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Genetic Analyses of the Red-footed Falcon (*Falco vespertinus*) – Population Structure and Alternative Reproductive Strategies

PhD Thesis

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Introduction

According to the Red List of the International Union for Conservation of Nature (IUCN), currently 226 bird species are considered critically endangered and in total 14% of them are believed to be threatened by extinction (1). Today, in serious situations conservation efforts do not rely only on habitat protection, reintroduction, and breeding programmes, but also involve genetic tools. Application of genetic markers has become highly important in studying the population structure, behavioural ecology, mating system and even in wildlife forensics, as this kind of 'molecular vision' gives insight to a world invisible via conventional field observation methods. In this way, conservation genetics has become an interdisciplinary field of science (Frankham 2003, Frankham 2005, Selkoe & Toonen 2006).
Nowadays, one of the most important tools in conservation genetics is microsatellite markers (SSRs, STRs) and SNPs. Although, many uncertainties have arisen about using STRs in population genetics and parentage analyses, they are still used keeping in mind their limitations (Putman & Carbone 2014, De Barba et al. 2017, Flanagan et al. 2019).

Present dissertation focuses on the conservation genetics of the Red-footed Falcon (*Falco vespertinus,* RFF) by the application of microsatellite (STRs, SSRs) markers.

RFF is a small raptor species that occupies large open grasslands across Eastern Europe and Asia, while the westernmost border of its range is Hungary (2, Palatitz et al. 2018, Purger 1998, Krištín et al. 2017). Interestingly, the species is a facultative colonial breeder and does not build its own nest, but usually uses the nest of other species from the family Corvidae (e.g. *Corvus frugilegus*, *Pica pica*, *Corvus cornix*). It has a high conservation value both in Hungary and internationally due to global population decline (Birdlife International 2015, 2). In the 20th century, it went through a dramatic population decrease in the Carpathian Basin as well, due to the expanding agriculture and the eradication of rooks. As a result, the population reduced from 2200-2500 breeding pairs to 500-600 pairs by 2006 and vanished from the Transdanubia region. Besides, most of the remained breeding pairs were forced to breed solitary (Keve & Szijj 1957, Fehérvári et al. 2008, Palatitz et al. 2015). Since then, many Hungarian and international programs were launched to prevent the decline (3). As part of the first LIFE program between 2006 and 2009, artificial nestboxes and colonies were introduced to offer favorable breeding sites and stabilise the population. Due to the conservation measures the number of breeding pairs started to increase and currently there are 1200-1300 pairs in Hungary (Palatitz et al. 2018). Also, the successful artificial nestbox scheme provided better

opportunities to sample and observe the individuals, study the species and get better understanding of population structure and reproduction biology. Despite the fact that the Red-footed Falcon is a well-studied species, there was no genetic knowledge available about it.

Aims

In our study, the population structure analyses initiated by a LIFE project (Conservation of the Red-footed Falcon in the Carpathian Basin, LIFE11/NAT/HU/000926) were completed with the assessment of the alternative reproductive strategies the species might exhibit. We performed the analyses with the usage of microsatellite markers, thus the aims of the present dissertation were the following:

1. Marker development
    1.1. Create a powerful marker set of new species-specific microsatellites for the RFF

2. Population structure
    2.1. Compare the genetic structure of the Hungarian breeding sites
    2.2. Compare the genetic structure of colonies in the Carpathian Basin and beyond
    2.3. Conduct a population assignment of individuals from a Romanian pre-migration site

3. Alternative reproductive strategies
    3.1. Confirmation of extra-pair paternity (EPP)
    3.2. Confirmation of intraspecific brood parasitism (IBP)
    3.3. Detecting other types of alternative reproductive strategies

Materials and Methods

Samples

All samples of RFFs were collected by the members of the Red-footed Falcon Conservation Workgroup of MME/Birdlife Hungary between 2008 and 2017. Permits have been assured by the Hungarian authorities: OKTF-KP/56-26/2015, PE-KTFO/1867-10/2018, PE-KTFO/1867-11/2018, PE-KTFO/1867-9/2018.

