

Genetic variability and population structuring in the European Lanner Falcon *Falco biarmicus feldeggii*

MAURIZIO SARÀ^{1,3,*}, CHIARA MENGONI², NADIA MUCCI², ENRICO GUZZO³, MILAN RUZIC⁴, MIRKO AMATO⁵, NICOLA ANTIOCO³, GIOVANNI BOANO⁶, SALVATORE BONDI³, GIOVANNI LEONARDI⁷, ANGELO NARDO³, ROSARIO MASCARA³, ADUA OSSINO⁵, ELISA VITALE³, LAURA ZANCA³

¹ Section Animal Biology, STEBICEF Department - Via Archirafi 18, 90123 Palermo, Italy

² Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA) - Via Ca' Fornacetta 9, 40064 Ozzano dell'Emilia (BO), Italy

³ Falcon Conservation - Via Maqueda 110, 90134 Palermo, Italy

⁴ Bird Protection and Study Society of Serbia - Vladike Cirica 24, 21000 Novi Sad, Serbia

⁵ Studio-Natura Mediterraneo, Environmental Studies - Via Cannellazza V strada 3, 96013 Carlentini (SR), Italy

⁶ Museo Civico di Storia Naturale di Carmagnola - Torino (Italy)

⁷ Hierofalcon Research Group - Via Stazzone 235, 95124 Catania (Italy)

* Corresponding author: maurizio.sara@unipa.it

Abstract – We analysed variation in 10 polymorphic microsatellites and a variable portion of control region of mtDNA in 24 specimens from 3 populations of European Lanner Falcon *Falco biarmicus feldeggii* living in Sicily, continental Italy and the Balkan area to assess species' genetic diversity and population structure in the poorly investigated range of this threatened subspecies. We considered also a dataset of previously published mtDNA sequences of the other Lanner Falcon subspecies and of *Hierofalco* subgenus members (*F. cherrug*, *F. rusticolus* and *F. jugger*) to outline the genetic variation in the region on a wide-ranging basis. Regard with mtDNA we identified 6 haplotypes from our 24 European Lanner Falcon specimens, 3 of which were new and unique (1 Sicilian, 2 Balkans) and the 3 others already known and shared with other Hierofalcons. The 62.5% of our sample, including 14 of Sicilians and one Apulia specimen, belonged to haplotype H_24 shared with *F. c. cherrug*, *F. rusticolus* and *F. jugger*. MtDNA analyses of European Lanner Falcons showed a dispersed pattern of our specimens inside the main *Hierofalco* clades and haplo-groups in a way congruent to what found in recent literature. These analyses confirmed that none of the Hierofalcons form a monophyletic group, nonetheless the Lanner Falcons can be subdivided in two major Palaearctic (*F. b. feldeggii*, *F. b. erlangeri* and *F. b. tanypterus*) and sub-Saharan African (*F. b. biarmicus* and *F. b. abyssinicus*) clades. Microsatellites analysis yielded a first outline of population genetic structure, with genetic identity between continental Italy and Sicily and a moderate degree of differentiation of the Balkan area with Sicily and continental Italy. The 3 populations did not show significant departure from Hardy-Weinberg equilibrium, with low values of the inbreeding coefficients and had allele richness and haplotype diversity consistent with literature. Microsatellites analysis (N_m , frequency of private alleles) suggests a gene flow among the three examined populations and the connection of Sicilian population to those of mainland.

Key-words: genetic structuring, *Hierofalco*, Lanner phylogenesis, microsatellites, mtDNA.

INTRODUCTION

Genetic factors affect the extinction risk of small animal populations and their investigation has gained a predominant role in conservation biology in the last fifty years (e.g. Frankel & Soulé 1981). Levels of population inbreeding and their harmful effects on reproduction and survival, loss of genetic diversity and hence of ability to evolve in response to environmental change, and reduction in gene flow due to fragmentation of populations, are just some of the indispensable topics that researchers and manag-

ers need to quantify in order to preserve and restore endangered species (Frankham *et al.* 2002). In addition, the collection of detailed information at the genetic level is a mandatory prerequisite for the correct planning of potential restocking/reintroduction programmes for severely threatened species (IUCN/SSC 2013).

The Lanner Falcon *Falco biarmicus* is a polytypic species with a Mediterranean and Afrotropical distribution, whose phenotypic variability has been currently described in five geographically defined subspecies across the distribution range. The nominate *F. b. biarmicus* covers south-

ern Africa, *F. b. abyssinicus* western, central and eastern Africa, *F. b. tanypterus* Israel, Arabian Peninsula, extending to the eastern part of northern Africa, *F. b. erlangeri* north-western Africa (from Morocco to western Libya), and finally *F. b. feldeggii* lives in Italy (south to the Po plain, from Emilia Romagna to Sicily), Balkan areas (Croatia, Bosnia, Serbia, Macedonia and Greece), Turkey and Southern Caucasus countries (Armenia, Georgia and Azerbaijan) (Ferguson-Lees & Christie 2001, Nittinger *et al.* 2005). The latter subspecies currently known as ‘European Lanner Falcon’ reaches in the Balkan and Italy the species’ northernmost limit of distribution and it is of conservation concern for the dramatic decline since 1950’s (Tucker & Heath 1994, BirdLife International 2004, 2015, 2017). The European Lanner Falcon has been included in Annex I of the EU Bird’s Directive (79/409/CEE and 2009/147/CEE), and is classified as Endangered in Europe (BirdLife International 2015). National Red Lists in Italy (Peronace *et al.* 2012) and in Bulgaria (Boev & Iankov 2011) consider the respective populations as Endangered and Critically Endangered. In 1999, BirdLife international, on behalf of the European Commission, draw an International Species Action Plan for Lanner falcon (Gustin *et al.* 2002), later followed by the Italian Action Plan (Andreotti & Leonardi 2007). None of the other countries included in the *F. b. feldeggii* range have defined similar action plans, to the best of our knowledge. Nonetheless, the global conservation status of the Lanner Falcon is of ‘Low Concern’ (BirdLife International 2017) and ‘SPEC 3’ (Tucker & Heath 1994, BirdLife International 2017) because the European populations are marginal (< 1%) with respect to the species’ global distribution area and population stocks. Recently, Massa & Borg (2018) suggested to separate the European Lanner Falcon and to list it as SPEC 2 to confer a category of threat more consistent with the subspecies’ current status.

