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# Cross-amplification in Falcons and Their Hybrids: A New Implemented Multi-locus Panel for Conservation and Forensic Purposes

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## Abstract

Falcons and their hybrids are among the most traded raptor species, with a significant proportion of international market involving countries with a strong falconry tradition. The trend in legal trade of falcons is strongly increased likewise the illegal international market is expected to swell due to the growing interest in wild-caught rather than captive-bred individuals. Biomolecular investigations represent a practical tool to foster actions finalized in tracking any wildlife trade, so contributing to the prevention of species overexploitation too. Contextually, they can inform about wild populations allowing the monitoring of genetic variability and structure. Microsatellites loci (STR) are amongst the most popular markers in molecular ecology. Despite the availability of species-specific microsatellite loci in some *Falco* species, a newly defined panel permitting the genetic analysis across the mostly traded *Falco spp*. and their hybrids has not been validated yet. Here we report the characterization of a panel of 21 highly polymorphic microsatellite loci selected from literature and evaluate its reliability for conservation and forensic purposes. We included in the study 163 captive-bred individuals belonging to in six species of falcons (*Falco biarmicus*, *Falco cherrug*, *Falco pelegrinoides*, *Falco peregrinus*, *Falco rusticolus* and *Falco tinnunculus*) and three hybrids (*Falco cherrug* x *Falco peregrinus*, *Falco peregrinus* x *Falco rusticolus* and *Falco cherrug* x *Falco rusticolus*). We identified two sample sets, including individuals from pure species belonging to 46 parental groups tested for genetic variability and differentiation and parentage analysis, and another enclosing individuals from pure species *F. cherrug*, *F. peregrinus, F. rusticolus* and their F1 hybrid individuals tested in the analysis of hybrid detection.

The proposed STR panel could be of value in monitoring genetic diversity and differentiation in wild populations and describing mating systems and gene flow; moreover, it has the potential to perform individual identification and parentage analysis, so contributing to investigate parental claims, illegal transfer or suspected smuggling in *Falco* species and their hybrids.

**Keywords:** *Falco* • Falcon hybrids • Cross-amplification • Microsatellites • Forensic • Illegal trade • Kinship • Genetic differentiation • Hybrid detection

## Introduction

The global loss of biodiversity is mediated by a synergistic combination of several important factors such as habitat destruction, overexploitation of wild populations, pollution and climate changes [1], driven ultimately by human-induced demographic, economic and societal factors [2]. According to the International Union for the Conservation of Nature's (IUCN) Red List of Threatened Species, 13% of bird species are now threatened with extinction [3] and their trade still represents one of the main drivers of extinction globally [4-7]; indeed, the demand on international trade for bird species remains high, increasing the pressure on their conservation [8].

Particularly, the international trafficking of raptors is a highly profitable industry [9]. Birds of prey have been since ever subject to human persecution in the forms of killing, trapping, or "laundering" into the captive- bred population to meet the international demand [10,11]. Furthermore, in the last fourth decades, it has been also recorded a significant increase in the legal raptor market [12], mainly fuelled by pet trade and socio-cultural reasons. The trafficking of raptors for the pet trade is indeed an emerging problem; for example, the owl trade increased in the last decades due to the growing popularity as pets [13]. *Falco* spp. and their hybrids are among the most traded raptor species, with the United Arab Emirates who import the largest number of captive-bred raptors for falconry [12]. Indeed, this practice is one of the oldest known human activities [14] and remains commonly in use as a sport and art form, especially in the Middle East [15] where it is preserved as part of cultural and sporting heritage [16]. Because of a soaring global market, a persistent decline in some falcon populations has been documented, impacting both population numbers and geographic distribution [10-17]. The population trend of *F. biarmicus*, *F. cherrug* and *F. tinnunculus* is decreasing and, particularly, Saker Falcon has most recently been assessed for the IUCN Red List of Threatened Species and listed as endangered [3].

