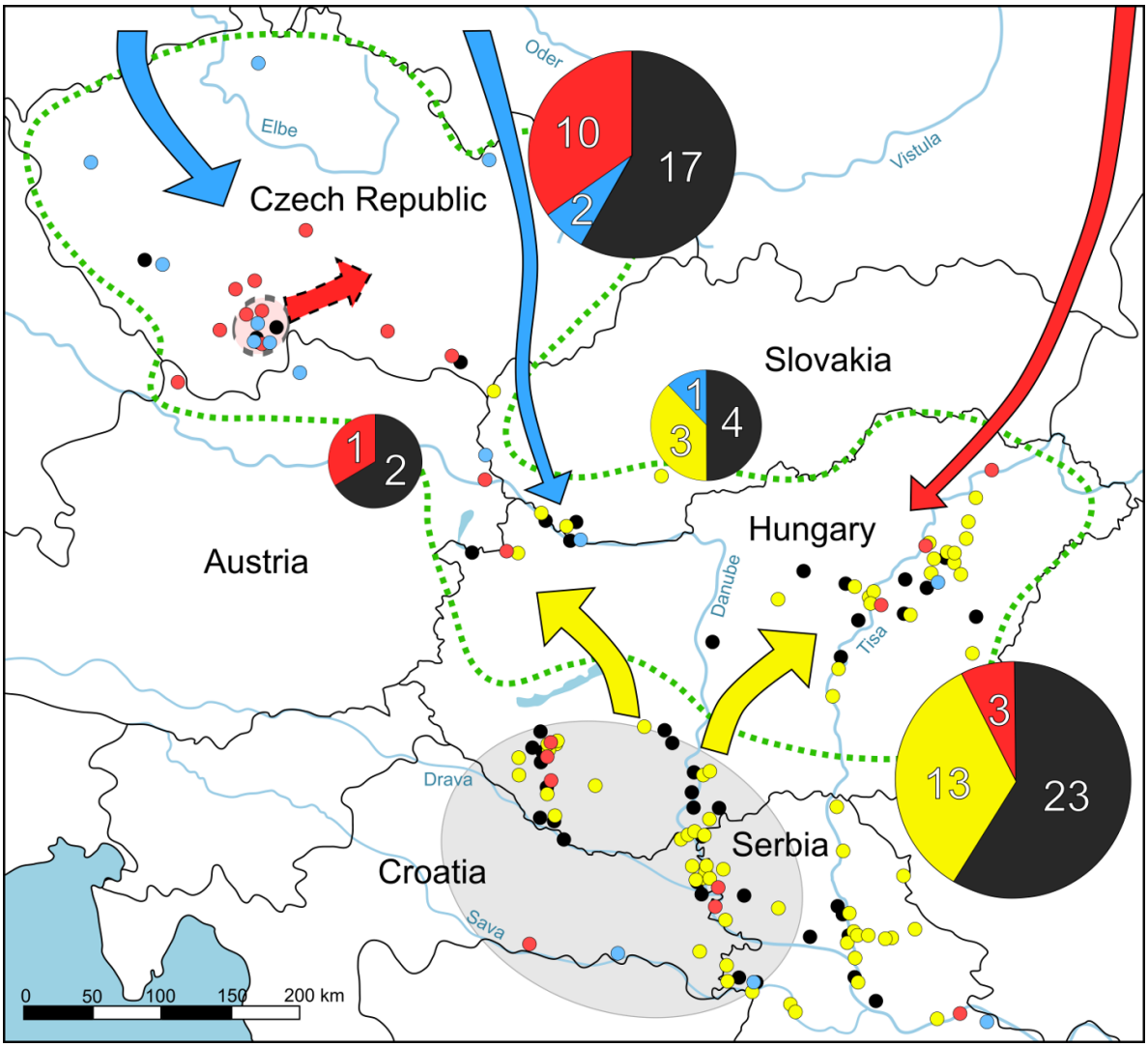

Figure II.3. Recolonization of the Carpathian Basin and Czech populations. During the bottleneck in the 1970s, breeding was not recorded at the northern and eastern parts of the Carpathian Basin. The refugial region of WTEs within the Carpathian Basin during this period is highlighted with a ***light grey oval*** (approximately overall 30-50 pairs). Samples within the area demarcated by the ***green dotted line*** were assigned to northern (Finland and Lithuania) / central (Germany and Poland) / southern (rest of the Carpathian Basin) geographical populations by GeneClass2. Individuals according to the Structure clusters (***coloured dots***, 60% cut-off criterion) and proportion of individuals with confirmed origin based on assignment tests in Structure (50% cut-off criterion) and GeneClass2 together (***pie charts***, numbers refer to the sample sizes from the recolonized regions of each country) are indicated by ***red*** (northern), ***blue*** (central), ***yellow*** (southern) and ***black*** (not classified). The ***light red oval*** surrounded by a dashed line depicts the area where 11 birds (offspring of two breeding pairs) were released in the Czech Republic (1978-1989). Main directions of suggested dispersion towards the recolonized areas are indicated by ***arrows*** (colours refer to the Structure clusters).

II.4. Discussion

The present study extends our previous knowledge about the genetic structure of the European WTE populations by analysing more samples from the Carpathian Basin and comparing genetic data from populations which have not been analysed together so far. More than half of the samples analysed in the present study were used in previous publications as well: samples collected in Germany and Austria (Honnen et al. 2010; Langguth et al. 2013), Poland (Langguth et al. 2013), the Czech Republic and Slovakia (Literák et al. 2007), Hungary and Croatia (Nemesházi et al. 2013) and Finland (Ponnikas et al. 2013). Studies of Literák et al. (2007), Ponnikas et al. (2013) and Nemesházi et al. (2013) concentrated on WTE populations on a local or national scale. The major differences between the present and Langguth et al. (2013)'s study are the sampled northern and southern countries and the set of loci (out of 11 microsatellites used here, five loci are common to both studies). While our northern samples mainly came from Finland (plus four samples from the Baltic countries), they studied birds from the Swedish coast and Estonia. In the southern region, where only a few countries were studied earlier, we extended the sampling area to the Carpathian Basin.

The distribution of genetic clusters proposed by Structure suggests that our samples from the studied European countries can basically be divided into three major populations: southern (Carpathian Basin countries: Hungary, Croatia, Serbia and Slovakia, south-eastern Czech Republic and north-eastern Austria), central (Poland, Germany, northern Austria and probably autochthonous Czech birds, see below), and northern (Finland, Lithuania and probably Estonia).

Using seven microsatellite loci, earlier studies (Honnen et al. 2010; Langguth et al. 2013), found evidence for a similar structure but a simpler subdivision into two clusters was favoured: a "western" and an "eastern" group, where the western was equivalent to our central and the eastern one equivalent to our northern and southern populations combined. High proportions of the B haplogroup found in the Carpathian Basin is in accordance with Langguth et al. (2013) who held that after the Pleistocene period the northern (except for Norway, Greenland and Iceland) and southern parts of Europe were mainly recolonized from a more eastern refugium, while central Europe was recolonized more from the west (Hailer et al. 2007).

Using information on the geographical distances between the sampling locations, Geneland (Guillot et al. 2008) suggested a further subdivision, separating the northern region into a coastal (Finnish Baltic coast) and an inland (Finnish Lapland, Lithuania and probably Estonia) subpopulation. This subdivision is in agreement with the findings of Ponnikas et al. (2013) who analysed 489 individuals from Finland, including 30 of the 32 Finnish samples used here, but based on fewer and mostly different microsatellite loci. Separation of the Baltic coast and the

interior mainland populations in northern Europe is supported by both genetic (Cederberg et al. 2003) and ringing data (Helander 2003) in Sweden, and it is possibly caused by differences in habitat preference (sea or fresh water). Since mt-hvr1 haplotype C01 is frequent along the Swedish (Hailer et al. 2007) and the Finnish (Ponnikas et al. 2013) Baltic coast, but is absent from any other population investigated so far, emigration from the Fennoscandian Baltic coast population to other areas seems to be less frequent, at least for females. Similarly, radio-telemetry data from Norway suggest that birds hatched at the coast stay close to their natal area (Nygård et al. 2003). Immigration from other populations is probably also rare (Ponnikas et al. 2013), although it cannot be ruled out completely. Allelic richness values in the northern Geneland clusters were found to be higher than that of the cluster of Germany and Poland, despite the stronger bottleneck in the former. This could be caused by immigration to the northern Geneland clusters from unsampled populations (e.g. Sweden or Russia; a connection between the Baltic coast breeding populations of Finland and Sweden has been shown by Helander 2003). However, high genetic diversity of the northern populations can be caused by the historical overlap of eastern and western lineages as well (see also Hailer et al. 2007; Ponnikas et al. 2013).

Based on microsatellite genotypes, Structure suggested a unique genetic cluster for the Carpathian Basin population. However, individuals assigned to clusters with basically central and northern European distribution are also present in the area (also confirmed by assignment tests in GeneClass2). As all the birds belonging to the northern or central Structure clusters were sampled as nestlings (except for one in Hungary and one in Austria), the presence of all three clusters in the Carpathian Basin breeding population is certain. We can therefore rule out that moulted feathers from vagrant individuals have artificially caused this pattern.

The sequencing of an increased number of Carpathian Basin WTEs in the present study for the mt-hvr1 indicated a potential further subdivision within the Carpathian Basin. While haplotype B12 is absent from any other studied population of the species distribution range so far, it is frequent in the western and north-eastern and rare in the south-eastern parts of the Carpathian Basin. This pattern suggests that the small number of breeding pairs (estimated to 10-30) remaining in the southwest Carpathian Basin during the 1970s preserved this haplotype and that extant Carpathian Basin eagles carrying B12 may be descendants of these breeding pairs.

Both the distribution of the Structure clusters and the exclusive occurrence of haplotype B12 support the assumption that population recovery in the Carpathian Basin occurred predominantly from a small surviving local population after the bottleneck period in the 20th century, similarly to other European populations (e.g. Hailer et al. 2006). However, assignment analyses on the individuals sampled in the recolonized areas suggest that birds originating

from more distant populations also settled in this region. The Carpathian Basin provides important wintering places for WTEs from different regions. In Hungary, observation data suggest that wintering birds come from Poland, Estonia, Lithuania, Latvia, Finland, Sweden and Russia, as well as from other Carpathian Basin countries (Horváth 2010; Horváth 2012). Therefore, we assume that (probably ongoing) immigration from the central and northern European populations also affected the genetic composition of the Carpathian Basin. We cannot rule out that even the west Asian population could contribute to its genetic diversity; however it has not been sampled for this study.

Although in the Carpathian Basin population nucleotide and haplotype diversities were found to be relatively high compared to those of the other European populations, values of heterozygosity based on microsatellites were relatively low. This contradiction is probably a consequence of different individuals investigated for microsatellites in our study from those with singleton haplotypes sequenced earlier.

Our results, together with other studies, suggest that despite the predominantly philopatric behaviour of the WTE, a considerable number of birds may breed far away from their natal area (e.g. 450 km: Struwe-Juhl and Grünkorn 2007). In the reintroduced Scottish population it has been shown that vagrant juveniles that cover larger distances are also likely to start breeding further from their natal area (Whitfield et al. 2009b). According to the known movement patterns based either on ringing (Helander 2003; Struwe-Juhl and Grünkorn 2007; Bělka and Horal 2009; Horváth 2009; Horváth 2010) or radio-telemetry (Nygård et al. 2003), the main direction of dispersal should be from the North to the South, especially through individuals that stay in their wintering area for breeding. The geographical distribution of the Structure clusters confirms this assumption, as do our data on allelic richness.

Although the WTEs are predominantly philopatric, we found signs for considerable large distance dispersal as well. Occasional long-distance migration is also known in other mainly philopatric large raptors – golden eagles carrying the same mtDNA haplotype were found as far apart as the European Alps and Japan (Nebel et al. 2015). Similarly, investigating mammal and bird species, Sutherland et al. (2000) found that majority of the individuals usually stay closer, while some of them disperse much further from their natal area. They found that carnivorous species with large body size usually show longer dispersal distances and it is suggested to be influenced by dynamics of resource availability in different landscapes as well. Although background of natal dispersal needs more exploration, difference in habitat value could possibly explain our findings. For WTEs originating from typical wintering places (with suitable food sources throughout the year, e.g. the Carpathian Basin or the Fennoscandian Baltic coast) it is a suitable option to stay close. Contrarily, birds hatched in other areas are forced to search for more distant places during the winter (e.g. Helander and Stjernberg 2003;

Nygård et al. 2003). They perhaps choose more often to stay in a new habitat with permanent resources instead of travelling back to their natal area.

In the Czech Republic, eleven young birds were released between 1978 and 1989 (ten in south Bohemia and one in south Moravia), all of them hatched in captivity from two breeding pairs (Claus Fentzloff pers. comm.). Geographically, the Czech population is expected to be part of the central European population, with a similar genetic composition to that found in Germany and Poland. This assumption is supported by the almost exclusive distribution of an inheritable feather abnormality in these three countries (Müller et al. 2007) and the relatively high proportion of the mt-hvr1 haplotype A02 in the area (Honnen et al. 2010) which is very frequent in Germany and Poland but absent or rare in other populations. Contrary to this, Geneland defined the Czech samples as a separate cluster, presumably due to the high number of birds with a northern European genetic signature. Although birds that were ringed in Poland, Germany, Estonia and Finland have been recorded in the Czech Republic (Literák et al. 2007), it is unlikely that such a high proportion of northern birds would have settled naturally in the area. Instead we assume that some of the parents of the released birds originated from the northern European populations, carrying genotypes which would be otherwise rare or absent in this area.

Similarly to the other recovered populations, natural recolonization of the Czech Republic could have been mainly local, but largely influenced by an anthropogenic factor, the reintroduction of birds of presumably northern origin. This may have affected the genetic composition of neighbouring areas as well, although ringing data have not confirmed such an impact on the Carpathian Basin population so far. While no negative effects of this artificial modification of the gene pool are known, the Czech WTE population highlights the importance of information on the relationships between natural populations before designing similar reintroduction programs.

III. The effects of genetic relatedness on mate choice and territorial intrusions in a monogamous raptor

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III.1. Introduction

Inbreeding can cause several negative effects on the offspring (e.g. Pusey and Wolf 1996; Oh and Badyaev 2006); therefore, avoiding kin during mate choice should be an adaptive strategy in several taxa. Ability of kin recognition has previously been shown in some birds (Sharp et al. 2005; Bonadonna and Sanz-Aguilar 2012); however, only a few studies found evidence for avoiding mating with kin so far (Wheelwright and Mauck 1998; Oh and Badyaev 2006; but see also Rudnick et al. 2005; Foerster et al. 2006; Kawano et al. 2009; Sardell et al. 2014). Some polyandrous or socially monogamous species with high divorce rate and high frequency of extra-pair offspring seem to avoid inbreeding by post-copulatory mechanisms (Foerster et al. 2006; Kawano et al. 2009) or considerable breeding dispersal (Oh and Badyaev 2006) rather than by mate choice.

Territorial intrusions occur in several raptor species. Conspecific intruders can approach even the nest site of a resident pair (Rutz 2005; Meyburg et al. 2007; Turrin and Watts 2014). Intrusions can be explained by several causes (see Penteriani et al. 2011). Here we concentrate on three common explanations, and for simplicity, we will refer to them in the manuscript by three short, arbitrary hypothesis names:

i) 'Pairing hypothesis': intruders may gain opportunity to mate (Garcia and Arroyo 2002; Rutz 2005; Mougeot et al. 2006) and possibly replace a resident bird (Ferrer 1993). If the intrusion serves mate choice, an intruder's relatedness to the opposite-sex resident may influence whether it spends time at close vicinity of the resident pair's nest. Nest site intrusions are frequently performed by juveniles (Rutz 2005; Turrin and Watts 2014), and it is also known that sometimes juveniles attempt breeding as well (Helander and Stjernberg 2003).

ii) 'Acquisition hypothesis': intruders may gain opportunity to acquire a good quality territory (see van de Pol et al. 2007; Ferrer et al. 2015). Several authors suggested that juvenile raptors

possibly assess potential breeding sites during their vagrant movements (Ferrer 1993; Helander and Stjernberg 2003; Whitfield et al. 2009b; Ferrer et al. 2015)

iii) 'Homecoming hypothesis': it is also known, that juveniles of several raptor species visit their natal area from time to time (e.g. Ferrer 1993; Nygård et al. 2003; Whitfield et al. 2009b). This behaviour may be explained by advantages of the familiar home field, such as knowledge on the food availability.

The white-tailed eagle (*Haliaeetus albicilla*, hereafter WTE) is a large raptor species with wide distribution range across the Palearctic and Greenland (BirdLife International 2015). WTEs live long (Helander 2003; Helander and Stjernberg 2003) and are known to be socially monogamous, territorial birds with individuals maturing around their 6th calendar year (Helander and Stjernberg 2003). Adults are faithful to their territory (Helander 2003; Helander and Stjernberg 2003; Krone et al. 2013) and in most populations stay nearby even during the winter. Juveniles are typically vagrant (e.g. Helander and Stjernberg 2003; Nygård et al. 2003; Whitfield et al. 2009b) and can explore populations for several hundred kilometres from their natal area (Nygård et al. 2003; Horváth 2009; Whitfield et al. 2009b). Still, the species is overall considered as philopatric (Nygård et al. 2003; Struwe-Juhl and Grünkorn 2007; Whitfield et al. 2009a; Nemesházi et al. 2016): long-distance dispersal events between the natal and breeding area rarely occur (e.g. Struwe-Juhl and Grünkorn 2007; Nemesházi et al. 2016). If intrusions are part of the breeding strategy, differences in the intrusion patterns may occur among sexes. Whitfield et al. (2009b) suggested that juvenile WTEs may search for potential breeding sites long before maturation. It has also been shown that male WTEs tend to start breeding at a younger age (Whitfield et al. 2009a) and, as breeding age approaches, move closer to their natal area than females do (Whitfield et al. 2009b).

We lack information on the background of mate choice in natural populations of both WTEs and other raptors, and studies on the possible functions of nest site intrusions are also scarce. As conventional methods (i.e. capture and marking of adults) are rarely suitable for investigating raptors, inference from non-invasively collected DNA samples can be an appropriate alternative (Rudnick et al. 2009; Vili et al. 2013a). This method allows researchers to identify and track individual birds across time and space, and to reconstruct their genetic relationships.

In this study, we had two main objectives. (1) We tested the hypothesis that in WTEs relatedness is considered when choosing mate. We expected that, in favour of inbreeding avoidance, the relatedness observed in breeding pairs should be lower than the relatedness predicted by random mating. (2) We investigated whether nest site intrusions can be explained by (i) the 'pairing', (ii) the 'homecoming' or (iii) the 'acquisition' hypothesis. We presumed that

(i) is supported if the pattern of relatedness between intruders and opposite-sex residents is similar to that found in actual pairs. If intruders turn out to be likely offspring of the resident pairs in the study population, we accept (ii). Finally, (iii) predicts that intrusions to high-quality territories are more frequent than expected by chance.