Information on population status and distribution in most of the Balkan countries is scarce and/or outdated. In Italy, several contributions addressed the ecology, distribution (e.g. Massa *et al.* 1991, Andreotti *et al.* 2008, Brunelli & Sarrocco 2012, Amato *et al.* 2014, Sarà 2014, Leonardi 2015, Sarà *et al.* 2016), reproductive biology and population trends (e.g. Allavena *et al.* 2015, Pezzo *et al.* 2016, Di Vittorio *et al.* 2017, Mascara & Nardo 2018) of this endangered species, focusing on conservation implications. Nonetheless, one basic information still lacking is those regarding the genetic variability and structuring of the Italian, Balkan and Greek populations. To the date, only a few researches (e.g. Wink *et al.* 2004, Nittinger *et al.* 2005, 2007, Fuchs *et al.* 2015) explored this topic, but they aim more at the phylogeographic reconstruction of the *Hierofalco* lineage to which the Lanner Falcon belongs together with the

Gyr Falcon *F. rusticolus*, the Saker Falcon *F. cherrug*, and the Laggar Falcon *F. jugger* (Nittinger *et al.* 2005); and at the Saker Falcon position within the *Hierofalco* (Nittinger *et al.* 2007).

This paper deals with a genetic exploration of Italian and Balkan Lanner Falcons. Our main purpose is to check whether the inclusion of more samples would confirm the previous phylogenetic relationships within the *Hierofalco* lineage. In addition, we are interested in assessing the genetic structure of these populations, with special attention to the degree of genetic diversity and identity in the island population of Sicily, and to the presence of gene flow between this latter and the continental populations of Italy and the Balkans. Most island populations have strong genetic identity paired with low genetic diversity, and are often more inbred than mainland populations (Frankham *et al.* 2002), and the Sicilian population should behave accordingly. However, thanks to recent data on the European Lanner Falcon’s dispersal (see paper of Sarà *et al.* in this same issue), we expect some connectivity among these populations and therefore a limited genetic identity and relatively higher genetic diversity of the Sicilian population. The position of Italian and Balkan Lanner Falcons within the *Hierofalco* lineage and their population genetic structuring may help the correct planning of conservation actions.

MATERIAL AND METHODS

We extracted DNA from quills of nuchal feathers (Horvath *et al.* 2005) and muscle tissues of 24 individuals, 18 coming from Italy (16 from Sicily, 1 from Emilia Romagna and 1 from Apulia) and 6 coming from the Balkan area (Table 1). Italian samples of both the continental (Emilia Romagna plus Apulia) and island (Sicily) populations came from alive/dead nestlings or from birds rescued alive or found dead in the wild near to sites of documented breeding. Their exact sampling locations are not specified here for the sake of conservation caution. The Balkan birds came from Serbian localities near the Kosovo border (FB10-15), and were captive birds maintained in aviary for falconry (Table 1). We used ZR Genomic DNA II Kit™ for solid/liquid samples (Zymo Research) to extract and purify genomic DNA from samples. DNA samples were genotyped using 10 microsatellite markers originally designed for *Falco peregrinus* (Nesjic *et al.* 2000, Mengoni *et al.* 2018): Fp13, Fp31, Fp46_1, Fp54, Fp79_4, Fp86_2, Fp89, Fp92_1, Fp107; and for *Accipiter gentilis* (Topinka *et al.* 2004): Age5. We carried out two independent PCR replicates to check the absence of Allelic Drop Out (ADO) or

Table 1. List of the 24 specimens considered in the study of genetic diversity of the European Lanner. Sample: MU = muscle tissue, FE = feather. Museum collections: 1. Istituto Nazionale per la Protezione e la Ricerca Ambientale (ISPRA); 2. Civic Museum Natural History of Carmagnola (Turin).

ID	Area	Region	Recovery date	Sex	Age	Sample	Fate/Sampling locality	GenBank Accession N
FB1	Italy	Sicily (Caltanissetta)	May 2016	M	JUV	FE	Alive/Nest	MK790074
FB2	Italy	Sicily (Caltanissetta)	May 2016	F	JUV	FE	Alive/Nest	MK790084
FB3	Italy	Sicily (Caltanissetta)	May 2016	F	JUV	FE	Alive/Nest	MK790093
FB4	Italy	Sicily (Palermo)	Mar 2016	M	SAD	FE	Alive/Rescue Centre	MK790094
FB5	Italy	Sicily (Palermo)	Aug 2014	F	AD	FE	Alive/Rescue Centre	MK790095
FB8	Italy	Sicily (Palermo)	May 2015	M	JUV	MU	Dead/Nest	MK790096
FB9	Italy	Emilia Romagna (Bologna)	Jan 2009	F	AD	MU	Dead/Museum collection 1	MK790097
FB10	Balkan	Serbia (Kosovo)	2010	F	AD	FE	Alive/Captivity Centre	MK790075
FB11	Balkan	Serbia (Kosovo)	2010	M	AD	FE	Alive/Captivity Centre	MK790076
FB12	Balkan	Serbia (Kosovo)	2010	F	AD	FE	Alive/Captivity Centre	MK790077
FB13	Balkan	Serbia (Kosovo)	2010	F	AD	FE	Alive/Captivity Centre	MK790078
FB14	Balkan	Serbia (Kosovo)	2010	F	AD	FE	Alive/Captivity Centre	MK790079
FB15	Balkan	Serbia (Kosovo)	2010	F	AD	FE	Alive/Captivity Centre	MK790080
FB16	Italy	Apulia (Foggia)	2013	M	JUV	MU	Dead/Museum collection 2	MK790081
FB17	Italy	Sicily (unknown)	2012	F	AD	FE	Dead/Rescue Centre	MK790082
FB18	Italy	Sicily (Palermo)	May 2015	M	JUV	MU	Dead/Nest	MK790083
FB20	Italy	Sicily (Palermo)	Jan 2015	F	AD	MU	Dead/Rescue Centre	MK790085
FB21	Italy	Sicily (Catania)	May 2017	M	JUV	FE	Alive/Nest	MK790086
FB22	Italy	Sicily (Catania)	May 2017	M	JUV	FE	Alive/Nest	MK790087
FB23	Italy	Sicily (Catania)	May 2017	M	JUV	FE	Alive/Nest	MK790088
FB24	Italy	Sicily (Catania)	May 2017	M	JUV	FE	Alive/Nest	MK790089
FB25	Italy	Sicily (Palermo)	May 2017	M	JUV	MU	Dead/Nest	MK790090
FB26	Italy	Sicily (Catania)	May 2017	F	JUV	MU	Dead/Nest	MK790091
FB27	Italy	Sicily (Enna)	May 2017	F	JUV	MU	Dead/Nest	MK790092

false alleles (FA). Furthermore, a 360 bp long fragment of the mtDNA control region was amplified and sequenced according to protocols described in Nittinger *et al.* (2005). In both cases we used the following PCR protocol: a first denaturation step at 94 °C for 3 min; 35 cycles at 94 °C for 40 s, 55 °C for 40 s, 72 °C for 40 s; and a final step at 60 °C for 30 min. PCR products were then processed in an ABI 3130XL sequencer.

We used Genalex 6.1 (Peakall & Smouse 2006) to estimate the allele frequencies by locus and population, mean number of alleles per locus (N_a), observed (H_o) and expected unbiased (U_{H_o}) heterozygosity and the related chi-square test (χ^2) for deviations from Hardy-Weinberg equilibrium. Pairwise F_{ST} (Weir & Cockerham 1984), which is a measure of among-population variance in allelic frequencies, and principal coordinate analysis (PCoA), which gave an ordination of all data points based on a covariance matrix with microsatellite data standardization, were calculated using Genetix 4.05 (Belkhir *et al.* 1996-2004) and F_{STAT} (Goudet 2001).