The marker development tests were conducted on 29 RFFs and 24 individuals of six closely related species (*F. peregrinus* (n=10), *F. tinnunculus* (n=8), *F. rusticolus* (n=3), *F. columbarius* (n=1), *F. subbuteo* (n=1) and *F. cherrug* (n=1)) using three types of samples: blood, feather and skin. In the case of RFFs, blood samples were collected between 2008 and 2015 from sites assigned by the former LIFE program: Conservation of *Falco vespertinus* in the Pannonian Region (LIFE05 NAT/H/000122). Feather and toe pad samples of the related species were provided by the Hungarian Natural History Museum, MME Birdlife Hungary and the Kecskemét Zoo.

Blood and/or feather samples of the population genetic analyses were collected from 120 RFF individuals (N_{female}=62 and N_{male}= 58) between 2015 and 2017. In total 21 adults, 99 chicks and juveniles were involved in the final analyses. They were categorised into eight major groups in Hungary and three in Romania according to their geographical location. Hungary: Borsodi mezőség (n=10), Csanádi-puszták (n=10), Cserebökény (n=10), Hevesi Füves Puszták (n=10), Hortobágy (n=10), Jászság (n=10), Kiskunság (n=10), Vásárhelyi-puszták (n=10). Romania: West region colonies (n=10) close to the Hungarian border, Southeast region colonies (n=10) over the Carpathians and Southeast Region pre-migration site close to the Black Sea (n=20).

For the study of alternative reproductive strategies, blood samples were collected from 128 chicks and 82 adults from 48 RFF families between 2008 and 2015 from the Vásárhelyi-Grasslands in the Körös-Maros National Park. After excluding multiple samplings 97 chicks and 74 adults from 37 families were used in the analyses.

In all cases, adults were captured by a net and identified using colour-rings. They were regarded as social (putative) parents if they regularly participated in incubation (including males), fed the chicks, behaved like a social pair and they were identified at least two independent occasions during the incubation and rearing period using telescope or camera traps.

All samples were stored at -20°C or in cryotubes in ethyl-alcohol (96%), also at -20°C in order to avoid degradation.

DNA extraction

DNA was extracted with the Genomic DNA Mini Kit (Geneaid®, New Taipei City, Taiwan), using the standard protocol provided by the manufacturer. To facilitate the digestion of feathers and toe pads, 10µl DTT (1.4-dithiothreitol, 1M) was added (Weigmann, 1968), and samples

were incubated at 60°C overnight and for 24 hours, respectively. Blood samples were incubated for 1.5 hours also at 60°C. In the end, DNA was eluted in 100 µl of elution buffer and stored at -20°C.

Microsatellite markers

In the study, cross-species and species-specific markers were used in different combinations. The latter type of markers was developed in the present study using the services of the Ecogenics GmbH. The institution designed the microsatellite candidates of the RFFs based on the samples of four *F. vespertinus* individuals. The library was analysed on an Illumina MiSeq platform using the Nano 2x250 v2 format (Balgach Switzerland). After getting the candidates, a PCR optimisation was performed by changing the concentration of $MgCl_2$ and DNA in the reaction mixes and the PCR conditions. From the original 44 tested primers pairs, ten (FalVes_04 FalVes_05 FalVes_13 FalVes_15 FalVes_26 FalVes_28 FalVes_30 FalVes_31 FalVes_38 FalVes_43) were applied and two additional markers (FalVes_03 and FalVes_34) were also published, but not used in the later analyses (Magonyi et al. 2019). The seven cross-species markers were: Fnd2.3 (Padilla et al. 2009), Fr34 (Nesje & Roed 2000), Fp82-2, Fp89, Fp54, Fp92-1 (Nesje et al. 2000) and Fp347 (Nittinger et al. 2007).

PCR analyses and conditions

All loci of the marker development were amplified in a 17 µl reaction mix, with slight differences in $MgCl_2$ and primer concentrations. All reactions happened in simplexes. Protocols and conditions are described in Magonyi et al. (2019).