III.2. Materials and methods

Study area and sample collection

We studied WTEs in the Boronka Landscape Protection Area and the associated contiguous forest, representing an overall 120 km^2 area (hereafter referred to as 'Boronka forest'). This forest is located in south-western Hungary (Figure III.1.), a region which served as a refugium for the Carpathian Basin WTE population during a bottleneck period in the 1970s (see also Horváth 2009 and Nemesházi et al. 2016). The Boronka forest provides suitable habitat for WTEs and maintains a population with outstanding breeding density: in 2015 there were 11 occupied territories within 120 km^2, with the closest neighbours (B-T6 and B-T7) successfully breeding at about 210 m distance from each other (Figure III.1.).

More than 250 freshly moulted feathers were collected within an approximately 100 m diameter area around each occupied nest (i.e. nests showing visual signs of reparation or breeding attempt; hereafter 'nest site') during the 3 breeding seasons between 2013 and 2015. In most cases, samples were collected twice a year: during the nestling's ringing process (late April) and after the fledging period (between late June and August). Moulted feathers were collected and stored as recommended by Vili et al. (2013). Identification of putative breeding individuals was verified by matching the genotypes of residents and nestlings from the same nest sites. For this purpose, body feathers were plucked from living (N=19) or dead (N=1) nestlings. These samples were collected during the ringing process and stored in 96% ethanol at 4°C until DNA-extraction.

In some analyses, the Boronka forest dataset was supplemented with other breeding WTE individuals sampled in other Hungarian breeding areas between 2010 and 2015 (Figure III.1.). Sampling and verification of these individuals as breeders was the same as described above.

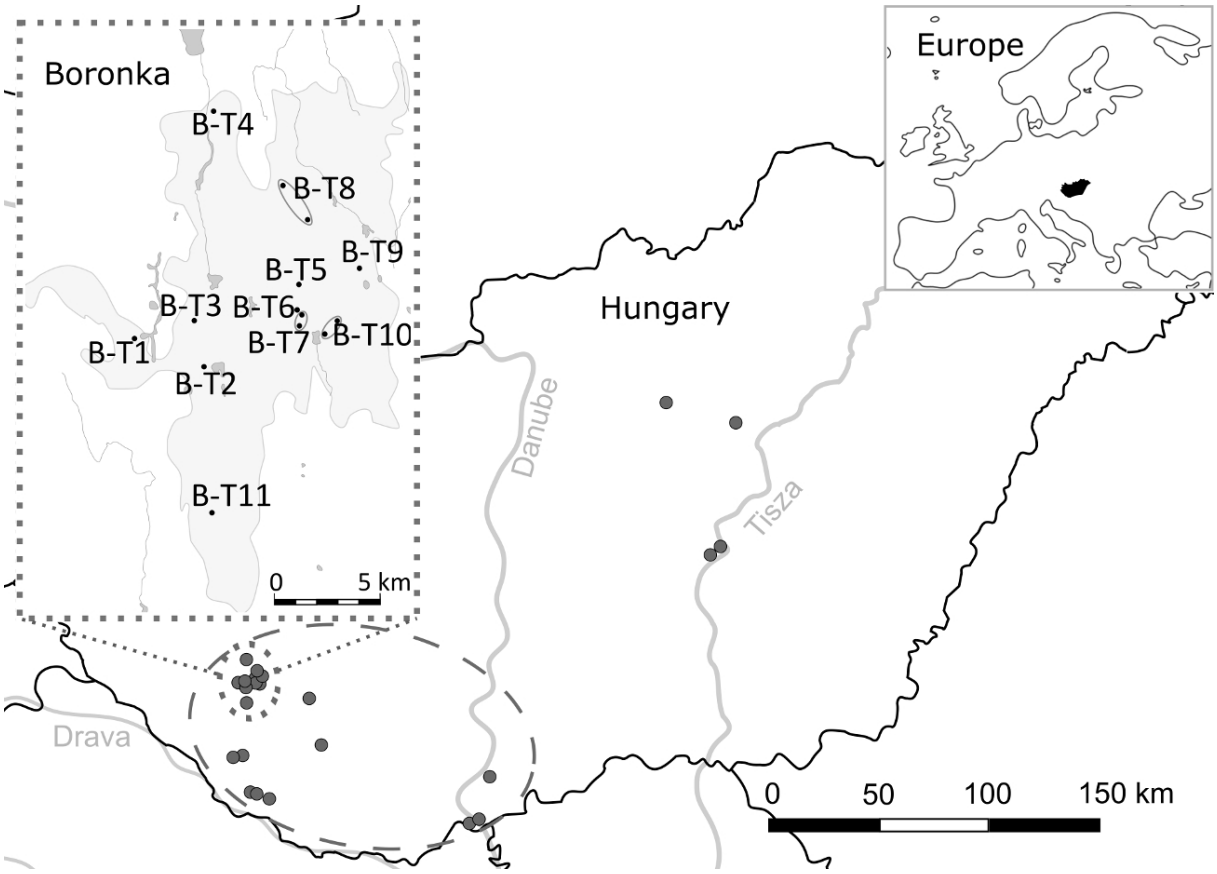

Figure III.1. Territories investigated in Hungary. Association of relatedness and mate choice was studied in south-western Hungary (demarcated by a ***dashed line***) and the Boronka forest (demarcated by a ***dotted line***). Nest site intrusions were investigated in the Boronka forest: position of the territories is shown enlarged (***Boronka***). Allele frequencies for both relatedness estimations and parentage analyses were calculated from consensus genotypes of resident WTEs from across Hungary.

Individual identification

When a greater number of feathers was collected at a nest site, we randomly chose 6 per year for genetic analyses. When colouration allowed (about 20% of the genotyped samples), age was estimated from each individual moulted feather (Figure I.3; but see also Forsman 1999 and Cieślak and Dul 2006). All feathers presumably belonging to juveniles and most of those belonging to adults were investigated, in addition to the randomly chosen feathers. DNA was extracted from the most suitable parts of each feather: the superior umbilicus (Horváth et al. 2005), or in small feathers the whole quill containing it. Molecular sexing was performed from each DNA sample (for details see appendix AIII. Methods). A DNA sample was chosen for further analyses if it was sufficient for molecular sexing, or otherwise in lack of more feathers available from the same nest site, if PCR products of microsatellite loci (see below) were visible on agarose gel.

Individual genotyping was performed using 13 nuclear microsatellite loci: Hal01, Hal03, Hal04, Hal09, Hal10, Hal13 (Hailer et al. 2005), Aa27, Aa35, Aa49 (Martínez-Cruz et al. 2002), IEAAAG04, IEAAAG05, IEAAAG12 and IEAAAG14 (Busch et al. 2005). The 5' end of each forward primer was labelled with a fluorescent dye (VIC™, FAM6™, PET ™, NED™, or HEX). For PCR profiles and conditions see appendix AIII. Methods. Fragment length analyses were performed on an ABI3130 sequencer (Applied Biosystems, using Gene Scan™ -500LIZ™ Size Standard). Each trace file was scored by two experts independently in Peak Scanner 1.0 (Applied Biosystems). Presence of genotyping errors, null alleles and linkage disequilibrium between pairs of microsatellite loci were checked as described in appendix AIII. Methods.

For moulted feathers with matching genotypes from the same nest site, consensus individual genotypes were prepared manually. In this process we allowed not more than 2, homozygote/heterozygote mismatches (that is, at the same locus, two alleles were amplified from one DNA sample, but only one of those was amplified from another DNA sample); accordingly, 13 out of 134 moulted feathers of multiply sampled individuals showed such mismatches with the assumed consensus genotypes, presumably due to allelic dropouts caused by poor DNA quality. Consensus genotypes were compared in Cervus 3.0.7. (Kalinowski et al. 2007), to reveal whether an individual was sampled at one or more nest sites. Probability of identity and probability of exclusion for combinations of increasing number of loci were calculated with Genalex 6.5. (Peakall and Smouse 2012).

Since WTEs are territorial, an individual was regarded as 'resident' when majority of the analysed feathers shed by same-sex individuals at a nest site belonged to it, and it was genotyped from at least three moulted feathers collected at the same nest site. This criterion is fairly conservative compared to previous studies on similar species (e.g. Rudnick et al. 2005). As extra-pair parentage is generally rare in raptor species (e.g. Mougeot 2004; Rudnick et al. 2005), we also considered an individual as 'resident' if its genotype matched with the genotypes of the nestling(s) hatched in the territory where it was sampled, even if the bird was identified from less than three feathers. In this case 'matching' meant that on each locus one of the two alleles of the putative parent is present in the genotype of the nestling. If both members of a putative resident pair were identified at two neighbouring nest sites in two succeeding years, we assumed that both nest sites belonged to the same territory. An individual was assumed as an 'intruder' at a nest site if another same-sex individual was known to be resident in the sampling year (either from moulted feathers or from matching nestling samples).

Relatedness and mate choice

Pairwise relatedness values were estimated via two methods: maximum likelihood relatedness (MLr) was calculated in ML-Relate (Kalinowski et al. 2006) and the relatedness estimator proposed by Lynch and Ritland (1999; LRr) was calculated in Spagedi 1.5. (Hardy and Vekemans 2002). As the Hungarian WTE population does not exhibit significant genetic structure (see Nemesházi et al. 2016 or SECTION II), allele frequencies for both estimations were calculated from genotypes of resident WTEs from across Hungary (N=48). Reliability of both estimators was checked in our data set with known parent-offspring (PO) pairs (N=67), where the expected value of pairwise relatedness is 0.5. Fisher's exact test was calculated in R 3.1.2. (R Core Team 2015; hereafter R) to compare the proportions of PO pairs with relatedness values between 0.4 and 0.6 according to MLr and LRr.

We used randomization tests in R to investigate whether mate choice is affected by relatedness. As our null hypothesis was random mating, we generated a null distribution of mean relatedness values calculated for randomly formed pairs of residents and potential mates. Lacking information on the actual pool of available mates at the time of mate choice for the resident WTEs, we used two approximations to create the set of potential mates from the males or females sampled close in space and time.

In the first dataset we concentrated on breeding pairs of the Boronka forest. Mean relatedness of these observed pairs was compared to null distributions of that of simulated pairs. We randomly chose mates for each resident male (N=11) and female (N=12) with known real-life mates. The set of available mates consisted of individuals sampled within the Boronka forest: residents, intruders and a single individual with uncertain role (in total 17 males and 21 females). In the second dataset, 16 south-western Hungarian resident pairs formed the observed set and mates were randomly chosen for both members of these pairs from the pool of opposite-sex resident individuals of the same region (17 males and 24 females; their median breeding distance was 27.5 km and the maximum was 125 km). For each dataset, randomizations were repeated 10 000 times.

To test the null hypothesis of random mating, we compared the observed mean relatedness of breeding pairs to the generated null distributions. P-values were calculated with the method proposed by Phipson and Smyth (2010) using the function 'permp' (conservative, two-sided version) in the R package 'statmod'.

Nest site intrusions

i) To test the 'pairing hypothesis', we calculated pairwise relatedness values between intruders and residents. We performed a randomization test in R, during which territories were randomly

chosen for each intruder to visit and pairwise relatedness values were considered between the intruder and the opposite-sex resident. For each intruder in this test, only those territories were available, where both residents were known in the year of revealed intrusion (8 out of 11 territories in each of the 3 sampling years). Randomizations were repeated 10 000 times and two-sided p-values (Phipson and Smyth 2010) were calculated for the difference of mean observed pairwise relatedness and the null distribution of means across random intruder-resident dyads.

Table III.1. Number of male and female intruders sampled in the Boronka forest between 2013 and 2015. Territory quality was measured as the number of years being occupied since 1987 (i.e. breeding attempt observed; ***'Occupied years'***). Total numbers of genotyped feathers are also shown. An intruder with unknown sex was found in B-T8 and a female with uncertain status (i.e. resident or intruder) was found in B-T4. These two individuals are not shown in the table.

Territory	Established	Occupied years	Genotyped feathers	Male intruders	Female intruders
B-T2	1987 [1]	29	12	-	1
B-T4	1987 [1]	29	20	4	3
B-T5	1991	25	8	-	-
B-T1	2003	13	14	1	2
B-T10	2004	12	6	-	1
B-T6	2006	10	14	-	-
B-T7	2008	8	10	-	1
B-T11	2008	8	6	-	-
B-T8	2012	4	12	-	-
B-T9	2012	3	2	-	-
B-T3	2013	3	11	-	2

[1] No information is available from before 1987.

ii) The 'acquisition hypothesis' suggests that intruders prefer to visit high quality territories. We presumed that during recolonization after a bottleneck in the 1970s (see also Horváth 2009 and Nemesházi et al. 2016), the best territories were occupied first and the less suitable ones became occupied later, as a consequence of increasing breeding density. We defined territory quality as the number of years being occupied since 1987 (similarly to Sergio and Newton 2003), based on data collected by the Somogy County Local Group of BirdLife Hungary and

the Danube-Drava National Park in the Boronka forest (Table III.1). To investigate whether territory quality affected the frequency of nest site intrusions, we calculated the ratio of the odds of intrusion in old territories to the odds of intrusion in new ones using Fisher's exact test in R. A territory was considered 'old', if it was occupied for more than 14.5 years (midpoint of the timescale of our dataset), and 'new' otherwise. We recorded whether intrusion was revealed or not across 27 'territory-years', that is, a given territory investigated in a given year was considered as a sampling unit. Note that this method assumes that intrusions in a territory across different years are as independent as those across different territories. We also investigated whether the odds of choosing an old territory differed between sexes based on 15 revealed intrusion events. We have calculated the ratio of the odds of choosing an old territory if the intruder was a male to the odds of that if the intruder was a female.

iii) To assess the 'homecoming hypothesis', we performed analyses of parentage, maternity and paternity in Cervus 3.0.7. (Kalinowski et al. 2007). These tests aimed to reveal whether an intruder is a likely offspring of any of the known resident pairs, males or females from the Boronka forest, respectively. Candidate parents were chosen from the known Hungarian resident WTEs, presuming that 50% of both the true mothers and fathers were sampled. Reliability of these analyses was verified on known parent-offspring pairs (N=67).

III.3. Results

Individual identification

DNA was extracted from overall 166 moulted feathers and 20 nestling samples from the Boronka forest, out of which 118 and 20 yielded sufficient DNA for further analyses, respectively. Furthermore we obtained DNA samples of additional 48 moulted feathers and 24 nestling samples previously collected in other Hungarian areas (for results on molecular sexing see appendix AIII. Results). Accordingly, microsatellite fragment analyses were performed for overall 166 moulted and 44 nestling DNA samples and all except for a single moulted feather were successfully genotyped for at least 8 loci. For a map showing the sampled territories see Figure III.1..

Genotyping errors overall did not significantly concern our dataset and no significant pairwise linkage disequilibrium was found after Bonferroni correction, but presence of null alleles in Hal10 could have affected our analyses (for details see appendix AIII. Results). Therefore, we excluded Hal10 from the further analyses and used a final dataset of 12 loci (data are available as electronic supplement ESM 3 of Nemesházi et al. 2017), consisting of altogether 56 alleles, with the number of alleles per loci ranging between 2 and 9.

This dataset proved adequate for individual identification and kinship estimations of WTEs. Based on residents from across Hungary (N=48, see below), probability of identity (P_{ID}) was estimated at 4.084×10^{-8} for 12 loci and 8.9×10^{-5} for 7 loci, suggesting that even the latter would be reliable considering a conservative threshold of $P_{ID} \leq 0.0001$ (Waits et al. 2001). For 12 loci, P_{ID} was about the magnitude of the threshold even with presence of siblings ($P_{ID} = 5.5\times10^{-4}$). Probability of exclusion values ranged between 0.9095 (when genotype of one parent is missing) and 0.9997 (when both parents are putative) for the 12 loci genotypes across the Hungarian breeding population.

Based on 165 moulted feathers, 63 individuals were identified (39 from the Boronka forest and 24 from other Hungarian areas) and each consensus genotype consisted of the complete set of 12 microsatellite loci. Altogether 48 (24 from Boronka, 24 other) individuals were considered as residents (identified from 1 to 10 moulted feathers at a given nest site) and 16 as intruders (each identified from only 1 moulted feather). All intruders were found in the Boronka forest, and each of them was identified at only one nest site as intruder. We were able to age a total of 17 residents (13 from the Boronka forest) and 8 intruders: all residents and 1 intruder were adults, while 7 intruders were juveniles. For more details on individual sampling in the Boronka forest see appendix Table AIII.3.

Out of 48 residents 41 were successful breeders as well (17 from the Boronka forest), as proven by genotypes matching with 44 nestlings. Out of 26 individuals identified from at least 3 moulted feathers, nestling samples from the same nest were available for 19, and all proved to be parents.

Among the intruders 10 were females, 5 were males and the sex of 1 individual remained unknown. When we tested hypotheses regarding sex, the latter intruder was excluded from the analyses.

Two females were known as residents and intruders as well. One of these was known as resident (B-T2) in the Boronka forest since 2013 and visited a neighbouring nest site (B-T10 in 6.6 km distance) in 2015, while both pairs were breeding successfully. The other female was first observed as an intruder in 2014 and appeared as a novel resident on the same nest site one year later, replacing the former resident female (B-T1). All these events were confirmed by matching nestling genotypes.

One individual's status remained unresolved: in one of the best territories in the Boronka forest (B-T4, known as occupied since 1987), the resident pair present in 2013 and 2014 disappeared from the nest site by 2015 and a new male seemed to occupy the territory (identified from 3 moulted feathers; the breeding failed). Feathers (N=2) from a juvenile female were also found at the nest site. This female's status was concerned as unknown because less than 3 of her

feathers were found; however, she might have been the mate of the new male as rare occurrence of breeding attempts by juveniles has been observed in the species (Helander and Stjernberg 2003).

Relatedness and mate choice

The maximum likelihood method (MLr) seemed to be more reliable in estimating real parent-offspring (PO) relationships than the Lynch-Ritland method (LRr). With MLr, 55 out of 67 real PO pairs got pairwise relatedness values between 0.4 and 0.6 (median=0.5, mean=0.52, SD=0.116). Contrarily, this ratio was 28/67 pairs with LRr (median=0.468, mean=0.479, SD=0.18) and reliability of the two estimators differed significantly according to Fisher's exact test ($p<0.001$). For comparative histograms across the two methods, see appendix Figure AIII.1. The results presented hereafter were obtained with MLr, and LRr results are shown only in appendix Table AIII.1.

In the Boronka forest, distributions of pairwise intersexual relatedness of resident females and resident/intruder males (i.e. the first dataset used to test random mate choice; Figure III.2a) was found as follows: majority of the individuals were estimated to be unrelated, but pairwise relatedness values up to ca. 0.6 occurred. Distribution of pairwise intersexual relatedness of resident females and resident males across south-western Hungary was similar (i.e. the second dataset to test random mate choice; appendix Figure AIII.2b).

Pairwise MLr values for observed resident pairs in the Boronka forest (N=12) ranged between 0 and 0.081 (mean=0.013, median=0, sd=0.03; Figure III.2b). The observed mean relatedness was significantly lower (Figure III.2c) than expected by random mating ($p= 0.01$ or 0.021, when choosing random mates for females or males, respectively). Randomization tests on all residents from across south-western Hungary gave similar results (appendix Table AIII.1).