The mtDNA sequences of our samples were aligned together with 56 haplotype sequences of Hierofalcons (29 samples of *F. biarmicus*, 14 *F. cherrug*, 8 *F. rusticolus* and 5 *F. jugger*), plus 2 Peregrine Falcons *F. peregrinus*, 3 Prairie Falcons *F. mexicanus*, 1 Hobby *F. subbuteo* and 1 Eurasian Kestrel *F. tinnunculus* retrieved from GenBank and published by Nittinger *et al.* (2005). The outgroups method is one of the most widely used techniques to root an evolutionary tree, assuming a sufficient knowledge of the evolutionary relationships between the outgroups and the candidate set, i.e. the ingroup formed by both our and published *Hierofalco* sequences (e.g. Kinene *et al.* 2016). To correctly root the tree, and furthermore to be consistent with the Nittinger *et al.*'s (2005) results, we employed as outgroups some sequences of Peregrine Falcon (sister group to the Hierofalcons), Prairie Falcon (sister group to the Peregrine – *Hierofalco* clade), plus of the Eurasian Kestrel and the Hobby (forming two other distinct monophyletic groups within the genus *Falco*) (Wink 2018). The mtDNA sequences were aligned with Bioedit (Hall 1999),

then the haplotypes were identified using Dnasp 5 (Librado *et al.* 2009). The Tamura-Nei genetic distance matrix was clustered using the Neighbour-Joining procedure in Mega 5 (Tamura *et al.* 2011) with internode bootstrap values determined after 1000 resampling steps. Eventually, the phylogenetic relationships among the mtDNA haplotypes were reconstructed using Median-Joining networks in Network 4.6 (Bandelt *et al.* 1999).

RESULTS

Mitochondrial DNA

We identified 6 haplotypes from the 24 European Lanner Falcon samples, of which 3 were unique and new (H_34, H_35 and H_36) and 3 were shared among two or more Lanner Falcon subspecies and/or other *Hierofalco* species, and were already stored in GenBank (H_8, H_21 and H_24). The Sicilian *feldeggii* sample split up among three haplotypes, one was unique (H_36, 1 individual), 14 individuals instead shared the same haplotype (H_24) with our South Italian *feldeggii* of Apulia and with 6 *F. c. cherrug*, 6 *F. rusticolus* and 1 *F. jugger*. This latter haplotype was the more common in our sample (62.5%). The other Sicilian specimen shared the same haplotype (H_8) with 2 *F. b. feldeggii* from Apulia and Tuscany and 1 *F. b. biarmicus* already described in Nittinger *et al.* (2005) and present in GenBank. Furthermore, the 2 other Sicilian specimens retrieved from GenBank had haplotypes (H_17 and H_18) not present in our collection, the first shared with 1 *F. rusticolus* and the last unique.

The six birds from the Balkan area had two unique and new haplotypes: individual FB14 had H_35, the 4 individuals FB11-12-13 and FB15 had H_34, while the last individual FB10 shared the same haplotype H_21 with the *F. b. feldeggii* from North Italy plus 4 *F. c. cherrug* and 1 *F. c. milvipes*. Fig. 1 accounts for the clustering of the specimens sampled by us plus those coming from literature and displays the clear distinction (93% of bootstrap replicates) between the used outgroups (*F. subbuteo*, *F. tinnunculus*, *F. peregrinus* and *F. mexicanus* specimens), and the large mix within the *Hierofalco* complex. Two large clades with a good percentage of bootstrap replicates (82%) emerge in the clustering by the Neighbour-Joining tree of Tamura-Nei genetic distances. Most of the continental Italian, Sicilian and Balkan *F. b. feldeggii* cluster in the first clade, however taking relationships with specimens of other Lanner Falcon subspecies (*F. b. tanypterus*, *F. b. biarmicus*, *F. b. erlangeri*) and specimens of the *Hierofalco* species (*F. rusticolus*, *F. jugger*, *F. c. cherrug*). The second clade clusters only three of our specimens (1 from Emilia Ro-

magna and 2 from Kosovo-Serbia) with some *F. b. biarmicus*, *F. b. abyssinicus*, and other specimens of Saker Falcon (*F. c. cherrug*, *F. c. milvipes*).

In spite of the large heterogeneity between the mtDNA clustering and the geographic origin (and hence, the taxonomic designation) of the *Hierofalco* representatives, a rather clearer arrangement of phylogenetic relationships comes out from the haplotype network reported in Figure 2. Several recurrent mutation events involving the main haplotype H_24 (common to 28 specimens, see details in Fig. 1) form a complex network of subordinate haplotypes, which includes most of the *Hierofalco* specimens considered. Almost all specimens from the three Palaearctic Lanner Falcons (*F. b. erlangeri* FBE, *F. b. tanypterus*, FBT, and *F. b. feldeggii* FBF medium grey, light grey, and white circles respectively, in Fig. 2) are connected in this network around H_24, and are well separated from the Afrotropical Lanner Falcons (*F. b. biarmicus* FBB and *F. b. abyssinicus* FBA, black and dark grey circles respectively in Figure 2). Afrotropical Lanner Falcons are interconnected among them by mutational steps starting from H_5. The three specimens forming the outgroups (FMEX and FPER, dark grey and white squares respectively, in Fig. 2) are well separated from the *Hierofalco* assemblage. Nonetheless, there are exceptions to such major structuring, with three Afrotropical Lanner Falcons (with H_4, H_8 and H_12) that prove to be related to the main haplotype H_24; and conversely a group of seven Saker Falcons (5 *F. c. cherrug* FCC and 2 *F. c. milvipes* FCM, with H_21, H_23 and H_25) plus three of our *F. b. biarmicus* (1 from Kosovo-Serbia and 1 from Emilia Romagna with H_21 and 1 from Kosovo-Serbia with H_35) that form a rather homogeneous haplo-group, although related to the haplotype network of Afrotropical Lanner Falcons.