In the case of kinship analysis, three different PCR protocols were used. The protocol and conditions for the cross-species markers (Fp89, Fp82-2, Fnd2.3, Fr34, Fp347 and Fp54) were identical to the one used for the first group of species-specific markers (FalVes_13, FalVes_26, FalVes_31 and FalVes_38) and they were amplified in simplexes. Additional information of PCRs used in the parentage analyses can be found in Magonyi et al. (2021).

In the case of population genetic analysis, the amplification of cross-species markers was performed in two different mixes containing multiple markers. *Mix1* contained Fp89, Fr34 and Fp54. *Mix2* contained Fp92-1, Fp347 and Fnd2.3 markers, while the Fp82-2 marker was amplified on its own. The species-specific markers, Falves_15 and Falves_26 were amplified in simplex reactions, as described in Magonyi et al. (2019), while FalVes_31 and FalVes_28

could be amplified in a duplex reaction. Both reaction mixes of the cross-species markers were amplifiable under the same PCR conditions differing from the one used for the species-specific marker.

In cases when molecular sex determination was needed, the intron 16 of CHD1 gene (chromodomain helicase DNA-binding) was used (Fridolfsson & Ellegren 1999, Suh et al. 2011).

PCR products were screened on 2% agarose gel by electrophoresis.

Programs and statistics

In order to identify misleading complex motifs, the candidate sequences of potential markers were checked with MEGA 7 (Kumar et al. 2016).

PCR products were analysed by capillary electrophoresis using the internal size standard GS500LIZ (Applied Biosystems, USA). Fragment lengths were determined using Peak ScannerTM Software v.1.0 (Applied Biosystems, Foster City, CA). Validation of peak reads in the electropherograms performed by re-genotyping of 30 RFF individuals on a different plate and scoring was done by two persons independently for all samples.

The presence of null alleles was estimated with the Micro Checker v.2.2.3 program (van Oosterhout et al. 2004). Basic statistics (N_a, H_e, H_o and PI) were calculated with GenAlEx v.6 (Peakall & Smouse, 2012). Deviation from the Hardy-Weinberg equilibrium (HWE) and possible linkage disequilibrium (LD) between loci were tested with Arlequin 3.5.2.2 (Excoffier & Lischer 2010).

For the estimation of genetic differentiation between breeding sites, F_{ST} (Weir & Cockerham, 1984) were calculated also by Arlequin 3.5.2.2 (Excoffier & Lischer 2010). Genetic structure was illustrated by Structure v.2.3.4 (Stanford University, USA, 2012). The map of sampling sites was created using the Free and Open Source QGIS 3.4.15 (http://qgis.org).

Parentage analysis was performed manually and with using Cervus 3.0 (Kalinowski et al. 2007). The program is based on the calculation of LOD (log of overall likelihood ratio) scores for construction of statistical tests in parentage analyses.

Results and Discussion

Marker development

Prior to the launch of the successful nestbox scheme, collecting reliable samples from RFFs proved to be problematic. Only field monitoring data or its combination with cross-species genetic markers could not provide enough information to conduct population structure and kinship analyses. Therefore, development of species-specific microsatellites was needed.

After PCR optimisation, out of 44 tested primer pairs 12 were reliably amplifiable and polymorph enough. In further analyses of population genetics and alternative reproductive strategies, ten were successfully used, while two remaining markers (FalVes_03 and FalVes_34) were excluded due to incomprehensible results.

The number of alleles per locus ranged from 6 to 26 (mean 13.4). The mean expected heterozygosity (H_e) was 0.82 (0.59-0.93) and the mean observed heterozygosity (H_o) was 0.69 (0.31-0.93). Significant linkage disequilibrium was found between FalVes_13 and FalVes_26 (p=0.0064), although it was not significant after Bonferroni correction (corrected alpha=0.0011). FalVes_04, FalVes_30 and FalVes_43 deviated significantly from Hardy-Weinberg equilibrium and Micro Checker also detected possible null alleles at these loci (F_{null}= 0.177-0.232). The combined probability of identity (PI) was 8.2×10^{-15} and the probability of identity between siblings (PI_{SIBS}) was 2.8×10^{-5}.