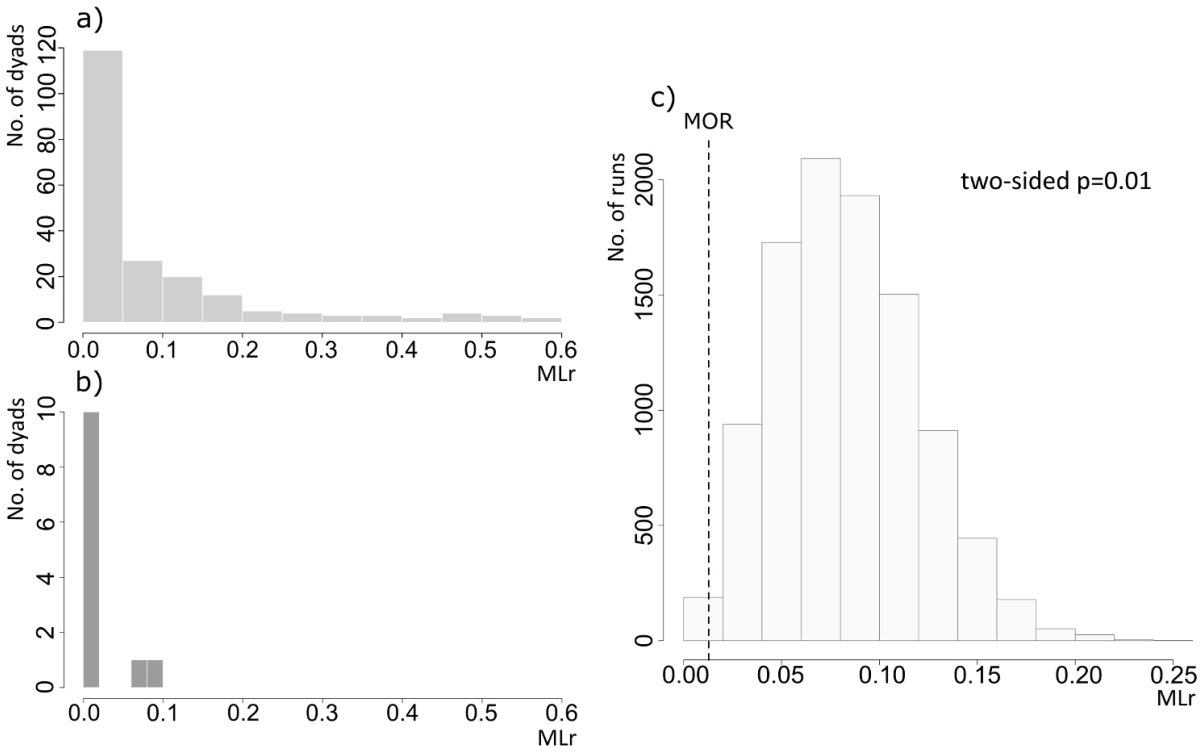

Figure III.2. Histograms of pairwise genetic relatedness of resident females and their observed or potential mates in the Boronka forest. a Intersexual pairwise maximum likelihood relatedness (***MLr***) values between resident females (N=12) and all males (residents and intruders; N=17). That is, all possible dyads considered for resident females under random mating. **b** Observed pairwise MLr of breeding pairs (N=12). **c** Null distribution of mean pairwise relatedness assuming that resident females (N=12) randomly choose a mate from males (N=17) sampled in the area between 2013 and 2015. Permutations were repeated 10000 times. The dashed line refers to the mean observed relatedness (***MOR***) of breeding pairs (N=12).

Nest site intrusions

i) 'Pairing hypothesis'. Relatedness (MLr) of observed dyads of intruders (males and females) and opposite-sex residents of the visited territories ranged between 0 and 0.161. Median observed pairwise relatedness for female intruders (N=9) and resident males of the visited territories was 0 (mean=0.037, SD=0.055). For observed dyads of male intruders (N=4) and resident females, median relatedness was 0 (mean=0.005, SD=0.01) as well. In cases of both female and male intruders, mean observed MLr was somewhat smaller than expected from the simulated null distributions, but this deviation was not significant (p= 0.189 for females and p=0.224 for males). The observed mean relatedness of intruders to opposite-sex residents was clearly smaller than the most frequent values in the null distribution for females (Figure

III.3), but this observed value for the male intruders was similar to their most frequent random values (appendix Figure AIII.3 and Table AIII.1).

ii) 'Acquisition hypothesis'. Female intruders (N=10) were found in 6 out of 11 territories of the Boronka forest, including the oldest and the most recently established territories as well (Table III.1). Contrarily, 4 out of overall 5 male intruders were found at the nest site of one of the two oldest (and therefore, presumably most suitable) territories and one was sampled in a later established, but also relatively old territory (Table III.1). Across a total of 27 investigated 'territory-years', intrusion was observed in 7 new and 4 old, and no intrusion was observed in 11 new and 5 old territories. Fisher's exact test showed that the odds of being chosen for intrusion is 1.246 times larger for old territories, but this difference was not significant ($p>0.999$, confidence interval (CI): 0.179 - 8.332). Among the 15 observed intrusion events we found 4 males and 4 females on old territories, while 1 male and 6 females were found on new ones. The odds of choosing an old territory was 5.305 times greater for male than for female intruders, however not significant ($p=0.282$, CI: 0.346 - 342.747).

iii) 'Homecoming hypothesis'. Both probabilities of exclusion (see above) and parentage analyses on known PO pairs (appendix Table AIII.2) confirmed reliability of our dataset to test the 'homecoming hypothesis'. None of the 15 intruders were offspring of the resident pairs sampled in the Boronka forest, as revealed by the parentage analysis in Cervus (appendix Table AIII.2). Since breeding WTEs could have been replaced after the intruders hatched, separate maternity and paternity analyses were performed as well. The most likely candidate mother or father with matching genotype was a resident from the Boronka forest for overall 5 intruders (3 males, 2 females). Only 2 of these candidate parent-offspring pairs had significant delta scores at a 95% confidence level, each of these 2 dyads contained a female intruder and a candidate father. Note that the results of the paternity analyses should be treated with caution because the majority of the sampled resident males (12 out of 20) belonged to the Boronka forest. Two additional dyads had significant delta scores: the most likely candidate mothers of 2 females were residents from south-western Hungary. Furthermore, 1 female intruder carried the allele 198 at locus IEAAAG04, which was found to be exclusive for the northern European WTE population by Nemesházi et al. (2016; see appendix Table AII.10).

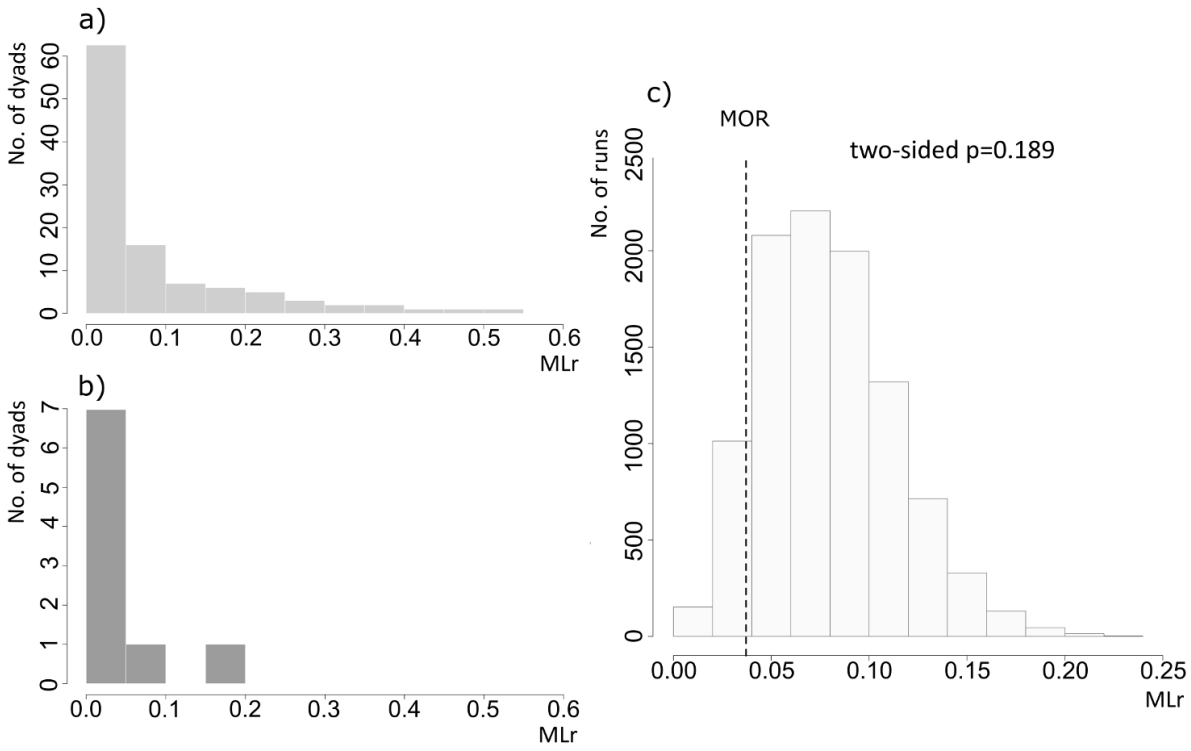

Figure III.3. Histograms of pairwise genetic relatedness of female intruders and resident males in the Boronka forest. a Pairwise maximum likelihood relatedness (***MLr***) values of each possible dyads of intruder females (N=9) and resident males (N=12). **b** Observed pairwise MLr of intruder female and resident male dyad in nest site intrusions. **c** Null distribution of mean pairwise MLr of intersexual intruder-resident dyads assuming that females randomly choose a territory to visit. Permutations were repeated 10000 times and in each run a single territory was randomly chosen for each intruder from available Boronka territories (8 in each year, where the resident pair was known in the year of its intrusion). The dashed line refers to the mean observed pairwise MLr (***MOR***) of dyads of intruder females and resident males.

III.4. Discussion

In the present study, we investigated (1) whether relatedness is considered when choosing a mate in the white-tailed eagle population of south-western Hungary. (2) We furthermore tested three non-exclusive hypotheses on the background of conspecific nest site intrusions: the (i) 'pairing', the (ii) 'acquisition' (iii) and the 'homecoming' hypotheses.

Our knowledge on mate choice and the background of conspecific territorial intrusion in large raptors is scarce. Investigation of such species is challenging and individual identification is mostly not applicable using capture-mark-resight methods. We identified resident and intruder WTEs using moulted feathers at occupied nest sites. This non-invasive DNA sampling method

is known as an appropriate alternative for conventional methods in territorial raptors (Rudnick et al. 2009; Vili et al. 2013a). The number of feathers found at a nest site may be influenced by several factors, such as the density of the underbrush, or the age (Forsman 1999) and condition of individuals present in the area. Individuals engaging in territorial talon fights may lose more feathers, but such interactions are relatively rare (Krone et al. 2013). We overall assumed that the number of feathers shed at a nest site by a particular individual should roughly refer to the time it spends in the area. As members of the breeding pairs spend considerable time at their nest site during the breeding season, the majority of moulted feathers should belong to them. We defined an individual as resident, if it satisfied two criteria: it was identified from the highest proportion of feathers shed by same-sex individuals in the same territory and it was identified from at least three moulted feathers. We assume that these criteria were reliable in our dataset: all those individuals which met these criteria and were compared with nestling genotypes from the same territory proved to be the parents.

Although the existence and mechanism of kin recognition in WTEs is unknown, our results suggest that WTEs can avoid kin when choosing a breeding partner: mean pairwise relatedness within actual breeding pairs was significantly lower than expected under random mating. Birds can avoid inbreeding by dispersal, extra-pair fertilization, sequential divorce, or kin avoidance in mate choice (Pusey and Wolf 1996; Wheelwright and Mauck 1998; Foerster et al. 2006; Oh and Badyaev 2006; Kawano et al. 2009). Abilities of kin recognition has been shown in some birds (Sharp et al. 2005; Bonadonna and Sanz-Aguilar 2012). Although kin avoidance in mating has rarely been demonstrated in natural bird populations so far (Wheelwright and Mauck 1998; Oh and Badyaev 2006), such a strategy may be especially crucial in raptor species which show long-term social monogamy and have few or no extra-pair offspring (Mougeot 2004; Rudnick et al. 2005). Sex-biased or long-distance natal dispersal might decrease inbreeding in WTEs (Struwe-Juhl and Grünkorn 2007; Whitfield et al. 2009a; Nemesházi et al. 2016). One of the two datasets used in our study to calculate null distributions of pairwise relatedness under random mating consisted of resident WTEs only, breeding within 125 km from each other, with a median distance of 27.5 km. We assumed that analysis of breeding individuals as potential mates in this spatial scale is relevant as WTEs explore even more distant areas before settling down (Nygård et al. 2003; Horváth 2009; Whitfield et al. 2009b), mean natal dispersal distance is around or over 100 km in several European WTE populations (Helander 2003; Struwe-Juhl and Grünkorn 2007), and even resident individuals move a few hundred kilometres away from their territories sometimes (Krone et al. 2013). All individuals in this dataset were sampled after natal dispersion, thus natal dispersal similarly affected the relatedness of randomly chosen pairs than that of actual breeding pairs. Still, the observed mean relatedness of actual breeding pairs was smaller than expected under random

mating. Therefore, our results suggest that besides sex-biased or long-distance natal dispersal, other**,** more direct mechanisms can also play a role in inbreeding avoidance of WTEs.

To our knowledge, the importance of relatedness in large raptors' mate choice was investigated in a single study beside ours. Rudnick et al. (2005) did not find evidence for a relatedness-based mate choice system in a wild population of eastern imperial eagles (*Aquila heliaca*). Discrepancies between our results and those of Rudnick et al. (2005) can be due to interspecific differences or different methodology; specifically, they used the Queller-Goodnight relatedness estimator. This estimator can exhibit reduced reliability for populations consisting of generally non-related individuals (Van De Casteele et al. 2001), and accordingly we found it inadequate in our study population (results not shown). In such populations, the Lynch-Ritland method (Lynch and Ritland 1999) performs better than other frequently used estimators (including the Queller-Goodnight). According to pairwise relatedness estimations for actual parent-offspring pairs, the maximum likelihood method (Kalinowski et al. 2006) performed even better in our dataset than the Lynch-Ritland method, similarly to the findings of Milligan (2003).

We investigated whether WTE nest site intrusions around the breeding season could be explained by (i) a relatedness-based pairing strategy, (ii) attempt to acquire high-quality territories, or (iii) homecoming of juveniles. Altogether 16 nest site intruders (10 females, 5 males and one individual with unknown sex) were detected across the Boronka forest between 2013 and 2015. Out of 8 intruders with known age, 7 were juveniles in the year of intrusion. Therefore, a significant proportion of the nest site intruders were presumably floaters with no territory and no mate. Observations suggest that in several raptor species, nest site intrusions of juveniles are more tolerated by residents than the intrusions of adult conspecifics (Mougeot et al. 2006; Turrin and Watts 2014).

Intersexual relatedness of resident-intruder dyads overall did not confirm the 'pairing-hypothesis', although both male and female intruders visited the nest sites of non-related opposite-sex residents. Possibly due to the larger sample size, a tendency for visiting less related resident males seemed somewhat stronger for female intruders. This pattern of intruders is similar to our results on within-pair relatedness of resident pairs and is consistent with the observations in several raptor species that suggest that territorial intruders could gain opportunities to reproduce (via replacement of a resident or even via extra-pair copulations; Garcia and Arroyo 2002; Rutz 2005; Mougeot et al. 2006). Observations also showed that juvenile WTEs sometimes attempt breeding (Helander and Stjernberg 2003).

In the Boronka forest, old territories were overall not preferred by intruders when sex was ignored. However, the odds of choosing an old territory over a new one was more than 5 times greater for males than for females. This effect was statistically non-significant, probably due to the small number of observations. Nevertheless, all male intruders visited relatively old territories, which is consistent with the 'acquisition hypothesis'. In the Boronka forest, neighbouring WTE pairs nest on average 2.75 km from each other (ranging between 0.21 and 6.85; unpublished data); this is an outstanding breeding density compared to most WTE populations in Europe (e.g. Radović and Mikuska 2009; Whitfield et al. 2009a). Previous studies on several raptor species showed that the frequency of territorial intrusions increases with breeding density (Ferrer et al. 2015) and recruiting individuals prefer to occupy territories in habitats of high breeding density (Hernández-Matías et al. 2010). Good-quality territories of such habitats can attract the floaters and recruiting individuals (Hernández-Matías et al. 2010; Ferrer et al. 2015), and even delaying the first year of breeding can be an adaptive strategy in prospect of a better quality territory (van de Pol et al. 2007).

Juveniles of large raptors can repeatedly return to their natal population (Ferrer 1993; Nygård et al. 2003). Yet, our results did not confirm that the nest site intruders originated from the Boronka forest as suggested by the 'homecoming hypothesis'. Parentage analyses revealed that none of the intruders were likely to be offspring of the recent breeding pairs of the Boronka forest. Although the majority of the intruders presumably hatched some time before our investigation started, it is unlikely that members of most of the breeding pairs would have been replaced over a few (presumably 1-4) years (Helander 2003; Helander and Stjernberg 2003). Instead of evidence for intruder returns to the natal population, we found one intruder which likely hatched in the northern European WTE population as it carried a characteristic allele (198 on locus IEAAAG04; Nemesházi et al. 2016, see appendix Table AII.10).

Since we could investigate only a small number of intruders, intrusion patterns found in our study cannot be considered representative for all WTEs. However, three recorded events were especially interesting: 1) one female intruder that became resident next year, 2) replacement of the resident male in a good quality territory frequently visited by intruders and 3) a resident female with successful brood visited the nest site of another successfully breeding pair.

A female visited a nest site in 2014 while the resident pair was successfully raising a nestling (with matching genotype) and by the next year this female became the mate of the resident male (also proven by matching nestling genotype), while the former resident female disappeared. This observation seems to confirm that nest site intruders can indeed gain opportunities to replace the same-sex member of a resident pair, as suggested by the 'pairing hypothesis'.

Another observation is consistent with the ‘acquisition hypothesis’: a new male seemed to acquire a territory (B-T4) in 2015, while the formerly resident pair disappeared from the nest site. Although this male was not recorded previously as an intruder, it is interesting that this was the most preferred territory by male nest site intruders during the study period and also it is one of the oldest and presumably most suitable territories in the Boronka forest. The most important difference between the two oldest territories (B-T4 and B-T2) may be their location in the forest (Figure III.1.). While B-T2 has a central position and its nest site is surrounded by closed forest and a lake in the vicinity, B-T4 is peripheral and is close to a route frequently used by floaters between the largest foraging lake in the area and the woods where they can spend the night. The relatively high number of nest site intruders found at B-T4 therefore may be a consequence of its position in addition to (or instead of) its quality. Nevertheless we do not know about any sex-biased foraging or roosting behaviour among floaters which could explain the relatively high proportion of males among these intruders compared to other territories.