Microsatellites

The population genetic diversity based of microsatellite analysis of the 24 specimens showed a number of alleles per locus ranging from 1 to 6 (Sicily: 1-4, continental Italy: 1-2; Balkan: 1-6). The allelic pattern across populations has been compared in Figure 3. The mean (\pm SE) allelic richness of the total sample was 2.30 ± 0.21 ; $N = 24$. Continental Italy had the lowest (1.9 ± 0.1) and Sicily the highest (2.8 ± 0.36) allelic richness, with these population means very likely depending from sample size (Fig. 3). All populations had a similar mean (\pm SE) of alleles, of different alleles with a frequency $\geq 5\%$ and of effective alleles (Fig. 3). The number and frequency of private alleles was rather low (Fig. 3, Table 2). Allelic diversity expressed by the Shannon information index, was relatively larger in Sicily (0.63 ± 0.13) than continental Italy ($0.59 \pm$

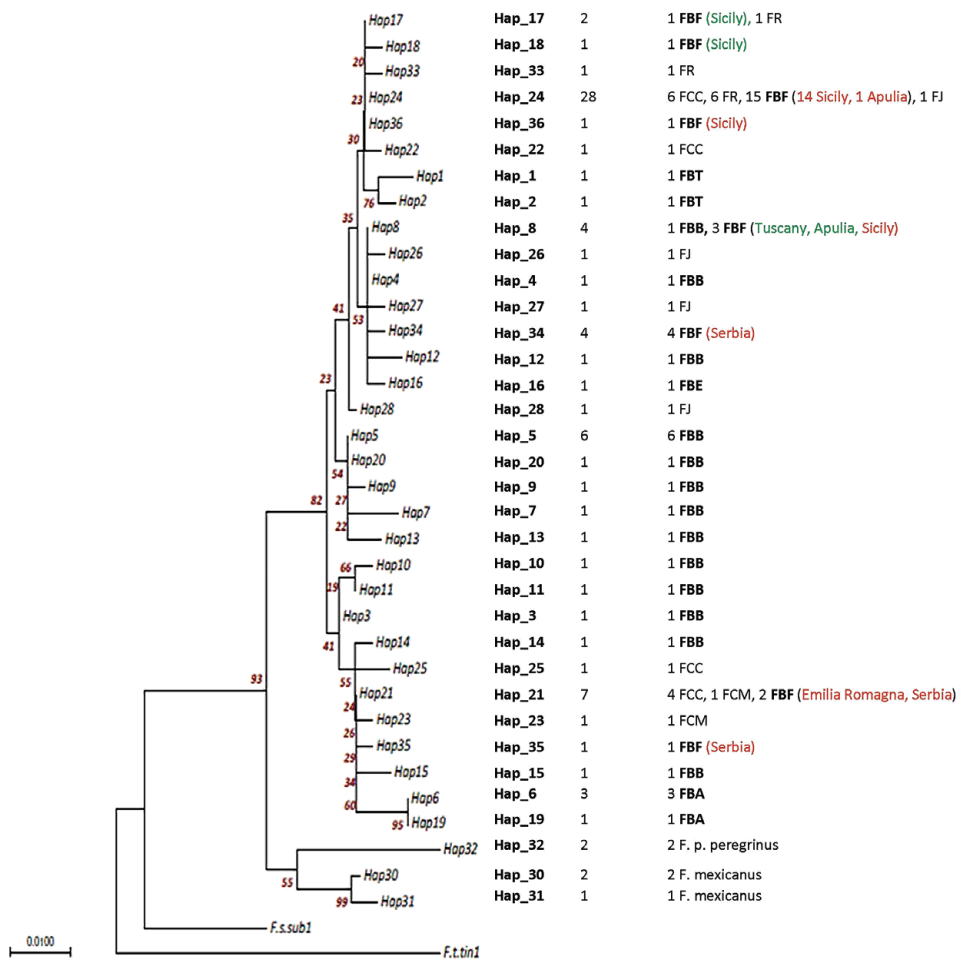


Figure 1. The clustering of mtDNA haplotypes by Neighbour-Joining of Tamura-Nei genetic distances among the Italian and Balkan (Kosovo-Serbia) individuals of *F. biarmicus feldeggii* (FBF) and the other Lanner Falcon subspecies (in bold FBB = *Falco biarmicus biarmicus*, FBE = *F. b. erlangeri*, FBT = *F. b. tanypterus*, FBA = *F. b. abyssinicus*) and species of the *Hierofalco* group (FR = *Falco rusticolus*, FCC = *F. cherrug cherrug*, FCM = *F. c. milvipes*, FJ = *F. jugger*). The localities of FBF individuals sampled in the present study have been reported in red between parenthesis, whereas the four specimens sampled in Nittinger *et al.* (2005) are indicated in green.

0.07) and the Balkan area (0.54 ± 0.16). Likewise, the Balkan Lanner Falcons have a relatively smaller quota (70%) of polymorphic loci than the 90% found in both continental Italy and Sicily. Observed heterozygosity in continental Italy (0.65 ± 0.13) was nearly twice the size in Sicily (0.34 ± 0.07) and the Balkan area (0.32 ± 0.12), accordingly the unbiased expected heterozygosity value in continental Italy (0.62 ± 0.09) was much larger than in Sicily (0.37 ± 0.07) and the Balkan area (0.34 ± 0.09). Total fixation index averaged -0.17 ± 0.11 , and the fixation index values per population were negative for continental Italy (-0.56 ± 0.21) and positive but around zero for the Balkan area (0.05 ± 0.17) and Sicily (0.06 ± 0.08). These fixation indexes combining the observed heterozygosity with unbiased expected heterozygosity show the low departures

from the Hardy–Weinberg equilibrium. With the cautions due to the limited sampling, none population showed significant reduction of heterozygosity with respect to Hardy–Weinberg expectations (Table 3).

Intra-population genetic estimation of the three studied populations is hardly comparable to other Lanner Falcon populations, for the lack of data. The only possible and partial comparison is with Nittinger *et al.* (2007) who, analysing the Saker Falcon phylogeography, brought together a ‘Mediterranean’ sample of Lanner Falcons (incorporating 8 specimens of *F. b. erlangeri* from Morocco, 2 *F. b. tanypterus* from Israel, and the same 4 *F. b. feldeggii* from continental Italy and Sicily we have retrieved from GenBank, see Figs. 1 and 2). Such a ‘Mediterranean’ group had 6 haplotypes and showed a total N of alleles = 33; N