All ten new markers and FalVes_03 were tested for cross-amplification on six closely related species (*F. peregrinus*, *F. tinnunculus*, *F. rusticolus*, *F. columbarius*, *F. subbuteo* and *F. cherrug*) and proved to be applicable, especially for *F. peregrinus* and *F. tinnunculus.*

In the population structure analyses four markers (FalVes_15, FalVes_26, FalVes_28 and FalVes_31) were used in combination with cross-species markers for genotyping 120 individuals. Except of FalVes_31 they were not in Hardy-Weinberg equilibrium (HWE) and showed the presence of null alleles. To verify the results of this complete marker set, the analysis was repeated after the exclusion of the two most problematic loci − FalVes_15 and Fp92-1 ($P \geq 0.1$) from the cross-species markers − giving the same findings.

In the analyses of alternative reproductive strategies all ten markers were tested on 210 individuals, although FalVes_04, FalVes_15 and FalVes_43 were excluded due to the presence of null alleles and unclear peaks in the electropherograms. In the case of FalVes_26 and FalVes_28, the signs of null alleles were also detected, although, due to their high

polymorphism and reliable amplification they were not excluded. Instead, the results were checked with the Cervus software allowing only one mismatch, while in the manual analysis two mismatches were needed to accept it as valid extra-pair fertilisation. Manual and software analyses provided the same results.

In addition to the analyses described above, our markers can be applied in wildlife forensics, as some of the tested species are commonly used in falconry and many times are concerned in forensic affairs. Some of the markers have already proved to be useful in one case of the Peregrine Falcon in an unpublished forensic case reported by the Hungarian authorities.

In summary, these new markers served as a base for studies to support the conservation management of RFFs making the species a potentially good candidate model of parental care, mate choice and avian coloniality. Even with their limitations, the markers could facilitate analyses of population structure, mating system, and in the future any further studies where it is unquestionably important to genotype every single individual.

Population structure

Compared to the global distribution of RFFs, the Hungarian colonies are located relatively close to each other, while the studied Romanian breeding site is separated by the Carpathian Mountains. In the analysis, 120 individuals were genotyped with ten microsatellite markers (six cross-species and four species-specific markers) in order to map the population structure of the Carpathian Basin and compare it with the Romanian breeding site.

Significant linkage disequilibrium was found by Arlequin 3.5.2.2 between several pairs of loci in several locations but without any consistency. Fp92-1 (p= 0.000428), FalVes_15 (p=0.00001199), FalVes_26 (p=0.0003471) and FalVes_28 (p=0.00227) deviated significantly from Hardy-Weinberg equilibrium. Micro Checker v.2.3.3 also detected possible null alleles at several loci: Fp92- 1 (F_{null}=0.2097), Fp89 (F_{null}=0.086), FalVes_15 (F_{null}=0.142) FalVes_26 (F_{null}=0.099), FalVes_28 (F_{null}=0.065). The probability of identity (PI) value of the marker set was between 2.3×10^{-11} and 3.6×10^{-10} for increasing locus combinations. The highest observed heterozygosity (H_o) was 0.71 in Hortobágy and the highest allelic diversity (A) was 7.2 in Csanádi-puszták. In this case of species-specific markers, the alleles found per locus ranged from 5 to 16 (mean 9 ± 4.05) and for species-specific markers from 11 to 39 (mean 21.25 ± 12.23). In total, the mean was 13.9 ± 9.95 and the mean of the alleles found per locations was 6.56 ± 0.4.

According to the AMOVA test done with Arlequin 3.5.2.2, the molecular variance among individuals is 15.44%, within individuals 84.31% and among populations 0.24%. Accordingly, the F_{ST} value was 0.00247 (p=1), while the F_{IS}=0.1548 (p=0). Among the pairwise F_{ST} values none was statistically significant. The Mantel test showed no correlation between pairwise F_{ST} values and pairwise geographical distances r = -0.188313 (p=0.795).
Similarly, the Structure v.2.3.4 (Stanford University, USA, 2012) analysis based on F_{ST} values could not differentiate the breeding sites. The number of possible populations (K) was set from 1 to 10, and the highest likelihood scores were given in case K=1.