The only known adult intruder was a female raising a nestling in 6.6 km from the visited nest, where the resident pair was breeding successfully as well. A similar behaviour was observed by Meyburg et al. (2007) in a lesser spotted eagle (*Clanga pomarina*) population, where a female rearing nestlings visited two different nest sites about 50 km from its own nest. These events are surprising given that these species are territorial and nestlings are exposed to predation risk while their parents are not around. For the time being, the reason for such behaviour remains unknown.

Most territorial bird species maintain a so called resource defence system (Greenwood 1980) where males compete for territories and mating occurs upon female choice. Nest-site intrusion patterns of WTEs found in the present study could be a consequence of such sex-biased strategies. Choosing an appropriate mate and territory has strong effect on reproductive success in all territorial bird species. As WTEs are monogamous and faithful to their territories, these decisions should be of outstanding importance for the individuals. Our results suggest that WTEs avoid inbreeding when choosing a mate and our observations on intrusion events, albeit few, are consistent with the ideas that female floaters might search for an appropriate mate while males search for a good-quality territory.

IV. Genetic structure confirms female-biased natal dispersal in the white-tailed eagle population of the Carpathian Basin

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IV.1. Introduction

Estimation of natal dispersal distance (NDD; i.e. distance between the natal and first breeding place of an individual: Greenwood and Harvey 1982) can provide important information related to evolution, ecology and conservation of a species. Long-distance natal dispersal for example can be a successful strategy to avoid inbreeding or competition with kin or more experienced adults for resources and mates (Greenwood and Harvey 1982; Perrin and Mazalov 2000). On the other hand, a strategy of short NDD (also referred to as philopatry) can be beneficial as well: moving within a restricted, well-known area, the individual can re-establish a territory just becoming vacant (Smith 1978); and the costs derived from crossing less suitable or even dangerous areas during dispersal may decrease as well (Yoder et al. 2004). Philopatry was found in several species with high migration capability, such as many birds and mammals (Greenwood and Harvey 1982; Pusey 1987; Alcaide et al. 2009; Whitfield et al. 2009a). Average NDD can differ between sexes and if so, it is usually male-biased in mammals and female-biased in birds (Greenwood and Harvey 1982; Pusey 1987). Sex-biased natal dispersal strategies may predominantly be consequences of intrasexual competition for territories, food resources or mates, and may serve inbreeding avoidance as well (Greenwood and Harvey 1982; Perrin and Mazalov 2000).

The white-tailed eagle (*Haliaeetus albicilla*; WTE) is a large raptor species distributed across the Palearctic and Greenland (BirdLife International 2015). While adult WTEs are in most populations sedentary, juveniles are vagrant and can move several hundred kilometres away from their natal area (Helander and Stjernberg 2003; Whitfield et al. 2009b; Nygård et al. 2010). Still, similarly to other large raptor species, WTEs are philopatric, as supported by both ringing data (Helander 2003; Helander and Stjernberg 2003; Struwe-Juhl and Grünkorn 2007; Whitfield et al. 2009a) and genetic structure of their wild populations (Hailer et al. 2007; Honnen et al. 2010; Nemesházi et al. 2016). However, NDD values published from different WTE

populations differ considerably, with mean values ranging from about 30 to 114 km (Helander 2003; Struwe-Juhl and Grünkorn 2007; Whitfield et al. 2009a). Whitfield et al. (2009a) reported that females dispersed on average two times further than males in western Scotland.

Investigation of large raptor species is challenging; capture of adults on the field is mostly not possible. Therefore, instead of conventional methods, genetic data recovered from non-invasive samples are increasingly used to investigate several topics of individual-based studies, such as annual turnover rate of breeding individuals, parentage, breeding dispersal, or territorial intrusions (Rudnick et al. 2005; Rudnick et al. 2009; Booms et al. 2011; Vili et al. 2013b; Nemesházi et al. 2017). Straightforward methods for investigation of natal dispersal require knowledge on both the hatching and first breeding place of the individuals sampled. Such investigations in large raptors require long-term investments, as several years pass between the hatching and recruitment of individuals (Helander and Stjernberg 2003; González et al. 2006). However, spatial genetic structure of breeders can reflect the extent of philopatry as well, as shown in wild populations of several bird species (Double et al. 2005; Temple et al. 2006; Ponnikas et al. 2013). As observations suggest that WTEs are long-term faithful to their territories (Helander 2003; Helander and Stjernberg 2003; Krone et al. 2013), we assume that breeding dispersal is negligible in this species. Therefore, the scale of their natal dispersal can be inferred indirectly from the genetic relatedness of breeding individuals, even if their hatching places are unknown.

We concentrated on two questions: 1) Is there a fine scale genetic structure within the WTE population of the Carpathian Basin as suggested by the philopatric behaviour of the species? 2) Is there a difference between sexes in pairwise breeding distance of close relatives? We assumed that if there is a sex bias in pairwise breeding distance of close relatives, that stands for a sex bias in NDD as well. Both questions were investigated using genotypes of non-invasively sampled breeding individuals across the Carpathian Basin.

IV.2. Materials and methods

We used DNA samples collected between 2010 and 2016 by fellow workers of assigned national parks in the Carpathian Basin WTE population (Figure IV.1). Moulted feathers were collected during the breeding season within approximately 100 m distance from nests where breeding attempts were observed. In most regions, moulted feathers were collected and stored as suggested by Vili et al. (2013a). Resident individuals of each territory were identified based on matching nestling genotypes or the largest number of matching moulted feathers (but at least three) shed by same-sex individuals at the same nest site (see also Nemesházi et al. 2017 or SECTION I and SECTION III).

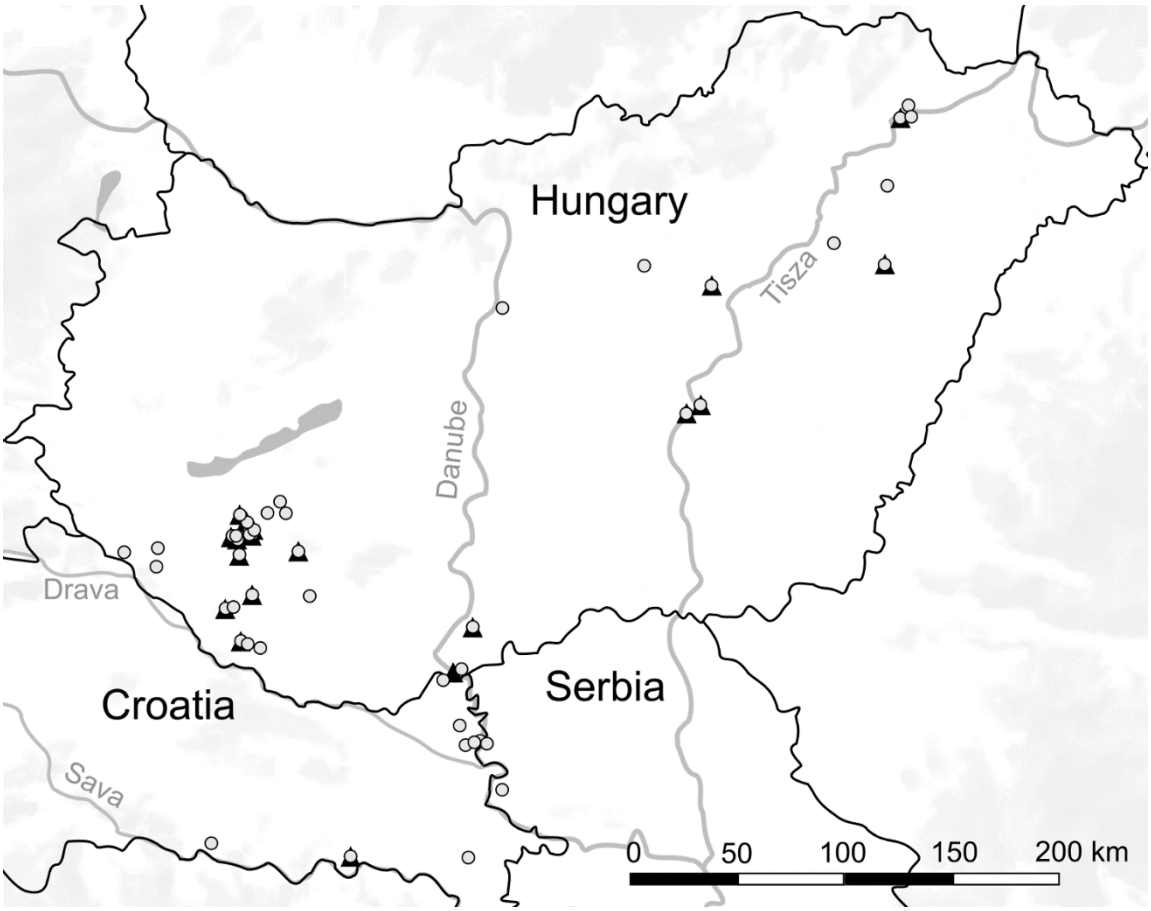

Figure IV.1. Nesting locations of breeding WTEs sampled across the Carpathian Basin. Black triangles indicate males and grey circles indicate females.

DNA was extracted from the superior umbilicus in large feathers (Horváth et al. 2005), and the whole quill below the vane in small ones. DNA purification kits (Quiagen – DNEasy Blood and Tissue Kit or Thermo - GeneJet genomic purification kit) were used following the manufacturers' instructions, with 10 µl of 1M dithiotreitol added during the digestion step.

Sex was identified from feather samples using the 2550F/2718R (Fridolfsson and Ellegren 1999) or the GEfUp/GErUp and GEfLow/GErLow primer pairs (Ogden et al. 2015), with PCR profiles following the original papers, but an an initial touch-down section was inserted in the latter (annealing temperature decreased from 65 to 60°C during 7 cycles). PCR products were visualized under UV light (2% agarose gel stained with ECO Safe; Pacific Image Electronics Co., Ltd.).

Each DNA sample was genotyped using 12 microsatellite loci: Hal01, Hal03, Hal04, Hal09, Hal13 (Hailer et al. 2005), Aa27, Aa35, Aa49 (Martínez-Cruz et al. 2002), IEAAAG04, IEAAAG05, IEAAAG12 and IEAAAG14 (Busch et al. 2005). PCR profiles followed the original papers for Aa and Hal loci, and Hailer et al. (2006) for IEAAAG loci, with some modifications (Nemesházi et al. 2016, or see SECTION II). Forward primers were 5' labelled with fluorescent

dyes (VIC™, FAM6™, PET™, NED™, or HEX) and fragment length analyses were performed on an ABI3130 Genetic Analyzer (Applied Biosystems, using Gene Scan™ -500LIZ™ Size Standard). Trace files were scored in Peak Scanner 1.0 (Applied Biosystems). We used Micro-Checker 2.2.3. (Van Oosterhout et al. 2004) to test for presence of null alleles and scoring errors due to large allele dropout or stutter bands. Genepop 4.2. (Rousset 2008) was used to estimate pairwise linkage disequilibrium across loci. Consensus individual genotypes were prepared manually, based on genotypes of feathers collected at the same nest site (as described in Nemesházi et al. (2017). Probability of identity for combinations of increasing number of loci was calculated with Genalex 6.5 (Peakall and Smouse 2012).

Fine scale genetic structure within the study population was investigated by testing spatial genetic autocorrelation (Smouse and Peakall 1999) based on individual genotypes of resident WTEs. Spatial genetic autocorrelation was investigated by two methods: using pairwise genetic distance calculated for co-dominant loci in Genalex 6.5 (Peakall and Smouse 2012), and kinship coefficient described by Loiselle et al. (1995) in Spagedi 1.5a (Hardy and Vekemans 2002). In these analyses, pairwise Euclidan distances between nest sites of sampled WTEs were used to test whether individual genotypes show random distribution in space; dyads of individuals were grouped into predefined spatial distance classes. Genalex calculated a genetic autocorrelation coefficient (*r*) for each distance class; the statistical significance was tested based on random permutation of individuals across distance classes (no. permutations=9999) and bootstrap estimates of *r* (no. bootstraps=10000). In Spagedi, the association between genetic relationship and spatial distances was assessed by averaging the pairwise genetic relationship for each distance class, and statistical significance for spatial genetic structure was tested by 10000 permutations of locations and gene copies. Both in Genalex and Spagedi, we performed calculations on three datasets: (i) all breeding individuals, (ii) females and (iii) males. Distance classes were definied aiming for each class to contain reasonably large number of pairwise data (minimum value was 380 for all breeding individuals, 117 for females and 37 for males). As spatial distribution of male-male dyads was aggregated (forming 4 groups: Figure IV.2a), upper border of each distance class was adjusted according to these aggregations, but the first group was divided in two distance classes. We performed this latter subdivision because two of the three datasets (i, iii) contained significantly more dyads between the borders of the first aggregation than between the others'. Accordingly, 5 subsequent distance classes were analysed for spatial autocorrelation, with upper borders of 20, 80, 180, 280 and 400+ km, respectively. Individuals breeding between 0 and 20 km from each other were grouped in the 20 km class, those breeding for more than 20 km up to 80 km to the 80 km class, and so on. The last distance class (referred as 400+) differred among

datasets, as the longest distance between two sampled male was 420 km, while it was 463 km between two females as well as between two sampled breeding individuals overall.

To test for a difference between sexes in pairwise breeding distance of close relatives, we calculated maximum likelihood pairwise relatedness in ML-Relate (Kalinowski et al. 2006) based on allele frequences across all sampled individuals. We previously found this a reliable estimate of real parent-offspring relationships in the Hungarian WTE population (Nemesházi et al. 2017). The expected value of relatedness for close relatives (i.e. siblings or parent and offspring) is 0.5. We assumed individuals to be closely related if their estimated pairwise relatedness was above 0.4, since these estimated values scatter around the expected values of relatedness: using the same 12 loci dataset, across 67 real parent-offspring dyads from Hungary, 85% got a pairwise relatedness value of at least 0.5, and 91% got a value greater than 0.4. In contrast, from 2516 dyads of presumably not related WTEs, 96% got values below 0.4 (calculated from the results of Nemesházi et al. 2017; see also appendix Figure AIII.1) Note that the value 0.4 is closer to 0.5, than to the expected value of the next level of relatedness (0.25 for half-siblings, grandparent and grandchild, etc.). One-tailed Wilcoxon rank sum test was calculated in R 3.1.2. (R Core Team 2015) to assess whether the distribution of geographic breeding distances of close relative females was shifted towards greater values than that of close relative males; using the function 'wilcox.exact' in the R package 'exactRankTests'. We used one-tailed test, because where NDD differs among sexes in birds, usually females disperse further (Greenwood and Harvey 1982), and female bias was found in another WTE population as well (Whitfield et al. 2009a). To test for difference in variances across the two sampled groups, we performed Levene's test for homogeneity of variances in geographic distances across closely related males and closely related females; using the function 'leveneTest' in the R package 'car'.

IV.3. Results

We used DNA samples extracted from a total of 214 moulted feathers belonging to 73 resident WTEs across the Carpathian Basin (24 males and 49 females; Figure IV.1). Each individual consensus genotype consisted of the full set of 12 loci.

A total of 59 alleles were found; the mean number of alleles across the 12 loci was 4.9, ranging from 2 on Hal03 to 10 on IEAAAG05. Analyses in Micro-Checker suggest that our dataset is not influenced by null alles, large allele dropout or stutter bands. No evidence for pairwise linkage disequilibrium was found across the loci after Bonferroni correction. Probability of identity was estimated at 3.3×10^{-8} for 12 loci genotypes, and that for siblings was estimated at

5.2×10^{-4}, suggesting that our dataset is reliable for individual-based genetic analyses (i.e. being below or about the magnitude of the threshold of 0.0001, following Waits et al. (2001).

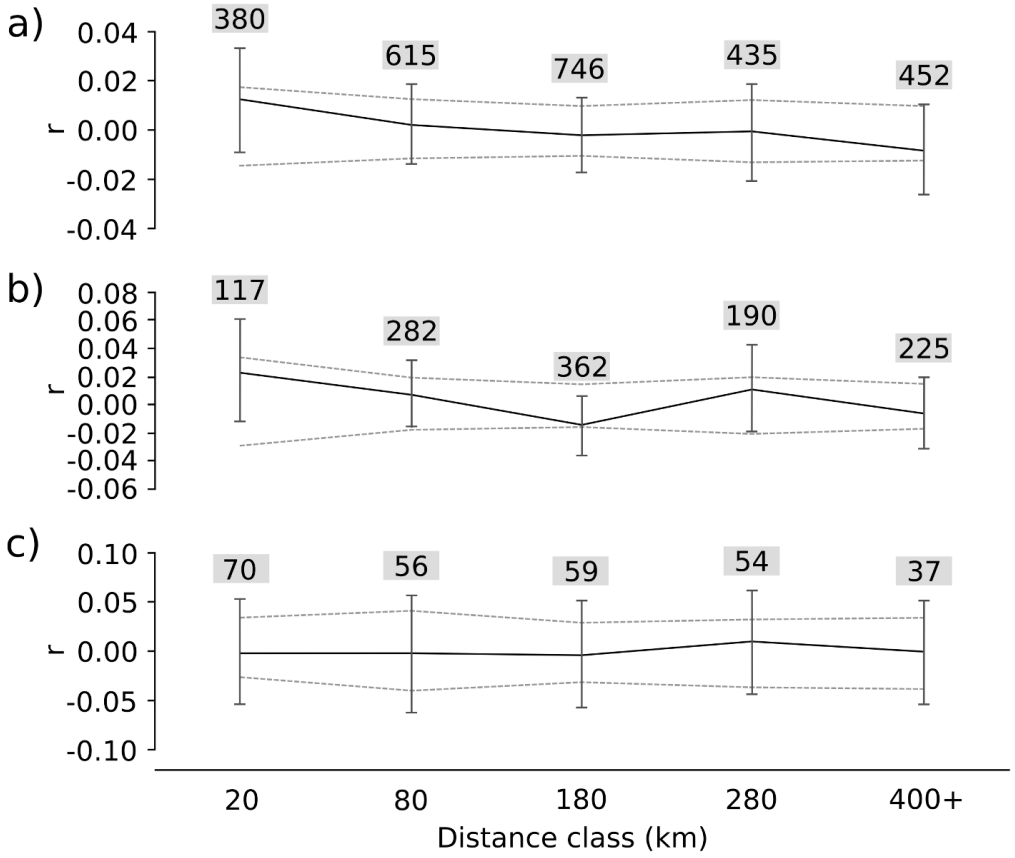

Figure IV.3. Correlogram plots of the degree of genetic correlation coefficient (*r;* calculated in Genalex) as a function of breeding distance. **a** Plot for dyads of all sampled breeders. **b** Plot for dyads of females. **c** Plot for dyads of males. ***Dashed lines*** indicate the permuted 95% confidence intervals. Bootstrapped 95% confidence ***error bars*** are also shown. ***Numbers with grey background*** indicate the numbers of pairwise comparisons (i.e. dyads) within each distance class. Note that the vertical axis scales differ among plots.