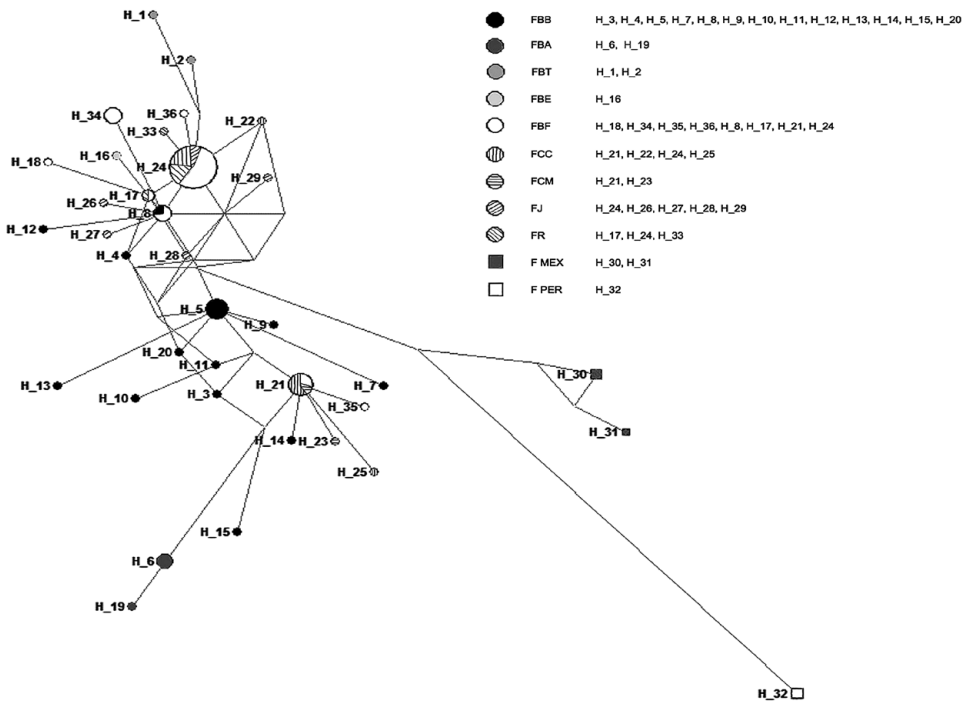


Figure 2. Haplotype network of 360 bp of mtDNA control region based on the 24 European Lanner Falcons (FBF) considered in this study plus the Nittinger *et al.* (2005) specimens retrieved from GenBank. The size of the nodes indicates the relative frequency of the corresponding haplotype in the dataset. Distances between internodes is proportional to the mutational steps occurring between adjacent haplotypes. Number of specimens and species/subspecies abbreviations as in Figure 1.

of effective alleles = 6; and unbiased expected heterozygosity = 0.83. In our European *F. b. feldeggii* sample we have found a similar number of haplotypes ($N = 6$) and total number of alleles ($N = 36$), but a smaller average value of N effective alleles (1.73 ± 0.14), and of unbiased expected heterozygosity (0.44 ± 0.05). These discrepancies are likely due to the presence of *F. b. erlangeri* and *F. b. tanypterus* specimens from Israeli and Moroccan populations that embrace more variety in the Nittinger *et al.* (2007) sample.

Genetic structuring

The amount of non-random mating was low in all population with F_{IS} values (after 999 allelic permutations per 10 loci in each population) negative in continental Italy [-0.083; with 95% confidence limits (CL95%) = -1.00 – -0.083] and positive in the other two populations (Balkan = 0.054; CL95% = -0.525 – 0.128 and Sicily = 0.093; CL95% = -0.078 – 0.181). The degree of genetic differentiation among populations as obtained by Genetix software

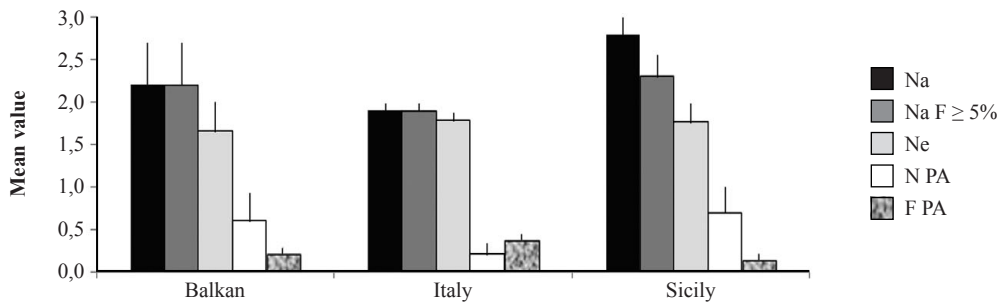


Figure 3. Allelic pattern across populations of European Lanner Falcon in continental Italy, Sicily and the Balkan area. Mean and SE values of $N_a = N$ of different alleles; $N_a (F \geq 5\%) = N$ of different alleles with a frequency $\geq 5\%$; $N_e = N$ of effective alleles; $N_{PA} = N$ of private alleles, i.e. unique to a single population (see list in Table 2); $F_{PA} =$ frequency of private alleles.

Table 2. List of private alleles per locus found in every population, with allelic frequency (FPA) and FPA population mean \pm SE (see Figure 3).

Population	Locus	Allele	FPA	FPA Mean \pm SE
Balkan	AGE5	149	0.083	0.22 \pm 0.06
Balkan	AGE5	156	0.167	
Balkan	FP92	108	0.083	
Balkan	FP79_4	147	0.417	
Balkan	FP79_4	153	0.250	
Balkan	FP79_4	155	0.333	
Continental Italy	FP92	100	0.250	0.38 \pm 0.13
Continental Italy	FP86	139	0.500	
Sicily	FP31	145	0.167	0.14 \pm 0.03
Sicily	FP54	100	0.292	
Sicily	FP54	102	0.125	
Sicily	FP107	191	0.125	
Sicily	FP107	207	0.031	
Sicily	FP79_4	141	0.094	
Sicily	FP79_4	149	0.156	

Table 3. Results of χ^2 test for deviations from Hardy-Weinberg equilibrium per every microsatellite locus in three European Lanner Falcon populations. In monomorphic loci all individuals are homozygous for the same allele. In bold, the only cases of statistical deviation from Hardy-Weinberg equilibrium.

Locus	Balkan	Continental Italy	Sicily
AGE5	$\chi^2_{15} = 18.00$; P = 0.263	$\chi^2_1 = 2.00$; P = 0.157	$\chi^2_6 = 3.20$; P = 0.783
FP13	monomorphic	$\chi^2_1 = 2.00$; P = 0.157	$\chi^2_1 = 0.52$; P = 0.473
FP31	$\chi^2_1 = 0.06$; P = 0.804	monomorphic	$\chi^2_3 = 5.42$; P = 0.144
FP54	monomorphic	$\chi^2_1 = 1.00$; P = 0.317	$\chi^2_6 = 4.23$; P = 0.645
FP89	$\chi^2_1 = 1.85$; P = 0.174	$\chi^2_1 = 0.22$; P = 0.637	$\chi^2_1 = 0.07$; P = 0.790
FP92	$\chi^2_3 = 0.67$; P = 0.881	$\chi^2_1 = 0.22$; P = 0.637	$\chi^2_1 = 0.55$; P = 0.460
FP107	$\chi^2_1 = 1.50$; P = 0.221	$\chi^2_1 = 0.22$; P = 0.637	$\chi^2_6 = 5.76$; P = 0.451
FP86	monomorphic	$\chi^2_1 = 2.00$; P = 0.157	monomorphic
FP46	$\chi^2_1 = 6.00$; P = 0.014	$\chi^2_1 = 2.00$; P = 0.157	$\chi^2_1 = 2.94$; P = 0.086
FP79_4	$\chi^2_1 = 1.260$; P = 0.739	$\chi^2_1 = 1.00$; P = 0.317	$\chi^2_6 = 17.15$; P = 0.009

is practically null ($F_{ST} = 0.005$) between continental Italy and Sicily, and relatively small in the other two cases, with $F_{ST} = 0.11$ between continental Italy and Balkan, and $F_{ST} = 0.08$ between Sicily and Balkan. The relative rate of gene flow among the three populations and averaged on the 10 microsatellite loci is $N_m = 4.80 \pm 2.22$. This value means that about 5 migrants per generation are exchanged among the European Lanner Falcon populations here considered. Analysis of molecular variance (AMOVA) showed that inter-population differences are much lower than intra-population differences and with statistical significance different from random and depending more from individual differences (Table 4).