Since the larger sample size shed light on the higher presence of null alleles both in cross-species and species-specific markers, to overcome the limitation of the marker set, results were verified with excluding the most problematic markers (Fp92-1 and FalVes_15, P≥0.1). This time, the AMOVA showed a slight differentiation (2.7% - 3.5%) between three Hungarian colonies, while the Mantel test showed no effect of distance again (r=-0.199337, p=0.759). Structure still showed no differentiation, and the highest likelihood scores were given in case K=1 again.

Since the RFF is long-distance migrant, the species is able to fly several thousands of kilometres and overcome many geographical barriers, like the Mediterranean Sea and the Sahara (Palatitz et al. 2018). Besides, ~3500 individuals were estimated on the biggest reported pre-migration site in Hungary (Borbáth & Zalai 2005) and in 2014 approximately 11 600 individuals were counted in the Carpathian Basin during the same period of autumn migration (Palatitz et al. 2015). Considering the current number of breeding pairs in Hungary (1200-1300) and slightly higher numbers in Romania (1300-1600 individuals), Hungary is presumably part of the autumn migration route of individuals living in the remote parts of their range (Palatitz et al. 2018). Additionally, considering the fact that they fly back through Western Africa during the spring migration, it is assumed, that the Carpathian Basin forms part of both the spring and the autumn migration (Palatitz et al. 2018). These findings highlight the conservation biological importance of the region, as it was the case for the Prairie Falcon (Doyle et al. 2018).

In summary, based on the AMOVA, the variance deriving from population differentiation is only 0.24% suggesting that the allele frequencies are similar in the sampled regions and we could not detect differences in genetic structure. Besides, since there was no correlation between the pairwise F_{ST} values and geographical distances, Mantel test supported that the

Carpathians − as a potential barrier − do not obstruct gene flow, nor do the geographical distances within the sampling area. With the usage of microsatellites, we can support that the Red-footed Falcons of the sampling area form a random mating population and conducting international conservation programs to manage them together is advised. However our findings could be further strengthened by the inclusion of additional genetic markers and samples from the eastern part of the breeding area in a subsequent study.

Population assignment was not feasible due to the lack of genetic differentiation between the studied colonies of the Carpathian Basin and the Southeast region colony over the Carpathians.

Alternative reproductive strategies

Raptors are seemingly monogamous species, but many of them use alternative reproductive strategies and based on observations, RFFs do so as well (Negro et al. 1996, Rosenfield et al. 2015). Since life history traits and mating system are important key factors in conservation, it is essential to study them in order to take effective conservation measures (Sutherland 1998, Caro 2007, Pryke et al. 2012, Garnier et al. 2012).

Our results are based on genotyping 171 individuals (74 adults and 97 offspring) from 37 RFF families with seven species-specific and five additional cross-species microsatellite markers.

The probability of identity (PI) value of the marker set of the 12 loci was $PI=9.8\times10^{-15}$ and for siblings it was $PI_{SIBS}=1.4 \times 10^{-5}$. The exclusion power (EP) value when both parents were known was $EP1=9 \times 10^{-9}$, when only one parent was known $EP2=9.3 \times 10^{-6}$ and $EP3=9 \times10^{-4}$ with no parent known (Calculated with GenAlEx 6.4, Peakall & Smouse 2012). The observed heterozygosity (H_o) was 0.738 (0.415-0.915) and the expected heterozygosity (H_e) was 0.745 (0.445-0.922). In total, 170 alleles were found (179 including the chicks), the mean was 14.167. The Micro Checker found one locus (FalVes_26 – F_{null}=0.1058) affected by null alleles because of homozygote excess. Cervus 3.0 (Kalinowski et al. 2007) software also found the social parents of every offspring to be identical with the genetic parents, except for the cases which were identified as EPP (extra-pair paternity), QP (quasi-parasitism) or IBP (intraspecific brood parasitism) manually as well.