In Spagedi, spatial autocorrelation analysis of all breeding individuals (N=73) showed a moderate, but significant negative relationship between average pairwise genetic similarity within each distance class and its logaritmic spatial distance (slope *ln(dist)* = -0.0027, *p*=0.044). However, no such relationship was found based on linear spatial distances, and the observed mean kinship values of each distance class did not significantly differ from that expected under random spatial distribution. Genalex also failed to find a relationship between the genetic autocorrelation coefficient *r* and spatial distance, and no significant deviation of *r* from zero was observed in any of the distance classes (Figure IV.3a). When analysing males

(N=24) and females (N=49) separately, both methods failed to find any spatial genetic structure and even the slope for logaritmic spatial distances was nonsignificant for each sex (correlogram plots of the Genalex results are shown in Figure IV.3).

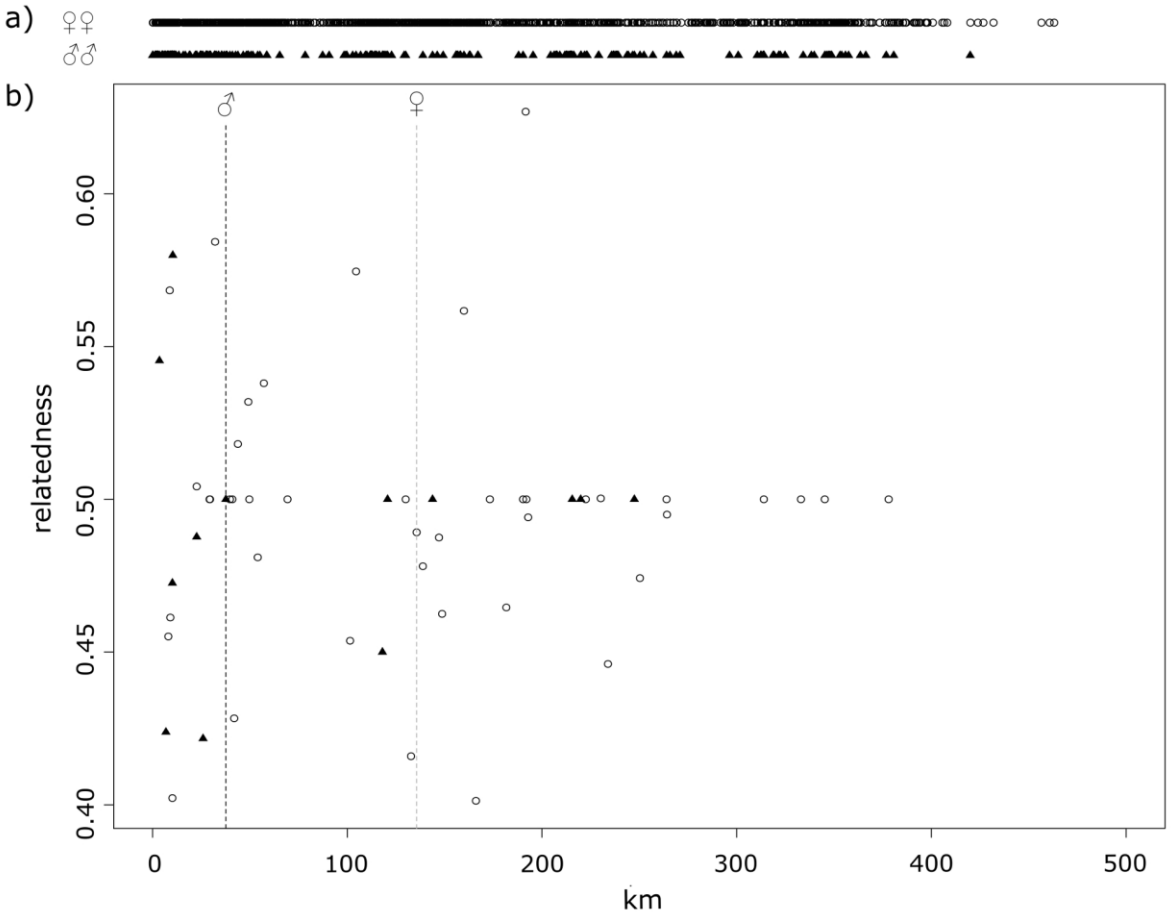

Figure IV.2. Pairwise breeding distance of WTEs. a Spatial distributions of all investigated same-sex dyads (spatial scale is shown in b). **b** Pairwise spatial distances (km) of individuals with a pairwise maximum likelihood relatedness (relatedness) of minimum 0.4. Male-male (***triangles***) and female-female (***circles***) dyads are shown separately. Vertical dashed lines indicate the median pairwise breeding distance of related males (***black***) and that of related females (***grey***).

Overall 43 female-female and 13 male-male dyads were assumed to consist of closely related individuals, based on their pairwise maximum likelihood relatedness being at least 0.4 (Figure IV.2b; note that these dyads were formed by overall 38 female and 18 male individuals). The maximum breeding distance was 378 km among related females and 247 km among related males; median values of breeding distance were 136 and 38 km, respectively. The Wilcoxon rank sum test revealed that distribution of pairwise geographic distances was significantly

shifted towards greater values for close relative females than for close relative males (p=0.038, *CI*: 2.609-Inf, W=371). Levene's test found no significant difference in the variance of geographic distances across related males and related females (p>0.6).

IV.4. Discussion

Mean natal dispersal distance (NDD) values reported in European white-tailed eagle (WTE) populations differ considerably, being below 60 km in the reintroduced population of western Scotland (Whitfield et al. 2009a) and exceeding 100 km in Sweden (Helander 2003). Observations in some WTE populations suggest that NDD can be female biased (Helander 2003; Whitfield et al. 2009a). Lacking sufficient information published from mark-resighting in the Carpathian Basin WTE population, we aimed to infer the spatial scale of natal philopatry based on microsatellite genotypes of breeding individuals. We had two objectives: 1) we investigated fine scale genetic structure within the study population by testing spatial genetic autocorrelation using two different methods, and 2) we tested whether close relative females can be found at larger distances than close relative males using Wilcoxon rank sum test.

First, spatial autocorrelation analyses were performed. This method is known to be effective in highlighting genetic spatial patterns expected in a philopatric population, or under sex-biased dispersal, the philopatric sex (Double et al. 2005; Temple et al. 2006; Banks and Peakall 2012; Ponnikas et al. 2013); that is relatively high genetic similarity among neighbouring individuals, or across the first few distance classes.

Although analyses in Spagedi revealed that the slope of the relationship of genetic similarity and logarithmic spatial distance (slope *ln(dist)*) was significantly negative for dyads of WTEs breeding in the Carpathian Basin, both Spagedi and Genalex failed to find significant deviation from a random spatial distribution of pairwise genetic relatedness values. When we analysed females and males separately, similar lack of fine-scale genetic structuring was found, with even slope *ln(dist)* being nonsignificant. Nevertheless, the species is philopatric, as shown by both mark-resight data (Helander 2003; Helander and Stjernberg 2003; Struwe-Juhl and Grünkorn 2007; Whitfield et al. 2009a) and presence of genetic structure at wide geographical range inferred from microsatellite data (Hailer et al. 2007; Honnen et al. 2010; Nemesházi et al. 2016). Moreover, the Carpathian Basin population maintains a local, unique mitochondrial haplotype as well (Honnen et al. 2010; Nemesházi et al. 2016). Similar contradictions of fine-scale spatial genetic and mark-resight data were reported in a lesser kestrel population (Alcaide et al. 2009), where the authors concluded that rare occurrence of long-distance dispersal events overrode the genetic structure expected in the otherwise philopatric

population. Long-distance dispersal events have been observed in WTEs as well (Struwe-Juhl and Grünkorn 2007).

In the Finnish WTE population, Ponnikas et al. (2013) reported strong genetic spatial autocorrelation for nestlings, and the mean distance of the highest distance class with significantly positive kinship coefficient corresponded to both ringing data from the Finland population and the known mean natal dispersal distances in the neighbouring population of Sweden. Using the same method for calculating genetic spatial autocorrelation, we failed to find such pattern among breeding WTEs in the Carpathian Basin. Investigating several WTE populations across Europe, Nemesházi et al. (2016) suggested that proportion of immigrants from distant populations may be higher in the Carpathian Basin than in populations to the North. All in all, we assume that the lack of fine-scale genetic structure in our study despite the known philopatric behaviour of the species is an outcome of some level of long-distance dispersal: if such events occur within the population, those increase the average genetic relatedness in higher distance classes, and immigrants may cause a background noise across all distance classes. Our limited sample size, especially in males which presumably should show stronger genetic structure, may have further increased difficulties for finding fine-scale genetic structure (Banks and Peakall 2012). Furthermore, the investigated spatial scale may be smaller than the extent of natal philopatry: in this case, extension of sampling to wider geographical range would be needed to find genetic structure with spatial autocorrelation analyses.

As a second objective, we tested by Wilcoxon rank sum test if close relative females can be found at larger distances than close relative males, as suggested by the typical pattern of female-biased natal dispersal in birds. Similar method was used by Haas et al. (2010) in a brown-headed nuthatch (*Sitta pusilla*) poulation. We calculated pairwise maximum likelihood relatedness (Kalinowski et al. 2006) to find closely related individuals, because it generally performs better than other relatedness estimators (Milligan 2003) and we found this method a reliable estimate of actual relatedness in our study population (see Material and methods and Nemesházi et al. (2017). Note, that we could only investigate the pairwise breeding distance of individuals, which is not equivalent to the natal dispersal distance (NDD), however related to that. For example, if two close relatives with a pairwise breeding distance of 200 km were parent and offspring, then the offspring probably indeed had an NDD value of 200 km; as observations showed that breeding WTEs are long-term faithful to their territory (Helander 2003; Helander and Stjernberg 2003; Krone et al. 2013). If they were siblings, then the fact that they bred 200 km apart means that one of them could have dispersed 200 km while the other one remained completely faithful to the natal area, or both could have dispersed 100 km, or even more, from an area located further from the bee line between the breeding sites of the

two individuals. Therefore, although our findings on close relatives with considerable breeding distance up to 378 km do not explicitly correspond to the NDD, they indicate the occurrence of long-distance natal dispersal. Note that the longest pairwise breeding distances of close relatives found in our study are comparable with the dimensions of the Carpathian Basin. This is in accordance with previous findings that the Carpathian Basin maintains one genetic WTE population based on nestling microsatellite genotypes (Nemesházi et al. 2016). We presumed that the directions of natal dispersal are not sex-biased (Whitfield et al. 2009a), and therefore the relationship of NDD and breeding distance of close relatives should be similar for both sexes.

Result of the Wilcoxon rank sum test suggests that long-distance dispersal events are female-biased in the Carpathian Basin WTE population. Similarly, female-biased natal dispersal was found in the reintroduced population of western Scotland based on mark-resight data, with females dispersing on average two times farther than males (Whitfield et al. 2009a). Helander (2003) also reported somewhat higher mean NDD for females than for males in Sweden. Female-biased dispersal is common in bird species, and may serve inbreeding avoidance, or may be a consequence of different sex roles derived from their resource defence system (Greenwood and Harvey 1982; Perrin and Mazalov 2000). Our findings suggest that sex-biased natal dispersal contribute to the generally low pairwise intersexual relatedness of WTEs breeding close to each other in southwestern Hungary (Nemesházi et al. 2017; appendix Figure AIII.2a).

Despite the species is known to be philopatric, our spatial autocorrelation analyses generally failed to show evidence of spatial genetic structure within the Carpathian Basin or gave only a very weak evidence of it. We assume that this contradiction is due to occurrence of long-distance natal dispersal events together with our limited sample size (and maybe the limited spatial scale). On the other hand, the comparison of pairwise breeding distances of closely related same-sex individuals revealed a significant female bias. We conclude that long-distance dispersal events are female-biased in the Carpathian Basin, in accordance with a limited number of previous reports on sex-biased NDD in other European WTE populations.

General conclusions and looking ahead

White-tailed eagles (WTEs) are known as philopatric (Helander and Stjernberg 2003; Struwe-Juhl and Grünkorn 2007; Whitfield et al. 2009b; Whitfield et al. 2009a), suggesting presence of genetic structure among (or within) breeding populations. We investigated the genetic structure of WTE populations by several methods in different spatial scales. All in all, these investigations highlighted that the Carpathian Basin maintains a WTE population with unique genetic composition, but no within-population genetic structure was found on a fine spatial scale. Previously published empirical data on natal dispersal distance (NDD) in other WTE populations suggest that average NDD should be smaller than the spatial scale investigated here (Helander 2003; Struwe-Juhl and Grünkorn 2007; Whitfield et al. 2009a). Therefore, we assumed that the lack of fine-scale genetic structure was not caused by an insufficiently chosen spatial scale of sampling. However, the limited sample size and immigration from other populations might have influenced our results. Overall, we suggest that the lack of fine-scale genetic structure within the Carpathian Basin is at least partly caused by female-biased long-distance natal dispersal events.

Understanding individual movements of floaters (future members of the breeding population) is important for species conservation (Penteriani et al. 2011). Here we provided some information on this topic, by investigating natal dispersal and nest-site intrusions in the Carpathian Basin WTE population. Our results confirmed that natal dispersal is sex-biased and suggest a sex-bias in territorial intrusions as well. We assume that sex-biased natal dispersal contributes to a generally low intersexual relatedness among potential mates in southwestern Hungary (i.e. reduced inbreeding probability), and nest-site intrusions may be related to territory choice and mate choice in WTEs.

Although the ability of kin recognition has been shown in some bird species lately (Sharp et al. 2005; Bonadonna and Sanz-Aguilar 2012; Krause et al. 2012), studies have rarely demonstrated kin avoidance in mate choice of birds so far (Wheelwright and Mauck 1998; Oh and Badyaev 2006). Based on pairwise genetic relatedness data, we gave the first report on kin avoidance in mate choice of a raptor species. We suspect that the long-term genetic monogamy could have promoted some direct mechanism for kin avoidance in WTEs. Therefore, future investigations of the ability and mechanism of kin recognition in WTEs and other genetically monogamous raptors could provide useful information for understanding the evolution of kin recognition.

Relevance for practical conservation biology

According to our results, the Carpathian Basin maintains a WTE population with unique genetic composition. For example, it preserved the mt-hvr1 haplotype B12 which, to our knowledge, is exclusively present in this population. Our findings suggest that the Carpathian Basin is important for the species' conservation not only because it maintains a significant abundance of breeding WTEs, but it is also important for the preservation of genetic diversity of the species.

Our results suggest that there is some level of gene flow from the North to the Carpathian Basin population. As northern European individuals generally visit this area during the winter, we assume that some of the wintering individuals could have settled here instead of going back to their natal population. Accordingly, not only the protection of breeding territories, but also the protection of the major wintering places in the Carpathian Basin can significantly contribute to the long-term preservation of the local breeding population.

We showed that using conservative criteria, a breeding WTE population can be non-invasively studied by using moulted feathers collected at occupied nest sites. The set of microsatellite DNA markers used in our studies proved to be adequate for individual identification in the Carpathian Basin population, providing an effective method for long-term studies related to the species' conservation, such as monitoring the space use (Bulut et al. 2016), and the turn-over (Vili et al. 2013b) or mortality rates in the breeding population.

Relevance for veterinary science

Conservation of WTEs can be important not only in terms of biodiversity preservation, but for wild animal health and public health as well. WTEs are top predators of water-related ecosystems, and can be useful tools for monitoring presence of persistent environmental pollutants (Helander et al. 2008; Eulaers et al. 2011). Presence of several harmful chemicals in an ecosystem can be detected from feather or egg samples of these raptors, as they accumulate those from lower trophic levels.

Besides being apex predators, WTEs may also have a significant role in clearing out carcasses from the wild: as facultative scavengers, they are known to regularly feed on mammalian carcasses as well (Selva et al. 2005; Sándor et al. 2015). Consequently, they can provide an important sanitary service for wild animal populations, including game animals, by preventing diseases from spreading from rotting carcasses left in the wild. According to a recent review, worldwide declines in vulture populations (the most important scavengers in many areas) can have far reaching consequences: with more carcasses being left in the wild, diseases can

more likely spread across mammalian scavenger species (Ogada et al. 2012). Although some vulture species were permanently present in the Carpathian Basin until the early 20th century, nowadays vultures are only rare visitors at most parts of the region: scavenging of carcasses is done by other species, and the WTE is probably one of the most important bird scavengers (Selva et al. 2005) in the area. Therefore, conservation of WTEs may have broader positive effect on wild animal health as well.

There are several mortality causes known in WTEs (Helander and Stjernberg 2003; Krone et al. 2006; Probst and Gaborik 2011). Ammunition remains in game animals cause lead intoxication in these scavenging raptors and can even result in death of the individuals (Krone et al. 2006; Helander et al. 2009). There is limited information on the prevalence of diseases and parasites in wild WTE populations; however, investigations of dead specimens found several species of ecto- and endoparasites (Gwiazdowicz et al. 2006; Krone et al. 2006).

The main distribution range of a feather abnormality disease (the pinching off syndrome) of WTEs concurs with the WTE population of Germany, Poland and the Czech Republic (Müller et al. 2007). With the recovery of the European WTE populations, it is likely that the genetic lineages of the neighbouring populations will increasingly overlap with time. If WTEs will disperse from this central European population to the neighbouring ones, occurrence of the so far locally distributed feather abnormality disease may become increasingly wider in the future, and may appear in the Carpathian Basin population as well.