The PCoA scatterplot in Figure 4 shows how all the specimens spread across the 2 axes, evidencing the feeble genetic structuring among the three studied populations. The Apulia specimen (FB16) lays in the middle of the Sicilian specimens. Three Sicilians (FB4, FB8, and FB18) are very close to the Kosovo-Serbian specimens (FB11-15). Then there are three distant individuals (FB3 from Sicily, FB9 from Emilia Romagna and FB10 from Kosovo-Serbia). Such a genetic structuring keeps equivalent even considering the third axis (not reported here for the sake of simplicity), with the cumulative percentage of variation explained by the first 3 axes equivalent to 49.65%.

Table 4. Analysis of molecular variance showing the low source of variation among (inter-population) and within (intra-population) the populations, with respect to the large intra-individual variability. Both the estimated intra-population ($\Phi_{IS} = 0.154$, $P = 0.002$) and estimated inter-population ($\Phi_{ST} = 0.117$, $P = 0.001$) variances are statistically significant from random (based on 999 standard permutations across the full data set).

Source	df	SS	MS	Estimated Variance	Estimated Variance (%)
Inter-population	2	10.792	5.396	0.265	11.7%
Intra-population	21	48.333	2.302	0.307	13.6%
Intra-individual	24	40.500	1.688	1.688	74.7%
Total	47	99.625		2.260	100%

DISCUSSION

The *Hierofalco*, formerly considered as a subgenus (Kleinschmidt 1901), is today better regarded as a superspecies, that is a complex of ecologically and morphologically similar species of large falcons replacing each other in various regions of the Old World and with close genetic relations (Ferguson-Lees & Christie 2001, Nittinger *et al.* 2005). The four species that form this lineage cluster as a sister group to the Peregrine Falcon (Wink *et al.* 2004, Wink 2018). Previous phylogenetic analysis (Nittinger *et al.* 2005, 2007) employed only a limited sample of the European Lanner Falcon (4 Italian specimens, see text above). Using the same protocols of Nittinger *et al.* (2005) for the non-coding fragment of the mtDNA control region, and of Nittinger *et al.* (2007) for microsatellites analyses, we examined a more exhaustive sample of the Sicilian population, the largest of this subspecies (Andreotti & Leonardi 2007), and for the first time a sample of specimens from the Balkan area, together with a new locality (Emilia Romagna) of continental Italy and a second specimen from Apulia. Our analyses thus added some lacking information on the phylogenetic relationships and the population genetic structure of the European Lanner Falcon in the broad framework of the *Hierofalco* superspecies.

MtDNA and microsatellites analyses of more European Lanner Falcons showed a dispersed pattern of our specimens inside the *Hierofalco* complex in a way congruent to what found in Nittinger *et al.* (2005, 2007). Specifically, in the NJ tree of mtDNA our specimens split in two main clades, similar to the A and B clades of Nittinger *et al.* (2005), with a minority (12.5%) of individuals populating the clade B, where *F. b. feldeggii* was not detected before. Accordingly, we reconstructed a similar network with three major haplotypes (H_24, H_21, H_5), mainly corresponding to haplo-groups I, II and III in Nittinger *et al.* (2005) and most of our specimens took their place in the haplo-group III.

The current explanation for such a heterogeneous ar-

rangement within the *Hierofalco* is that these falcons represent an assemblage of morphospecies not yet differentiated in the molecular markers used so far. The pattern of interspecific genetic variation and phylogenetic relationships are not in accordance with the current geographic distribution and taxonomy of the Hierofalcons because none of the four species form a monophyletic group. *Hierofalco* species are so closely related because they should have split up only recently (i.e. probably in the last interglacial period of Pleistocene, ~ 130,000 years ago) from an African ancestor very similar to *F. b. biarmicus* (Nittinger *et al.* 2005). The puzzling phylogenetic arrangement of Hierofalcons is not unique, as there are other raptor species, like for instance the Old World Buzzards (Jowers *et al.* 2019), poorly differentiated genetically as result of a Pleistocene recent radiation.

The genetic patterns currently observed on Hierofalcons may be the result of a variety of evolutionary processes, including incomplete lineage sorting of ancestral polymorphisms, and interspecific gene flow through hybridization during the different waves of colonization in Eurasia across time (Nittinger *et al.* 2005, 2007), and likely due also to the historical human use for falconry purposes (Nittinger *et al.* 2007).

Restricting the field to the Lanner Falcon, what the Bayesian clustering method based on allele frequencies strongly supports (see Nittinger *et al.* 2007), and our findings coherently show, is the existence of two major genetic groups of *F. biarmicus*, which correspond geographically to sub-Saharan African and Mediterranean populations. The three Palaeartic subspecies (i.e. *F. b. feldeggii*, *F. b. tanypterus* and *F. b. erlangeri*) have thus a strong genetic identity that recall the debate about their subspecific designation (see Massa *et al.* 1991, and a review in Leonardi 2015). Starting from Hartert (1915 in Leonardi 2015) several researchers have considered *erlangeri* and *tanypterus* indistinguishable, or at best *tanypterus* as an intermediate form between *erlangeri* and the more conspicuous and larger *feldeggii* (Massa *et al.* 1991). In this framework, is

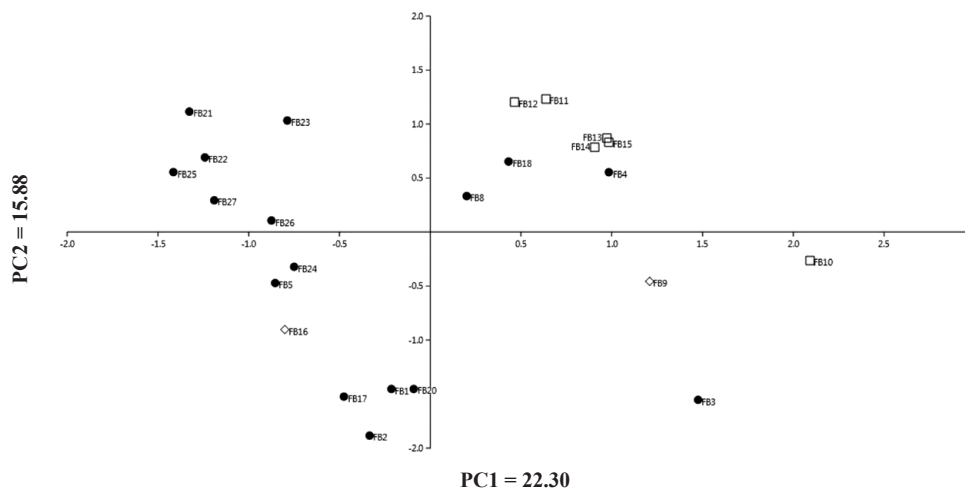


Figure 4. Scatterplot of the 24 European Lanner Falcons (same ID codes in Table 1) living in the three populations of continental Italy (white diamonds), Sicily (black dots) and the Balkan area (white squares) as resulting from the principal coordinate analysis on a covariance matrix with microsatellite data standardization. The cumulative variance explained by the two first components is 38.18%.