We found a low rate of EPP (2.7% of nests and 2.06% of chicks) fitting into the trend of other Falco species (Swatschek et al. 1993, Warkentin et al. 1996, Korpimaki et al. 1996, Negro et al. 1996, Villarroel et al.1998, Alcaide et al. 2005) and also a low rate of IBP (3.7% of nests

and 1.03% of chicks). Furthermore, we found the first case of QP in a Falco species and polygamy in the RFF.

Since we could find only one affected nest in each case of alternative strategy, we could only report them and provide some ideas for better understanding the mating system of the species and for future studies. We could only detect the successful cases of IBP, while burying the eggs or rolling them out of the nest is also common in the case of RFFs (Palatitz et al. 2018). Based on this fact, we suppose that IBP might be higher and a real strategy, instead of a mere coincidence. Besides, the fixed clutch size detected in RFFs, but not typical of Falcons might also play a role as a strategy against raising extra-pair chicks.

However, we might have to take into account the impact of human intervention as well, as the nestboxes have a different construction compared to a natural nest. There are already proven effects of the nestboxes in RFF colonies; Bragin et al. (2017) found earlier egg laying and − in the case of nestboxes which were placed on forest edges − higher offspring loss, too. The previously mentioned case of polygamy might indicate that the closed nest boxes have a role in the choice of strategy, since the same male was feeding in two nestboxes at the same time and on the same tree with the entrance holes in the opposite direction.

All things considered, it is essential to implement further studies about the natural colonies because at this point, we could only detect the presence of IBP but not confirm that it is a hidden natural strategy or the side effect of a successful conservation measure of the nestboxes. In the latter case, mate choice and parental care system of the species might be altered by human interventions resulting in long-term consequences on the mating system. Another possible outcome is the increased rate of desertion by females and the reduction of clutch size lowering the reproductive success affecting the population dynamic as well.

Regardless of the underlying mechanism, our current results are the first about EPP, IBP, polygamy and quasi-parasitism in the Red-footed Falcon, increasing the number of bird species where these phenomena were documented. Further research based on these new results about this colonial raptor might also help to understand how the mating system, life history traits and conservation management interact.

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Magonyi, N. M., Mátics, R., Szabó, K., Fehérvári, P., Solt, S., Palatitz, P., & Vili, N. (2019). Characterization of novel microsatellite markers for the red-footed falcon (*Falco vespertinus*) and cross-species amplification in other Falco species. *European Journal of Wildlife Research, 65*(4). https://doi.org/10.1007/s10344-019-1300-8

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1. Magonyi, N. M., Vili, V,, Szabó, K., Fehérvári, P., Kumar, S. R. & Mátics, R. (2018) **New microsatellite markers for a protected raptor species, *Falco vespertinus.*** Student Conference on Conservation Science (SCCS) , Centre for Ecological Research – (Poster presentation)

2. Magonyi (2019) **Fifty Shades of Blue.** Student Conference on Conservation Science: SCCS 2019, Centre for Ecological Research − (Presentation)

3. Magonyi (2019) **Extra-pair paternity and intraspecific brood parasitism in the Red-footed Falcon (*Falco vespertinus*) using species-specific and cross-species microsatellite markers**. International Symposium on Reproductive strategies - Reproductive strategies from genes to societies 2019 − (Presentation)

4. Magonyi, N. M. (2015) **Egyedi azonosítás − egyedi módszer: A DNS barcoding felhasználási lehetőségei (Unique identification – unique method: Possible uses of DNA barcoding)**, XX. Bolyai Conference, Eötvös Loránd University– (Presentation)

5. Magonyi, N. M. (2015) **Észak-Vietnámból származó denevérminták filogenetikai vizsgálata - Két új faj felfedezése (Phylogenetic study of bat samples from North Vietnam - Discovery of two new species)**, Students' Scientific Conference of Biology, Eötvös Loránd University – (Presentation)

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