Results new to science

The following results of the present dissertation are new to science:

1. Collection of **moulted feathers** at occupied nest sites during the breeding season is a reliable non-invasive method for sampling resident WTEs. However, due to potential influence of feathers lost by intruders conservative criteria are needed for addressing the sampled individuals as residents. [SECTION I]
2. The studied European countries can basically be divided into **three major WTE populations**: southern (Carpathian Basin countries: Hungary, Croatia, Serbia and Slovakia, south-eastern Czech Republic and north-eastern Austria), central (Poland, Germany, northern Austria and probably autochthonous Czech birds), and northern (Finland, Lithuania and probably Estonia). [SECTION II]
3. The previously described haplotype B12 at the mt-hvr1 is not only unique, but frequent in the Carpathian Basin WTE population. Our results on both the microsatellite genetic clusters and the mitochondrial haplotypes suggest that **recovery in the Carpathian Basin** occurred predominantly from a small surviving local population after the bottleneck period in the 1970s. [SECTION II]
4. Assignment analyses on the individuals sampled in the recolonized areas suggest that **immigration from the central and northern European populations** also affected the genetic composition of the Carpathian Basin WTE population. [SECTION II]
5. Genetic composition of the **Czech WTE population** suggests that although natural recolonization of the area could have been mainly local, it was largely influenced by the reintroduction of birds of presumably northern origin. [SECTION II]
6. Our results based on pairwise genetic relatedness suggest that **WTEs can avoid kin** in mate choice. To our knowledge, no studies found evidence for similar strategy for inbreeding avoidance in raptor species so far, however it should be of outstanding importance for species with long-term genetic monogamy. [SECTION III]
7. Spatial distribution of genetically related breeding individuals suggest that **long-distance natal dispersal** occurs within the otherwise philopatric WTE population of the Carpathian Basin, and these events are female-biased. [SECTION IV]

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Publications related to the dissertation

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Nemesházi E., Kövér Sz., Zachos F.E., Horváth Z., Tihanyi G., Mórocz A., Mikuska T., Hám I., Literák I., Ponnikas S., Mizera T., Szabó K.: **Natural and anthropogenic influences on the population structure of white-tailed eagles in the Carpathian Basin and central Europe**, J. Avian Biol., 47. 795-805, 2016. (**IF_{2016}**: 2.228)

Nemesházi E., Szabó K., Horváth Z., Kövér Sz.: **The effects of genetic relatedness on mate choice and territorial intrusions in a monogamous raptor**, J. Ornithol., 'online first', 2017. (**IF_{2016}**: 1.468)

2. Full text publications in peer-reviewed journals with no impact factor assigned

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Publications not related to the dissertation

1. Full text publications in peer-reviewed journals

Vili N., **Nemesházi E**., Kovács Sz., Horváth M., Kalmár L., Szabó K.: **Factors affecting DNA quality in feathers used for non-invasive sampling**, J. Ornithol., 154. 587-595, 2013. (**IF_{2013}**: 1.927)

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Appendices

- **AI: appendix for SECTION I.**

- **AII: appendix for SECTION II.** From the electronic supplement for Nemesházi E., Kövér Sz., Zachos F.E., Horváth Z., Tihanyi G., Mórocz A., Mikuska T., Hám I., Literák I., Ponnikas S., Mizera T., Szabó K.: Natural and anthropogenic influences on the population structure of white-tailed eagles in the Carpathian Basin and central Europe, J. Avian Biol., 47. 795-805, 2016.
  The original is available here:
  http://www.avianbiology.org/sites/avianbiology.org/files/appendix/jav-00938.pdf

- **AIII: appendix for SECTION III.** From the electronic supplement for Nemesházi E., Szabó K., Horváth Z., Kövér Sz.: The effects of genetic relatedness on mate choice and territorial intrusions in a monogamous raptor. Journal of Ornithology. doi: 10.1007/s10336-017-1494-z, 21017.
  The original is available here: https://link.springer.com/article/10.1007/s10336-017-1494-z#SupplementaryMaterial

AI: appendix for section I

Table AI.1. Moulted WTE feathers with colour patterns discriminatory for aging or somewhat different compared to most similar type feathers. Individuals were aged based on feathers highlighted with light grey background (following Forsman 1999 and Cieślak and Dul 2006). The last column shows the number of individuals with assigned age (adult or juvenile) and those with unknown age for each type-pattern category.

Feather type	Vane colour	Additional pattern	Tip colour	Diagnostic for	N feathers	N individuals
tail (large)	white	some brown mottling may be present in the lower half	same	adult	26	22
		extensive brown mottling	dark	juvenile	2	2
uppertail covert	white	variable level of brown mottling in the lower half	dark	-	3	2 adult 1 unknown
body covert	white or pale	any level of mottling may occur	dark	juvenile	6	6
		some level of mottling	mildly darker	-	1	1 unknown
		no mottling	mildly darker	-	2	1 adult 1 unknown
			paler	-	5	1 adult 4 unknown
	dark	some level of mottling	same or paler	-	7	2 adult 1 juvenile 4 unknown
		no mottling	same or paler	-	2	2 unknown
neck covert	pale	moderate mottling	same	-	1	1 unknown
		no mottling	same or paler	-	4	2 adult 2 unknown
flight feather (large)	dark	extensive white mottling	same	juvenile	2	2
		one large pale patch	same	-	1	1 adult
	paler	moderate white mottling	same	-	1	1 adult
flight feather (small)	dark	moderate white mottling	same	-	3	1 adult 2 unknown
		no mottling	paler	-	1	1 unknown

Table AI.2.The number of analysed feathers which were shed by residents or other individuals in each territory. Residents were assessed based on matching nestling genotypes from the same territory and year.

Territory	Year	Number of shed feathers				
		SUM	Resident Female	Resident Male	Intruder	Nestling
B-T1 *	2013	6	3	3	0	0
	2015	5	3	1	1	0
B-T7	2015	7	5	2	0	0
B-T10	2015	6	3	1	1	1
B-T8	2015	12	9	1	1	1
B-T2	2015	8	5	2	1	0
B-T5	2015 **	4	3	1	0	0
B-T6 *	2014	8	4	4	0	0
	2015	4	3	1	0	0
C5	2010	5	3	2	0	0
C9	2010	6	6	0	0	0
D10	2015	6	4	2	0	0
D11	2015	4	4	0	0	0
D13	2013	4	3	1	0	0
D14	2015	4	4	0	0	0
D21	2016	4	3	1	0	0
D2	2015	6	6	0	0	0
D4	2012	4	4	0	0	0
D5	2015	4	4	0	0	0
D6	2015	6	4	2	0	0
D8	2012	5	5	0	0	0
T3	2015	7	5	2	0	0
T4	2015	5	3	2	0	0
T5	2016 **	6	4	2	0	0
T6	2016	6	5	1	0	0
T7	2016	4	4	0	0	0
T8	2016	6	6	0	0	0

* Moulted feathers collected in two different years were analysed from B-T1 and BT6.

** In B-T5 and T5, nestling samples were available from previous years only, but breeding was successful in the year of moulted feather collection as well.

AII: appendix for section II

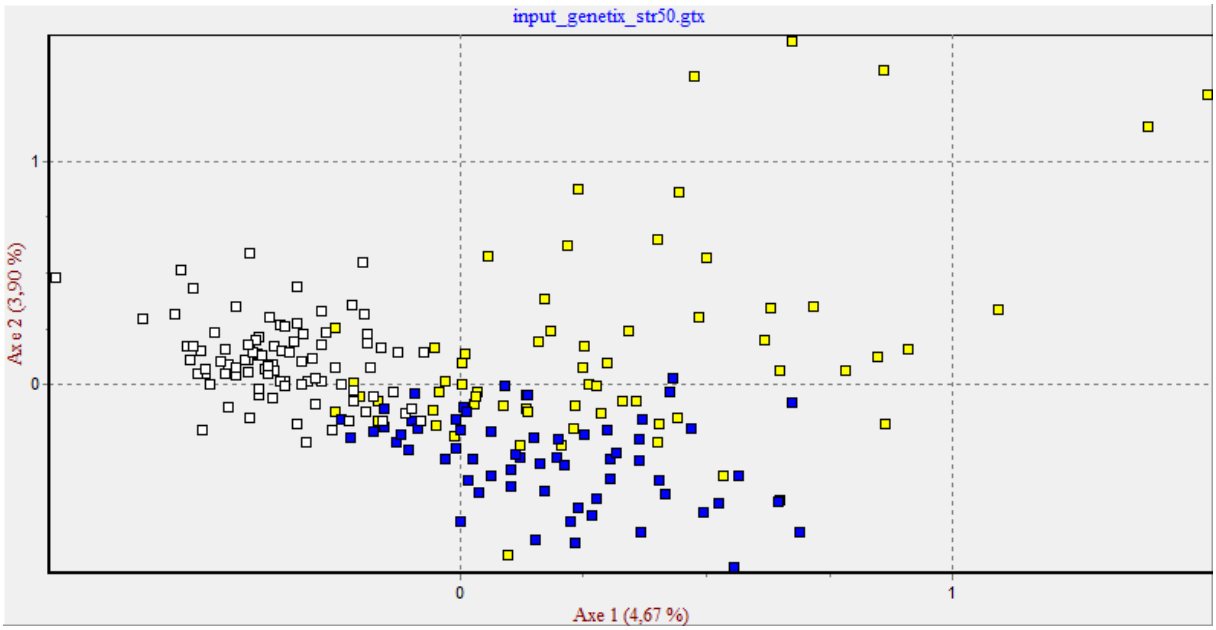

Figure AII.1. Factorial correspondence analysis (FCA) performed in Genetix 4.05.2. based on 218 individual microsatellite genotypes (11 loci). Squares with different colours indicate individuals assigned to different genetic clusters by Structure 2.3.4. (according to the 50% cut-off criterion): northern (***yellow***), central (***blue***) and southern (***white***). The three clusters show overall segregation, but there is some overlap (mostly of northern eagles with those from the other two clusters).

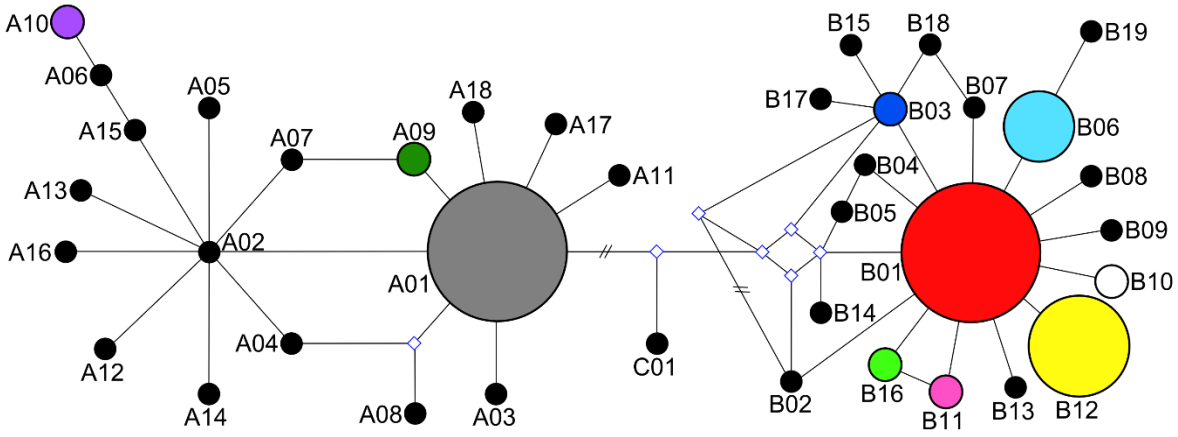

Figure AII.2. Median-joining network of all 38 mt-hvr1 haplotypes found in the global distribution range of the species. Haplotypes found in the Carpathian Basin so far are in colour, and the area of each circle is proportional to their frequency within the Carpathian Basin. Colour code is identical with Figure II.2. Links between the haplotypes indicate a single mutational step except for the ones marked with double dashes where two mutations occurred. In the present study we only found the haplotypes A01, B01, B06 and B12.

Table AII.1. Ln probability and ΔK values for the number of genetic clusters (K) according to the Structure analysis. Calculation of the ad hoc statistic we used to decide the accepted number of clusters is: ΔK = (|mL(K + 1) – 2mL(K) + mL(K – 1)|) / sdL(K), where L(K) is the natural logarithm of the probability that K is the correct number of clusters, m is the mean and SD is the standard deviation of the 10 replicate runs for the same K value. (For the lowest and highest K values in a comparison, ΔK is not applicable.)

Replicate	K=1	K=2	K=3	K=4	K=5	K=6	K=7
1	-6170.2	-6094.3	-6045.7	-6307.2	-6356.6	-6302.9	-6500.0
2	-6169.9	-6092.5	-6039.4	-6092.2	-6420.9	-6143.4	-6488.8
3	-6170.2	-6090.7	-6037.3	-6072.6	-6563.8	-6240.5	-6659.1
4	-6170.0	-6099.4	-6049.8	-6062.5	-6611.8	-6166.0	-6556.6
5	-6170.1	-6095.5	-6037.1	-6080.1	-6259.2	-6161.3	-6350.8
6	-6170.2	-6097.0	-6038.1	-6118.8	-6525.3	-6219.9	-6483.2
7	-6170.2	-6082.1	-6040.9	-6236.1	-6467.1	-6209.3	-6378.0
8	-6169.9	-6089.4	-6043.8	-6079.6	-6339.8	-6186.1	-6415.1
9	-6170.4	-6094.6	-6042.0	-6082.3	-6381.2	-6224.7	-6447.9
10	-6170.1	-6100.5	-6039.5	-6200.5	-6702.9	-6240.3	-6433.9
Mean	-6170.12	-6093.6	-6041.36	-6133.19	-6462.86	-6209.44	-6471.34
SD	0.155	5.348	4.072	84.483	137.538	47.264	89.534
ΔK	-	4.540	35.384	2.815	4.239	10.903	-

Table AII.2. Percentage of sampled individuals assigned to the genetic clusters proposed by Structure within geographically separable units, using three different cut-off criteria. ***N****:* number of sampled individuals. Lower-case letters refer to the three Structure clusters, according to their predominant distribution (***n****:* northern, ***c****:* central, ***s****:* southern), ***u*** denotes non-classified individuals. ***Spatial units***: clusters suggested by Geneland (Finnish Baltic coast (***B. coast***), Finnish Lapland and Lithuania (***Lap, Lit***), Germany and Poland (***Ger, Pol***), Czech and Carpathian Basin (***CB***)), sorted into three major groups according to their geographical location (***Northern, Central, Southern***). The most frequent Structure clusters in each unit are bold. Both Structure and Geneland results are averages across 10 replicate runs.

Spatial unit	N	50% cut-off				60% cut-off				70% cut-off			
		n	c	s	u	n	c	s	u	n	c	s	u
Northern	35	**77**	11	3	9	**71**	9	0	20	**63**	6	0	31
B. coast	23	**74**	17	0	9	**74**	13	0	13	**61**	9	0	30
Lap, Lit	12	**83**	0	8	8	**67**	0	0	33	**67**	0	0	33
Central	71	30	**63**	1	6	24	**59**	0	17	18	**48**	0	34
Ger, Pol	43	12	**81**	2	5	7	**79**	0	14	5	**72**	0	23
Czech	28	**57**	36	0	7	**50**	29	0	21	**39**	11	0	50
Southern (CB)	143	13	9	**62**	17	8	5	**55**	32	4	3	**43**	50

Table AII.3. Number of individuals within the recolonized regions with probable origin. These birds were classified consistently to one population according to assignment tests of GeneClass2 and genetic clusters suggested by Structure (for the latter, we used three different cut-off criteria: 50, 60 and 70%). ***N****:* number of sampled individuals in each recolonized region. Lower-case letters in the columns refer to the assumed origin (***n****:* northern, ***c****:* central, ***s****:* southern).

Recolonized region	N	50% cut-off			60% cut-off			70% cut-off		
		n	c	s	n	c	s	n	c	s
Austria	3	1	0	0	1	0	0	0	0	0
Slovakia	8	0	1	3	0	1	3	0	1	3
Hungary	39	4	0	13	3	0	13	2	0	11
Czech Republic	29	12	2	0	10	2	0	8	1	0
SUM	79	17	3	16	14	3	16	10	2	14

Tables AII.4-AII.7: Pairwise F_{ST} (below diagonal) and unbiased Nei's genetic distance (above diagonal) for the Geneland and Structure clusters based on microsatellite data. All comparisons were statistically significant ($p<0.001$ for Structure and $p<0.003$ for Geneland clusters). AII.5-AII.7: 'individual' clusters with 50, 60 and 70% cut-off criteria, respectively.

AII.4.

Geneland cluster	Finnish Baltic coast	Finnish Lapland and Lithuania	Germany and Poland	Czech	Carpathian Basin
Finnish Baltic coast	0	0.074	0.064	0.104	0.091
Finnish Lapland and Lithuania	0.043	0	0.060	0.030	0.038
Germany and Poland	0.040	0.041	0	0.064	0.062
Czech	0.056	0.018	0.039	0	0.064
Carpathian Basin	0.088	0.051	0.072	0.052	0

AII.5.

Structure cluster - 50%	*northern*	*central*	*southern*
northern	0	0.070	0.078
central	0.041	0	0.110
southern	0.067	0.099	0

AII.6.

Structure cluster - 60%	*northern*	*central*	*southern*
northern	0	0.090	0.091
central	0.053	0	0.123
southern	0.084	0.118	0

AIII.7.

Structure cluster - 70%	*northern*	*central*	*southern*
northern	0	0.103	0.111
central	0.059	0	0.145
southern	0.097	0.135	0

Table AII.8. Number of individuals sampled in the Carpathian Basin that were successfully analysed for both markers (mt-hvr1 and microsatellites), and their assignment to the Structure clusters based on the three cut-off criteria (*n:* northern, *c:* central, *s:* southern).

Haplotype	N	50% cut-off			60% cut-off			70% cut-off		
		n	c	s	n	c	s	n	c	s
A01	24	4	0	18	2	0	16	1	0	14
B01	18	7	0	7	4	0	5	2	0	3
B06	6	1	1	3	1	0	3	1	0	2
B12	12	0	1	10	0	1	10	0	0	9
B03	1	0	1	0	0	1	0	0	0	0
B10	1	0	1	0	0	1	0	0	1	0
SUM	62	12	4	38	7	3	34	4	1	28

Table AII.9. Number of mt-hvr1 haplotypes occurring in at least two countries and found throughout the breeding range of the species so far. Sampled countries are sorted according to their geographical region: northern/central/south-central Europe (***NE***, ***CE*** and ***SE***, respectively), North Atlantic Ocean (***NA***) and Asia (***A***). For further information see the original papers (Hailer et al. 2006; Hailer et al. 2007; Honnen et al. 2010; Langguth et al. 2013; Ponnikas et al. 2013; Treinys et al. 2016).

Re-gion	Geneland cluster	Country or part	N	Haplogroup			Frequent haplotypes						Other distributed haplotypes								
				A	B	C	A01	A02	A03	B01	B12	C01	B02	B03	B04	B05	B06	B07	B11	B18
NE	**Finnish Lapland and Lithuania**	Finnish Lapland	23	8	15	-	8	-	-	9*	-	-	1	-	0-1*	0-1*	2	2	-	-
		Lithuania	45	26	19	-	20	6	-	14	-	-	-	2	-	-	2	1	-	-
		Estonia***	24	5	19	-	4	1	-	9	-	-	-	5	-	-	-	3	-	2
		Overall %	**92**	***42.4***	***57.6***	**-**	***34.8***	***7.6***	**-**	***34.8***	**-**	**-**	***1.1***	***7.61***	***0-1****	***0-1****	***4.4***	***6.5***	**-**	***2.2***
	-	Swedish Lap**	22	12	10	-	12	-	-	5	-	-	1	-	2	1	1	-	-	-
	Finnish Baltic coast	Finnish coast	63	20	31	12	18	2	-	30*	-	12	-	1	-	-	-	-	-	-
		Overall %	**63**	***31.7***	***49.2***	***19***	***28.6***	***3.2***	**-**	***47.6***	**-**	***19***	**-**	***1.6***	**-**	**-**	**-**	**-**	**-**	**-**
	-	Swedish coast**	51	24	21	6	22	1	-	21	-	16	-	-	-	-	-	-	-	-
CE	**Germany and Poland**	Germany	85	78	7	-	17	58	-	7	-	-	-	-	-	-	-	-	-	-
		Poland	55	46	9	-	13	25	-	2	-	-	-	-	-	-	-	-	-	1
		Overall %	**140**	***88.6***	***11.4***	**-**	***21.4***	***59.3***	**-**	***6.4***	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	***0.7***
	Czech	Czech Republic	9	3	6	-	-	2	-	3	-	-	-	-	-	-	1	-	1	-
		N Austria	3	1	2	-	-	1	-	1	-	-	-	-	-	-	-	-	-	-
		Overall %	**12**	***33.3***	***66.7***	**-**	**-**	***25***	**-**	***33.3***	**-**	**-**	**-**	**-**	**-**	**-**	***8.3***	**-**	***8.3***	**-**
SE	**Carpathian Basin**	SE Austria	10	2	8	-	1	-	-	5	1	-	-	1	-	-	-	-	-	-
		Slovakia	6	2	4	-	1	-	-	1	-	-	-	-	-	-	2	-	1	-
		Serbia	30	11	19	-	11	-	-	15	2	-	-	-	-	-	1	-	-	-
		Hungary	44	18	26	-	18	-	-	9	12	-	-	-	-	-	5	-	-	-
		Croatia	9	3	6	-	3	-	-	4	2	-	-	-	-	-	-	-	-	-
		Overall %	**99**	***36.4***	***63.6***	**-**	***34.3***	**-**	**-**	***34.3***	***17.2***	**-**	**-**	***1***	**-**	**-**	***8.1***	**-**	***1***	**-**
NA	**-**	Norway	33	32	1	-	32	-	-	1	-	-	-	-	-	-	-	-	-	-
		Greenland	8	8	-	-	7	-	1	-	-	-	-	-	-	-	-	-	-	-
		Iceland	26	26	-	-	7	-	19	-	-	-	-	-	-	-	-	-	-	-
A	**-**	Japan	8	-	8	-	-	-	-	7	-	-	-	-	1	-	-	-	-	-
		Kazakhstan	26	3	23	-	-	-	-	13	-	-	-	-	-	-	1	-	-	-
		Kola Peninsula	10	1	9	-	1	-	-	2	-	-	2	1	-	1	1	2	-	-

* Haplotypes in Finland were specified based on 473 bp instead of 499 bp. Therefore, B04/B05 and B01/B10/B14 could not be distinguished. As B10 was found only in one Austrian and B14 in one Czech sample, higher proportions of these haplotypes in Finland are unlikely, and we therefore assigned these Finnish birds to B01.