not surprising that the specimens of our three populations mix together (e.g. Fig. 4) and that their genetic arrangement does not show any geographic pattern, with a low to moderate degree of genetic differentiation between Sicily, continental Italy and the Balkan area. Furthermore, the value about the number of migrants (N_m) suggested a gene flow among the three populations. We need to remark here that N_m is an approximation of migration rates that populations have if they follow the island model, and this estimate reflects historical evolutionary rates of gene flow in equilibrium circumstances; so it may not reflect current gene flow (Frankham *et al.* 2002). Nonetheless, the low number and frequency of private alleles would support the findings on N_m , because the frequency of private alleles in different subpopulations is inversely related to gene flow (Slatkin 1985).

Overall, statistics from microsatellite analyses on population genetic structure indicated an acceptable value of allele richness and haplotype diversity, in a conservation perspective. All three populations did not show significant departures from Hardy-Weinberg equilibrium, with low values of the inbreeding coefficients (non-random mating). In the Sicilian population, that had the relatively higher amount of non-random mating with respect to the other two populations, the F_{IS} value is however low and allelic diversity high. First evidence of natal dispersal outside the island (see paper of Sarà *et al.* in this same issue) points in the same direction, supporting our expectations about the little isolation of Sicilian Lanner Falcons and the occurrence of gene flow between Sicily and continental Italy, particularly with Apulian breeding area.

Until recently, inbreeding depression and reduction in heterozygosity do not seem to have been a threat for the Italian and Balkan populations. This occurred likely because the loss of genetic diversity can be delayed and slow in small populations of long-lived species (Frankham *et al.* 2002); as those of the European Lanner Falcon, where the longevity record into the wild is seventeen years (Leonardi 2015). The detrimental factors hitting the European Lanner Falcons (e.g. Sarà 2014, Di Vittorio *et al.* 2017) would have been acting on few generations of breeders in the last 20-30 years (Andreotti & Leonardi 2007) and without still evident consequences on population genetic diversity. They would become of much higher concern in next years, persisting the numerical and breeding performances decline (e.g. Boev & Iankov 2011, Allavena *et al.* 2015, Mascara & Nardo 2018). For instance, in a population of some 50 breeding pairs, corresponding to an effective size $N_e = 11$, we expect a dramatic ~37% and ~61% loss of heterozygosity in the next 10 and 20 generations, respectively (M. Sarà *pers. obs.*).

It is worth to remind here that our findings are preliminary and need to be taken with caution, as they could not be fully representative of the genetic profile of the studied populations, due to the sex and age heterogeneity of sampling, and to the low number of continental Italy and Balkan specimens. Further analyses on genetic structure, including senescence and presence of past/recent population bottlenecks, employing more Italian, Balkan and Greek specimens would be thus necessary for the definitive genetic assessment of the European Lanner Falcon.

Nevertheless, the results we have found have some im-

mediate conservation implications. The evidences that the Sicilian population is not closed but is connected to mainland, and that a 'pure' European Lanner Falcon pedigree does not exist, suggest a metapopulation approach to conservation programmes, so to include the largest possible genetic stock of this populations' network.

Acknowledgements – This study was supported by the Nando & Elsa Peretti Foundation (project n° 442 - Practical actions for preserving the main European population of Lanner Falcon). All field activities were conducted in accordance with relevant guidelines and regulations. Specifically, captures were carried out under prior favourable opinion of Istituto Nazionale per la Protezione e la Ricerca Ambientale (ISPRA) n. 50952/2013, 20267/2014 and 18013/2016 and under authorizations n. 1076/2012, 1616/2014, 30321/2016 and 2599/2018 issued by Regione Siciliana. Mr. Tibor Buza kindly agreed to access and sample his captive falcons. We thank Ettore Randi and an anonymous reviewer for the useful comments to earlier versions of the manuscript.