** We did not investigate any Swedish samples for microsatellites. However, based on the similar haplotype-distributions to the neighbouring Finnish areas, the Swedish Lapland and Baltic coast are likely part of the northern inland and Baltic coast populations, respectively. Moreover, birds originating from the Finnish coast were found as breeders at the Swedish coast (Helander 2003).

*** Classification by Geneland of the only Estonian sample analysed for microsatellites was uncertain. However, mtDNA haplotype composition in Estonia (B03 and B07; Hailer et al. 2007; Langguth et al. 2013) is most similar to that of adjacent population of Finnish Lapland and Lithuania and its geographical position suggests as well that Estonia belongs to this population.

Table AII.10. List of microsatellite alleles and their frequencies within each Geneland and Structure clusters. Geneland clusters: Finnish Baltic coast (***BC***), Finnish Lapland and Lithuania (***FL***), Germany and Poland (***GP***), Carpathian Basin (***CB***) and Czech (***Cz***). Structure clusters (with 50, 60 and 70% cut-off criteria): northern (***n***), central (***c***) and southern (***s***). Alleles with presumable diagnostic information (by presence) on a bird's origin are marked with ***red*** (northern), ***blue*** (central) or ***yellow*** (southern).

Locus	Allele	Geneland clusters					Structure – 50%			Structure – 60%			Structure – 70%		
		Bc	FL	GP	CB	Cz	n	c	s	n	c	s	n	c	s
Aa27	N	23	12	43	76	26	56	56	44	47	49	35	37	39	27
	93	0.02	-	-	-	-	0.01	-	-	0.01	-	-	0.01	-	-
	97	0.04	0.21	0.26	0.16	0.10	0.10	0.23	0.14	0.07	0.22	0.13	0.05	0.24	0.15
	99	0.83	0.58	0.71	0.78	0.52	0.63	0.73	0.83	0.63	0.73	0.84	0.59	0.72	0.83
	101	0.09	0.04	-	-	0.15	0.11	-	-	0.13	-	-	0.15	-	-
	103	0.02	0.17	0.03	0.06	0.23	0.16	0.04	0.03	0.16	0.04	0.03	0.19	0.04	0.02
Aa35	N	23	12	39	140	27	65	58	87	54	50	76	41	38	59
	239	0.11	-	0.01	0.18	-	0.05	0.03	0.23	0.05	0.01	0.25	0.04	-	0.28
	241	0.72	0.83	0.77	0.72	0.96	0.84	0.78	0.68	0.85	0.79	0.67	0.87	0.78	0.64
	245	0.17	0.17	0.22	0.10	0.04	0.12	0.18	0.09	0.10	0.20	0.08	0.10	0.22	0.08
Hal01	N	23	12	35	137	27	61	54	88	51	44	77	40	33	60
	132	0.04	0.17	-	0.01	-	0.04	-	0.01	0.05	-	0.01	0.06	-	0.01
	134	0.13	0.25	0.46	0.61	0.46	0.26	0.51	0.66	0.22	0.55	0.68	0.19	0.52	0.68
	136	0.15	0.08	0.0	0.08	0.11	0.16	0.02	0.06	0.17	-	0.05	0.19	-	0.04
	138	0.33	0.29	0.41	0.30	0.19	0.28	0.38	0.27	0.27	0.34	0.27	0.28	0.42	0.27
	140	0.33	0.17	0.11	0.004	0.22	0.23	0.08	-	0.25	0.10	-	0.25	0.06	-
	142	0.02	0.04	-	0.01	0.02	0.03	0.01	-	0.04	0.01	-	0.04	-	-
Hal04	N	23	12	39	142	27	66	57	90	54	48	78	41	38	61
	150	-	-	0.01	-	0.19	0.07	0.01	-	0.07	0.01	-	0.09	0.01	-
	154	-	0.08	-	0.03	-	0.04	0.02	-	0.05	-	-	0.05	-	-
	156	0.09	0.04	0.06	0.11	0.11	0.10	0.05	0.11	0.10	0.04	0.12	0.11	0.05	0.12
	158	0.07	0.17	0.10	0.01	0.04	0.05	0.07	0.01	0.05	0.07	0.01	0.04	0.09	0.02
	160	0.85	0.71	0.82	0.71	0.54	0.68	0.81	0.72	0.68	0.84	0.70	0.67	0.83	0.66
	162	-	-	-	0.13	0.13	0.06	0.04	0.16	0.06	0.03	0.17	0.05	0.01	0.20
Hal09	N	23	12	40	142	26	65	57	90	53	47	7	40	38	61
	127	0.04	0.13	0.09	0.04	0.06	0.10	0.05	0.01	0.10	0.04	0.01	0.13	0.05	-
	133	0.22	0.25	0.29	0.32	0.33	0.28	0.31	0.32	0.25	0.30	0.35	0.24	0.30	0.36
	137	0.02	0.08	0.01	0.01	-	0.05	0.01	-	0.06	0.01	-	0.04	0.01	-
	139	0.04	0.08	0.01	0.02	-	0.04	-	0.02	0.04	-	0.01	0.05	-	0.02
	141	0.30	0.25	0.31	0.36	0.31	0.26	0.32	0.39	0.30	0.35	0.40	0.28	0.32	0.43
	143	0.13	0.17	0.26	0.12	0.31	0.18	0.25	0.09	0.16	0.26	0.09	0.18	0.28	0.07
	145	0.24	0.04	0.03	0.12	-	0.08	0.06	0.16	0.08	0.04	0.15	0.10	0.04	0.11
Hal10	N	23	12	40	143	28	66	59	90	54	49	78	41	37	61
	232	0.48	0.08	0.54	0.16	0.23	0.24	0.61	0.06	0.20	0.63	0.04	0.22	0.65	0.02
	234	0.04	0.04	0.01	0.05	-	0.02	0.01	0.07	0.03	0.01	0.08	0.04	0.01	0.08
	236	0.07	0.29	0.03	0.14	0.18	0.20	0.06	0.08	0.21	0.05	0.08	0.20	0.04	0.06
	238	0.41	0.58	0.41	0.65	0.57	0.52	0.32	0.79	0.55	0.31	0.80	0.55	0.30	0.84
	240	-	-	0.01	-	0.02	0.02	-	-	0.01	-	-	-	-	-
Hal13	N	23	12	40	143	26	65	58	90	53	48	78	41	37	61
	149	0.28	0.08	-	0.01	-	0.14	0.01	-	0.16	0.01	-	0.15	-	-
	151	0.13	0.04	-	0.08	-	0.07	-	0.08	0.08	-	0.07	0.07	-	0.06
	155	0.04	-	0.08	0.01	0.08	0.02	0.09	0.01	0.03	0.09	0.01	0.04	0.08	-
	157	0.33	0.63	0.35	0.58	0.37	0.40	0.35	0.70	0.35	0.36	0.71	0.35	0.36	0.74
	159	-	-	0.04	0.02	0.08	0.05	0.03	0.01	0.06	0.04	0.01	0.06	0.04	-
	161	0.17	0.25	0.51	0.29	0.35	0.25	0.51	0.19	0.24	0.48	0.19	0.22	0.51	0.19
	163	0.04	-	0.03	-	0.04	0.05	-	-	0.06	-	-	0.07	-	-
	165	-	-	-	0.01	0.10	0.03	0.01	0.02	0.04	0.01	0.01	0.04	-	0.02
IEAAAG04	N	23	12	39	142	26	64	59	89	52	50	78	41	38	61
	198	-	0.08	0.03	-	-	0.03	-	-	0.03	-	-	0.04	-	-
	202	-	0.04	-	0.05	-	0.03	-	0.05	0.03	-	0.05	0.02	-	0.03
	206	0.61	0.42	0.58	0.68	0.62	0.62	0.61	0.66	0.61	0.60	0.65	0.57	0.57	0.66
	210	0.20	0.21	0.26	0.26	0.27	0.20	0.24	0.28	0.21	0.24	0.28	0.21	0.26	0.30
	214	0.07	0.08	0.13	0.01	0.10	0.04	0.14	0.01	0.03	0.15	0.01	0.04	0.16	0.02
	218	0.13	0.17	-	-	-	0.06	0.01	-	0.08	0.01	-	0.10	0.01	-

	222	-	-	0.01	-	0.02	0.02	-	-	0.02	-	-	0.02	-	-
IEAAAG 05	N	23	12	41	142	26	65	60	88	53	50	77	41	39	61
	113	-	-	0.01	-	0.04	0.02	0.01	-	0.01	0.01	-	0.01	-	-
	121	-	0.04	-	-	-	0.01	-	-	0.01	-	-	0.01	-	-
	125	0.11	0.04	-	-	-	0.05	-	-	0.05	-	-	0.06	-	-
	133	0.02	0.04	-	0.02	0.02	0.05	-	0.01	0.05	-	0.01	0.05	-	-
	137	0.04	0.04	0.29	0.01	0.10	0.02	0.24	0.01	0.02	0.29	-	0.02	0.29	-
	141	0.04	0.08	0.06	0.08	0.27	0.17	0.05	0.06	0.18	0.05	0.06	0.17	0.05	0.07
	145	0.39	0.29	0.45	0.61	0.27	0.27	0.51	0.68	0.27	0.50	0.68	0.26	0.53	0.69
	149	0.28	0.04	0.04	0.03	0.21	0.13	0.09	0.03	0.14	0.07	0.03	0.16	0.06	0.03
	153	0.04	0.17	0.11	0.07	0.10	0.12	0.09	0.03	0.13	0.07	0.04	0.12	0.06	0.02
	157	-	0.13	0.01	0.01	-	0.03	-	0.02	0.02	-	0.01	0.02	-	0.02
	161	0.02	0.08	-	0.06	-	0.05	-	0.05	0.06	-	0.05	0.06	-	0.06
	165	-	-	0.01	-	-	-	0.01	-	-	0.01	-	-	-	-
	169	0.04	-	0.01	0.07	-	0.04	-	0.09	0.04	-	0.10	0.02	-	0.11
	180	-	-	-	0.01	-	0.01	-	0.01	-	-	0.01	-	-	-
	188	-	0.04	-	-	-	0.01	-	-	0.01	-	-	0.01	-	-
	206	-	-	-	0.01	-	0.02	-	0.01	0.02	-	-	0.01	-	-
	210	-	-	-	0.01	-	0.01	-	0.01	-	-	-	-	-	-
IEAAAG 12	N	23	12	43	97	25	59	56	64	51	49	53	39	38	43
	95	0.04	0.04	-	0.01	0.02	0.03	0.01	-	0.04	0.01	-	0.04	-	-
	97	0.48	0.50	0.50	0.60	0.60	0.63	0.43	0.61	0.65	0.43	0.65	0.63	0.43	0.67
	101	0.09	0.04	-	-	-	0.03	-	-	0.04	-	-	0.04	-	-
	104	0.15	0.21	0.15	0.15	0.20	0.13	0.19	0.16	0.12	0.19	0.13	0.15	0.18	0.14
	108	0.09	0.17	0.16	0.18	0.14	0.14	0.18	0.16	0.11	0.16	0.15	0.12	0.16	0.14
	112	0.15	-	0.19	0.06	0.04	0.03	0.20	0.07	0.04	0.20	0.07	0.01	0.22	0.05
	116	-	0.04	-	-	-	0.01	-	-	0.01	-	-	0.01	-	-
IEAAAG 14	N	23	12	36	140	24	63	54	89	52	46	77	41	37	60
	174	0.30	0.46	0.43	0.36	0.48	0.43	0.45	0.31	0.46	0.49	0.32	0.46	0.49	0.32
	178	0.24	0.42	0.29	0.34	0.29	0.30	0.21	0.39	0.28	0.22	0.38	0.26	0.23	0.36
	182	0.46	0.13	0.28	0.28	0.23	0.26	0.33	0.28	0.26	0.29	0.27	0.28	0.28	0.30
	186	-	-	-	0.02	-	0.01	-	0.02	-	-	0.02	-	-	0.03

Table AII.11. Number of private alleles in each Geneland and Structure cluster and geographical region.

Type	Unit name	Private allele
Structure 50%	northern	11
	central	1
	southern	0
Structure 60%	northern	13
	central	1
	southern	2
Structure 70%	northern	17
	central	0
	southern	1
Geneland	Finnish Baltic coast	1
	Finnish Lapland and Lithuania	3
	Germany and Poland	1
	Czech	0
	Carpathian Basin	4
Geographical regions	East European Plain	7
	Great European Plain	5
	Carpathian Basin	4

Table AII.12. Optimized volumes of microsatellite primers for PCR reactions and fragment length analysis on a sequencer. Different amplification reactions are separated by dotted lines. All primers were diluted to 5 pikomol/µl and forward primers were 5'-labeled with fluorescent dyes. Each PCR reaction was performed in 16µl volume. All primers were published earlier (Hal: Hailer et al. 2005), Aa: Martínez-Cruz et al. 2002; IEAAAG: Busch et al. 2005).

Primer pair	Dye	PCR reactions		Run in sequencer	
		primer (µl)	type	Volume of PCR product (µl)	Run
IEAAAG04	FAM	1.6	multiplex	9	1
Hal04	NED	2.4			
Hal01	FAM	2.3	multiplex	7	
IEAAAG12	HEX	1.7			
IEAAAG14	VIC	2.57	singleplex	4.5	
IEAAAG05	PET	2.57	singleplex	4.5	
Hal13	VIC	1.8	multiplex	9	2
Hal10	NED	2.2			
Hal09	FAM	2.57	singleplex	6	
Aa35	PET	2.1	multiplex	10	
Aa27	FAM	1.9			

AIII: appendix for section III

AIII. Methods

DNA extraction and molecular sexing

In moulted feathers we preferentially used the superior umbilicus as DNA source (Horváth et al. 2005), but if it was not visible (e.g. in small coverts), the whole quill below the vane was digested. In nestling feathers, DNA was extracted from the tip of the quill. DNA extraction was performed using Quiagen – DNEasy Blood and Tissue Kit or Thermo - GeneJet genomic DNA purification kit, following the manufacturers' instructions and adding 10 µl of 1M dithiotreitol during the digestion step.

Molecular sexing was performed in each DNA sample using the 2550F/2718R primer pair (Fridolfsson and Ellegren 1999) or the GEfUp/GErUp and GEfLow/GErLow primer pairs (Ogden et al. 2015). Bands from each PCR product were visualized under UV light following an electrophoresis process on a 2% agarose gel containing ECO Safe (Pacific Image Electronics Co., Ltd.).

PCR reactions for molecular sexing and microsatellites

PCR reactions were performed in a 16 µl volume. Reactions were singleplex or multiplex, depending on the used primer pairs (see below). Singleplex reactions contained 1.6 µl PCR buffer (10 µl Dream Taq™, Fermentas), 16.25 mM $MgCl_2$ (Promega), 1.30 mM dNTP-mix (Fermentas), 0.33 units of DNA-polymerase (Dream Taq™, Fermentas), 6.43 pmol of each primer and 10-70 ng of template DNA. Multiplex reactions were similar, just the amount of each primer changed.

For molecular sexing, the 2550F/2718R primer pair (Fridolfsson and Ellegren 1999) was used in a singleplex reaction. Amplification with the GEfUp/GErUp and GEfLow/GErLow primer pairs (Ogden et al. 2015) was performed in a multiplex reaction, with 3.75 pmol of each primer.

Microsatellite fragment analyses were performed in two pools of PCR products. In the first pool, primers IEAAAG05 and IEAAAG14 were amplified separately, while IEAAAG04 (4-4 pmol forward and reverse) and Hal04 (6-6 pmol), and IEAAAG12 (4.26-4.26 pmol) and Hal01 (5.75-5-75 pmol) were amplified in mupltiplex reactions. The second pool contained products of three singleplex (Aa35, Hal03 and Hal09) and two multiplex reactions, where Hal10 (5.5-5.5 pmol) and Hal13 (4.5-4.5 pmol), and Aa27 (3-3 pmol) and Aa49 (5.25-5.25 pmol) were amplified together.

For molecular sexing, PCR profiles followed the suggestions of the original papers for each sexing system (Fridolfsson and Ellegren 1999; Ogden et al. 2015), but the profile described by

Ogden et al. (2015) was completed with an initial touch-down section where annealing temperature decreased from 65 to 60°C during 7 cycles.

Similarly to (Nemesházi et al. 2016), we used a modified PCR profile following Hailer et al. (2006) for IEAAAG and Hal loci, where reactions were repeated in 37 cycles and both annealing and amplifications steps lasted for 45 seconds. Amplification of Aa loci was performed as described by Martínez-Cruz et al. (2002). Annealing temperatures for Aa and Hal loci were set as described in the original papers (Martínez-Cruz et al. 2002; Hailer et al. 2005). As suggested by Hailer et al. (2006), we used 56°C for loci IEAAAG04, IEAAAG05 and IEAAAG14, and following Nemesházi et al. (2016), annealing was performed in 60°C for IEAAAG12 .

Testing for genotyping errors and pairwise linkage disequilibrium

To minimise genotyping errors, PCRs were repeated when they yielded uncertain results (e.g. low or ambiguous peaks). Reliability of the assumed genotypes was tested with two methods. First, we randomly repeated 100 PCRs and their fragment length analyses. Second, we compared genotypes from 134 moulted feathers with the assigned individual's consensus DNA profile (see below, N=33). Presence of null alleles and scoring errors due to large allele dropout or stutter bands was estimated in Micro-Checker 2.2.3. (Van Oosterhout et al. 2004). Pairwise linkage disequilibrium across loci was estimated in Genepop 4.2. (Rousset 2008).

AIII. Results

DNA extraction and molecular sexing

DNA was extracted from overall 242 moulted and 52 nestling feathers (166 and 20 from the Boronka forest, respectively). Out of these 197 and 46 were successfully sexed, respectively. Nestling sex ratio was 0.413 (19 males, 27 females). Among the succesfully sexed moulted feathers, 74% belonged to females.

Testing for genotyping errors and pairwise linkage disequilibrium

Reliability of our genotype data was estimated to 96.5% and 99%, based on randomly repeated PCRs and multiple samples from one individual, respectively. Results of Genepop suggested that linkage disequilibrium may exist between 7 out of the 78 pairs of loci, but none of these were significant after Bonferroni correction. Micro-checker did not find evidence for genotyping errors due to stutter bands or large allele dropout, but suggested a significant null allele presence in Hal10. As genotypes of some homozygote residents did not match their nestlings' genotype, but exclusively on this locus, we excluded Hal10 from the further analyses and used a final dataset of 12 loci. We overall assumed that genotyping errors did not considerably influence our results.

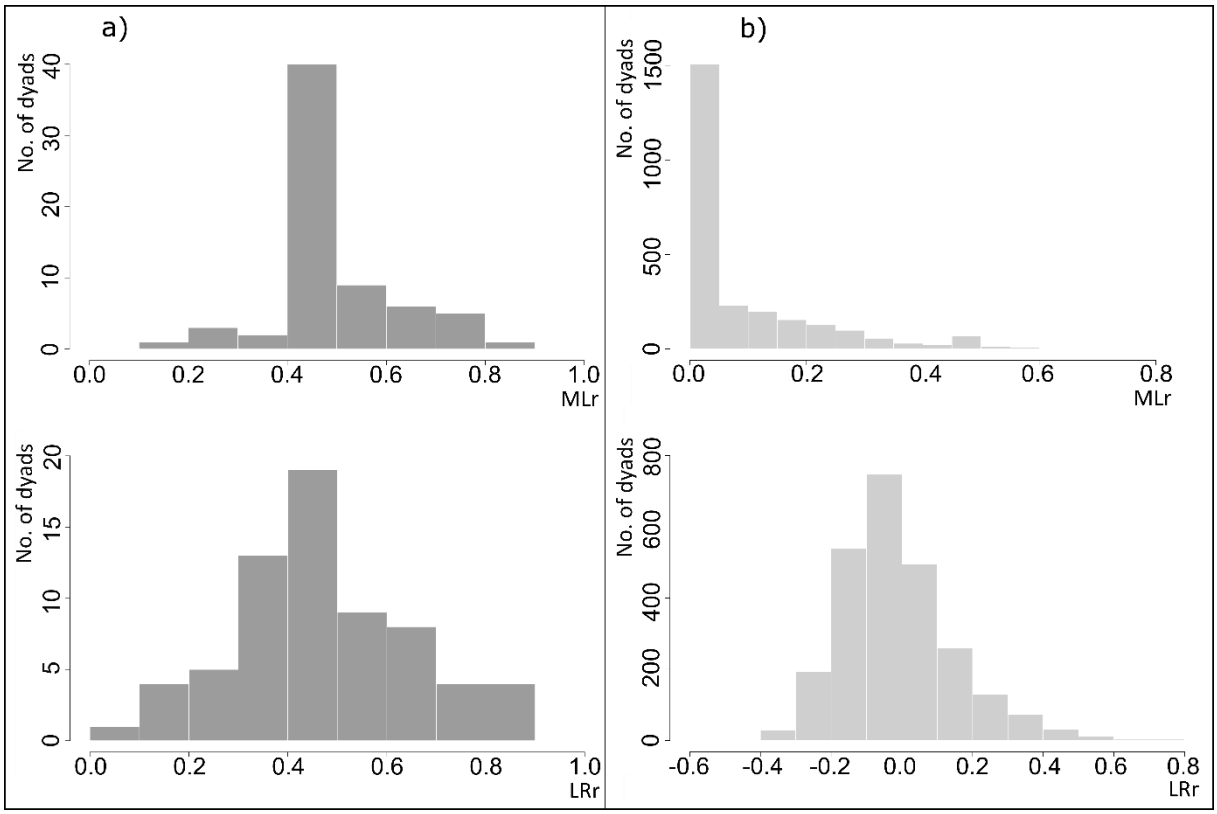

Figure AIII.1. Histograms illustrating the reliability of two relatedness estimators in the Hungarian WTE population. a Pairwise relatedness values of known parent-offspring dyads (N=67) from Hungary estimated with the maximum likelihood (***MLr***) and Lynch-Ritland (***LRr***) methods. **b** Pairwise relatedness (MLr, LRr) values of dyads of nestlings and presumably not related individuals identified from moulted feathers across Hungary.

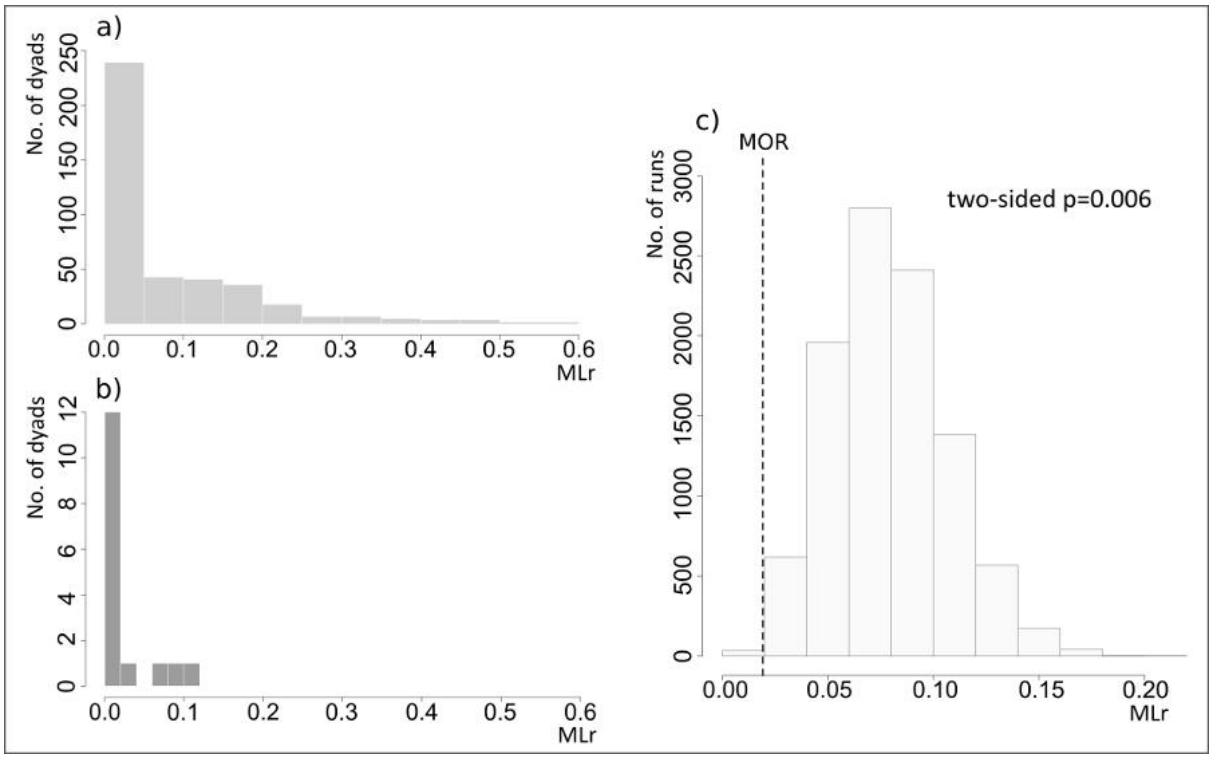

Figure AIII.2. Histograms of pairwise intersexual genetic relatedness of resident white-tailed eagles in south-western Hungary, including the Boronka forest. a Pairwise intersexual MLr values of each possible dyads of resident females (N=24) and resident males (N=17) in south-western Hungary. **b** Observed pairwise maximum likelihood relatedness (***MLr)*** of breeding pairs (N=16) across south-western Hungary. **c** Null distribution of mean pairwise MLr assuming that resident females with known mates (N=16) in south-western-Hungary randomly choose a mate from resident males (N=17) of the same area. Permutations were repeated 10000 times. The dashed line refers to the observed mean pairwise MLr (***MOR***) of breeding pairs (N=16) from south-western Hungary.

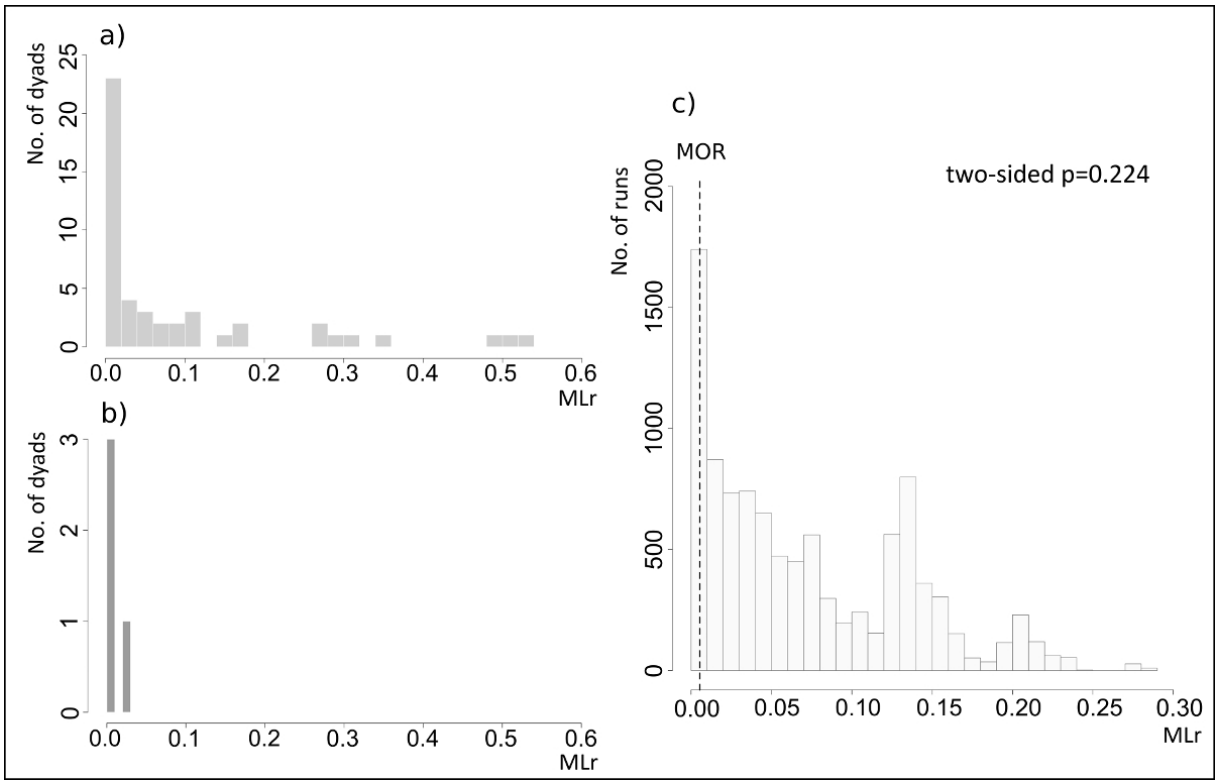

Figure AIII.3. Histograms of pairwise genetic relatedness of male intruders and resident females in the Boronka forest. a Pairwise maximum likelihood relatedness (***MLr***) values of each possible dyads of intruder males (N=4) and resident females (N=12). **b** Observed pairwise MLr of intruder male and resident female dyads in nest site intrusions. **c** Null distribution of mean pairwise MLr of intersexual intruder-resident dyads assuming that males randomly choose a territory to visit. Permutations were repeated 10000 times and in each run a single territory was randomly chosen for each intruder from available Boronka territories (8 in each year, where the resident pair was known in the year of its intrusion). The dashed line refers to the mean observed pairwise MLr (***MOR***) of dyads of intruder males and resident females.

Table AIII.1. Observed (*Obs.*) mean intersexual relatedness values and the direction of their deviation from most frequent values (*D.F.*) and median (*D.M.*) of values in the null-distributions assuming random mate choice (i.e. dyads are breeding pairs) and nest site intrusion (i.e. dyads consist of an intruder and an opposite-sex resident). Relatedness was estimated by the maximum likelihood (***MLr***) and Lynch-Ritland (***LRr***) methods. Two-sided p-values (***P***) were calculated following Phipson and Smyth (2010). For simplicity, we only show results on females' mate choice, but note that the results were similar when we assumed that resident males would randomly choose a mate.

		MLr					LRr				
Dyads	**N**	**Obs.**	**SD**	**D.F.**	**D.M.**	**P**	**Obs.**	**SD**	**D.F.**	**D.M.**	**P**
Breedig pairs (Boronka)	12	0.013	0.030	lower	lower	***0.010***	-0.099	0.074	lower	lower	*0.070*
Breding pairs (SW Hungary)	16	0.019	0.037	lower	lower	***0.006***	-0.083	0.077	lower	lower	*0.069*
Female intrusion	9	0.037	0.055	lower	lower	0.189	-0.034	0.11	lower	lower	0.401
Male intrusion	4	0.005	0.010	similar	lower	0.224	-0.064	0.014	lower	lower	0.284

Table AIII.2. Results of parentage, maternity and paternity analyses calculated in Cervus (Kalinowski et al. 2007). We tested the reliability of our assignments of parent-offspring relationships using the genotypes of all nestlings and all parents, without considering the information on true relationships in the analyses. Genotypes of each known parent-offspring dyad were matching across all genotyped loci. For nestlings, the table shows the number of dyads (***maternity*** or ***paternity***) and triads (***parentage***) where the true parent(s) of each nestling got the highest LOD score (***most likely candidate***) or the second highest LOD score (***second candidate***). To test whether 15 intruders sampled in nest sites in the Boronka forest may have originated from this population, we performed similar analyses: candidate parents were chosen from resident males (N=20) and females (N=27) from Hungary, out of which 12 and 11 were residents from the Boronka forest, respectively. Note that one female being an intruder in 2014 and resident in 2015 was considered as intruder, and another female which was observed as intruder but was already resident in another territory was considered as resident in these analyses. The table shows the number of intruder-resident dyads and triads with matching genotypes containing the most likely or second candidate parent(s), being either residents of the Boronka forest or another Hungarian area. The number of dyads and triads with significant Delta score at a 95% or 80% confidence level are also shown.

		Parentage	Maternity	Paternity
True parent(s)-offspting relationship	*No. of nestlings*	*25*	*35* [1]	*32*
	Most likely candidate	23	29	27
	Second candidate	2	4 [2]	5 [2]
	Significant with 95% confidence	3	13	10
	Significant with 80% confidence	12	26	21
Candidate parent(s) from the Boronka	*No. of intruders*	*15*	*15*	*15*
	Most likely candidate	0 [3]	1	5
	Second candidate	0	5	4
	Significant with 95% confidence	0	0	2
	Significant with 80% confidence	0	0	3
Candidate parent(s) from other Hungarian areas	Most likely candidate	1 [3]	11	3
	Second candidate	0	6	2
	Significant with 95% confidence	0	2	0
	Significant with 80% confidence	1	5	0

[1] Cervus failed to find the true mother as most likely or second candidate in maternity analysis for two nestlings.
[2] For one nestling the maternity analysis suggested a wrong individual (i.e. different from its true parent) as most likely candidate parent, with its Delta being significant with the relaxed (80%) confidence level. The paternity analysis similarly failed for another nestling.
[3] In the parentage analysis for a single intruder, the most likely candidate father, but not the mother was a resident from the Boronka forest. This result is therefore not shown in the table.

Table AIII.3. List of individuals identified from moulted feathers in the Boronka forest between 2013 and 2015 regarding territory, status and sex. The number of moulted feathers from which an individual was identified (***N feathers***), the number of sampled nestlings with matching genotypes from the same territory (***N nestlings***) and the years when a particular individual was sampled or its presence as resident was verified with matching nestling genotypes (***Years***) are also shown.

Territory	Individual	Status	Sex	Age	N feathers	N nestlings	Years
B-T1	B1-T-2013	resident	F [1]	adult	3	3	2013-2014
	B1-T-2015	resident	F [1]	-	4	2	2015
	B1-H-2013	resident	M	-	5	5	2013-2015
	HA a157	intruder	F	-	1	-	2014
	HA a328	intruder	M	-	1	-	2015
B-T2	B2-T-2013	resident	F [2]	adult	7	1	2013-2015
	B2-H-2013	resident	M	adult	4	1	2013-2015
	HA a351	intruder	F	juvenile	1	-	2015
B-T3	B3-T-2013	resident	F	adult	5	-	2013-2015
	B3-H-2013	resident	M	-	4	-	2013-2015
	Juv6-B3-2014	intruder	F	juvenile	1	-	2014
	HA a456	intruder	F	-	1	-	2015
B-T4	B4-T-2013	resident	F	adult	5	-	2013-2014
	B4-H-2013	resident	M	-	3	-	2013-2014
	B4-H-2015	resident	M	adult	3	-	2015
	Juv2-B4-2013	intruder	F	juvenile	1	-	2013
	Juv4-B4-2014	intruder	F	juvenile	1	-	2014
	HA a246	intruder	F	-	1	-	2014
	HA a51	intruder	M	-	1	-	2013
	Juv1-B4-2013	intruder	M	juvenile	1	-	2013
	Juv5-B4-2014	intruder	M	juvenile	1	-	2014
	HA a359	intruder	M	-	1	-	2015
	B4-JuvT-2015	unknown	F	juvenile	2	-	2015
B-T5	B5-T-2013	resident	F	adult	5	2	2013-2015
	B5-H-2013	resident	M	adult	3	2	2013-2015
B-T6	B6-T-2010	resident	F	adult	8	4	2013-2015
	B6-H-2010	resident	M	-	6	4	2013-2015
B-T7	B7-T-2013	resident	F	-	7	2	2013-2015
	B12-H-2015	resident	M	-	2	2	2015
	HA a70	intruder	F	-	1	-	2013
B-T8	B8-T-2013	resident	F	adult	10	3	2013-2015
	B8-H-2013	resident	M	-	1	3	2013-2015
	HA a369	intruder	-	juvenile	1	-	2015
B-T9	B9-T-2012	resident	F	adult	1	1	2013
	B9-H-2013	resident	M	-	1	1	2013
B-T10	B13-T-2015	resident	F	adult	3	2	2015
	B13-H-2015	resident	M	-	2	2	2014-2015
B-T11	B11-T-2014	resident	F	adult	3	-	2014
	B11-H-2014	resident	M	-	3	-	2014

[1] The female known as resident in 2013 and 2014 of BT-1 was replaced by a new female by 2015. The new female was already present in 2014 as intruder in the same nest site (identified from one moulted feather; this event is not shown in the table).

[2] The resident female of B-T2 visited the nest site of B-T10 in 2015 (identified from one moulted feather; this event is not shown in the table).