REFERENCES

- Allavena S., Andreotti A., Corsetti L. & Sigismondi A., 2015. Il Lanario in Italia: problemi e prospettive. Ed. Belvedere, Latina.
- Amato M., Ossino A., Brogna A., Cipriano M., D'Angelo R., Di pasquale G., Mannino V., Andreotti A. & Leonardi G., 2014. Influence of habitat and nest-site quality on the breeding performance of Lanner Falcons *Falco biarmicus*. *Acta Ornithol.* 49: 1–7.
- Andreotti A. & Leonardi G., 2007. Piano d'Azione nazionale per il Lanario (*Falco biarmicus feldeggii*). *Quad. Conserv. Natura*, 24. Min. Amb. & INFS, Bologna.
- Andreotti A., Leonardi G., Sarà M., Brunelli M., De Lisio L., De Sanctis A., Magrini M., Nardi R., Perna P. & Sigismondi A., 2008. Landscape-scale spatial distribution of the Lanner Falcon (*Falco biarmicus feldeggii*) breeding population in Italy. *Ambio* 37: 440–444.
- Bandelt H.J., Forster P. & Röhl A., 1999. Median-joining networks for inferring intraspecific phylogenies. *Molecular Biol. Evol.* 16: 37–48.
- Belkhir K., Borsa P., Chikhi L., Raufaste N. & Bonhomme F., 1996–2004. GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations. Laboratoire Génome, Populations, Interactions. CNRS UMR 5000, Univ. Montpellier II, France.
- BirdLife International, 2004. Birds in Europe: population estimates, trends and conservation status. BirdLife International. Cambridge, UK.
- BirdLife International, 2015. European Red List of Birds. Office for Official Publications of the European Communities, Luxembourg.
- BirdLife International, 2017. European birds of conservation concern: populations, trends and national responsibilities. BirdLife International, Cambridge, UK.
- Boev Z. & Iankov P., 2011. The Lanner. P. 81 in: Golemanski V. (ed.) *Red Data Book of the Republic of Bulgaria*, Vol. 2. Animals. Bulg. Soc. Protection Birds, Conserv. S., Book 10, Sofia.
- Brunelli M. & Sarrocco S., 2012. Caratteristiche ambientali dei siti di nidificazione del Lanario *Falco biarmicus* nel Lazio. *Riv. ital. Orn.* 82: 263–265.
- Di Vittorio M., Di Trapani E., Cacopardi S., Rannisi G., Falci A., Ciaccio A., Sarto A., Merlino S., Zafarana M., Greci S., Salvo G., Lo Valvo M., Scuderi A., Murabito L., La Grua G., Cortone G., Patti N., Luiselli L. & López-López P., 2017. Population size and breeding performance of the Lanner falcon *Falco biarmicus* in Sicily: conservation implication. *Bird Study* 64: 339–343.
- Ferguson-Lee J. & Christie D.A., 2001. *Raptors: Birds of prey of the world*. A. & C. Black Publ., London.
- Frankel O.H. & Soulé M.E., 1981. *Conservation and Evolution*. Cambridge Univ. Press, Cambridge, UK.
- Frankham R., Ballou J.D. & Briscoe D.A., 2002. *Introduction to Conservation Genetics*. Cambridge Univ. Press, Cambridge, UK.
- Fuchs J., Johnson J.A. & Mindell D.P., 2015. Rapid diversification of falcons (Aves: Falconidae) due to expansion of open habitats in the Late Miocene. *Molecular Phylog. Evol.* 82: 166–182.
- Goudet J., 2001. FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). <http://www.unil.ch/izea/software/fstat.html>.
- Gustin M., Palumbo G. & Corso A., 2002. International Action Plan for the Lanner Falcon *Falco biarmicus*. BirdLife International, Council of Europe, T-PVS/Inf, 16.
- Hall T.A., 1999. BioEdit. A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41: 95–98.
- Horvath M.B., Martinez-Cruz B., Negro J.J., Kalmar L. & Godoy J.A., 2005. An overlooked DNA source for non-invasive genetic analysis in birds. *J. Avian Biol.* 36: 84–88.
- IUCN/SSC, 2013. Guidelines for Reintroductions and Other Conservation Translocations. Version 1.0. IUCN Species Survival Commission, Gland, Switzerland.
- Jowers M.J., Sánchez-Ramírez S., Lopes S., Karyakin I., Dombrovski V., Qniba A., Valkenburg T., Onofre N., Ferrand N., Beja P., Palma L. & Godinho R., 2019. Unravelling population processes over the Late Pleistocene driving contemporary genetic divergence in Palearctic buzzards. *Molecular Phylog. Evol.* 134: 269–281.
- Kinene T., Wainaina J.M., Maina S. & Boykin L., 2016. Rooting Trees, Methods for. Pp. 489–493 in: R. Kliman (ed.), *Encyclopedia of Evolutionary Biology*. Vol. 3. Academic Press, Cambridge, MA.
- Kleinschmidt O., 1901. Der Formenkreis *Hierofalco* und die Stellung des ungarischen Wügfalken in demselben. *Aquila* 8: 1–48.
- Leonardi G., 2015. The Lanner Falcon. Printed and published privately, Catania.
- Librado P. & Rozas J., 2009. DnaSP v5 A Software for Comprehensive Analysis of DNA Polymorphic Data. *Bioinformatics* 25: 1451–1452.
- Mascara R. & Nardo A., 2018. Il lanario *Falco biarmicus feldeggii*, in un'area della Sicilia centro-meridionale (Italia). *Uccelli Italia* 43: 85–93.
- Massa B. & Borg J., 2018. European Birds of Conservation Concern: some constructive remarks. *Avocetta* 42: 75–84.
- Massa B., Lo Valvo F., Siracusa M. & Ciaccio A., 1991. Il Lanario (*Falco biarmicus feldeggii* Schlegel) in Italia: status, biologia e tassonomia. *Naturalista sicil.* 15: 27–63.
- Mengoni C., Zuberogoitia I., Mucci N., Boano G., Urban T. Guzzo E. & Sarà M., 2018. Genetic variability in Peregrine Falcon populations of the Western Palearctic region. *Ornis Hungarica* 26: 12–26.
- Nesje M., Røed K.H., Liffeld J.T., Lindberg P. & Steen O.F., 2000. Genetic relationships in the peregrine falcon (*Falco peregrinus*) analysed by microsatellite DNA markers. *Molecular Ecol.* 9: 53–60.
- Nittinger F., Haring E., Pinsker W., Wink M. & Gamauf A., 2005.

- Out of Africa: phylogenetic relationships between *Falco biarmicus* and the other Hierofalcons (Aves: Falconidae). *J. Zool. System. Evol. Res.* 43: 321–331.
- Nittinger F., Gamauf A., Pinsker W., Wink M. & Haring E., 2007. Phylogeography and population structure of the Saker Falcon (*Falco cherrug*) and the influence of hybridization: mitochondrial and microsatellite data. *Molecular Ecol.* 16: 1497–1517.
- Peakall R. & Smouse P.E., 2006. GenAlEx 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecol.* 6: 288–295.
- Peronace V., Cecere, J.G., Gustin, M. & Rondinini C., 2012. Lista rossa 2011 degli Uccelli nidificanti d'Italia. *Avocetta* 36: 11–58.
- Pezzo F., Chianciani G., Cutini S., Fabbrizzi F., Grilli G., Nardi R. & Paesani G., 2016. Rapporto sullo status del Lanario *Falco biarmicus feldeggii* in Toscana 2014-2016. *Picus* 42: 132–136.
- Sarà M., 2014. Spatial analysis of Lanner Falcon habitat preferences: Implications for agro-ecosystems management at landscape scale and raptor conservation. *Biol. Conserv.* 178: 173–184.
- Sarà M., Mascara R. & López-López P., 2016. Understanding the coexistence of competing raptors by Markov chain analysis enhances conservation of vulnerable species. *J. Zool. London* 299: 163–171.
- Slatkin M., 1985. Rare alleles as indicators of gene flow. *Evolution* 39: 53–65
- Tamura K., Peterson D., Peterson N., Stecher G., Nei M. & Kumar S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biol. Evol.* 28: 2731–2739.
- Topinka R.J. & May B., 2004. Development of polymorphic microsatellite loci in the northern goshawk (*Accipiter gentilis*) and cross-amplification in other raptor species. *Conserv. Genetics* 5: 861–864.
- Tucker G.M. & Heath M.F., 1994. Birds in Europe: their conservation status. BirdLife International. Conservation Series No. 3. Cambridge, UK.
- Weir B.S. & Cockerham C.C., 1984. Estimating *F*-statistics for the analysis of population structure. *Evolution* 38: 1358–1370.
- Wink M., Sauer-Gürth H., Ellis D. & Kenward R., 2004. Phylogenetic relationships in the *Hierofalco* complex (Saker-, Gyr-, Lanner-, Laggard Falcon). Pp. 499–504 in: Chancellor, R. D. & Meyburg, B-U. (eds.) Raptors Worldwide. Proc. 6th World Conf. on Birds of Prey and Owls. World Working Group on Birds of Prey, Berlin.
- Wink M., 2018. Phylogeny of Falconidae and phylogeography of Peregrine Falcons. *Ornis Hungarica* 26: 27–37.

Received on 12nd April 2019

Accepted on 20th June 2019

Associate editor: **Michele Panuccio**

This work is licensed under the Creative Commons Attribution-ShareAlike 4.0 International License. To view a copy of this license, visit <http://creativecommons.org/licenses/by-sa/4.0/>.