As a result, all *Falco spp*. have been listed in Appendices I and II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) that played a fundamental role in regulating and monitoring wildlife species international trade and, presently, represents a valuable tool for biodiversity conservation preventing overexploitation of wild species for market purposes [18]. The European Union (EU) joined CITES, whose directives have been applied in all the Member States through Council (EU) Regulations.

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Species subjected to maximum protection are listed in Annex A, that includes all CITES Appendix I-listed species and some species listed in Appendices II and III for which the EU has adopted stricter domestic measures. As included in Annex A, *Falco spp*. and their hybrids enjoy the maximum protection, and the trade is allowed only if strict conditions are respected and the captivity-bred is proved [19,20].

Biomolecular investigations represent a reliable tool to support actions finalized in preventing species overexploitation and in tracking any wildlife trade. Contextually, they can inform about wild populations, particularly on any changes in genetic variability or structure that could affect their viability. Microsatellites loci (STRs) are amongst the most popular markers in molecular ecology [21]. They allow the description of genetic variability [22], the absence of gene flow in the presence of barriers hampering the movements of individuals [23], the admixture and hybridization between different populations or species [24,25] the description of genetic dynamics during the genetic monitoring of carnivores [26]. Expertise in their use is widespread and the possibility of sharing information across different research groups is possible by the construction of an allele ladder [27,28]. Moreover, forensic genetics permits the identification of species and individuals [29], family clusters [30] illegal hybridization [31] thus making their use reliable in several crime resolutions.

In the past decades, many species-specific panels of STRs have been developed in some *Falco* species, such as *F. colombarius* [32], *F. naumanni* [33], *F. peregrinus* [34,35], *F. rusticolus* [36], *F. vespertinus* [37]. However, only five panels focused on more than one species and were tested across a restricted range of falcon species, sometimes resulting non performant in all of them [32-37]. The lack of a validated STR panel across a wide range of falcon species makes unfeasible a deep comparison of variability indices for monitoring and conservation projects, and the identification of hybrid individuals for forensic purposes. Such evidence calls for a newly defined panel permitting the genetic variability analysis across the mostly traded *Falco spp*. and their hybrids. In this study, we tested thirty-two polymorphic microsatellite loci selected from literature in six species of falcons (*F. biarmicus*, *F. cherrug*, *F. pelegrinoides*, *F. peregrinus*, *F. rusticolus* and *F. tinnunculus*) and three hybrids (*F. cherrug* x *F. peregrinus*, *F. peregrinus* x *F. rusticolus* and *F. cherrug* x *F. rusticolus*) with the aim of evaluating the reliability of a unique panel for the most critical species of falcons. Specifically, the present study is focused on using such a panel for conservation and forensic purposes i) to monitor genetic variability and differentiation, ii) to perform individual identification and track illegal trades through paternity tests; iii) to identify *Falco* hybrids.

# Materials and Methods

## Sampling

Samples included in this work were selected from the CITES sample database, managed since 1995 by the Italian Institute of Environmental Protection and Research (ISPRA) on behalf of the Italian Ministry of the Environment and Energy Security (MASE).

A total of 163 captive-bred individuals was used for the analysis (Table 1), belonging to the following species: *F. biarmicus* (n=24), *F. cherrug* (n=24), *F. pelegrinoides* (n=24), *F. peregrinus* (n=24), *F. rusticolus* (n=24), *F. tinnunculus* (n=24), *F. cherrug* x *F. peregrinus* (n=5), *F. peregrinus* x *F. rusticolus* (n=4), and *F. cherrug* x *F. rusticolus* (n=10).

## Marker choice

Twenty-five out of the 32 PCR primers used in this work were isolated from Peregrine falcon (*F. peregrinus*) [34,35] thirteen out of them have been originally tested in Gyrfalcon *F. rusticolus* and Saker *F. cherrug* too [35]. We also included seven microsatellite loci described in Merlin (*F. columbarius*) and characterized for cross-species amplification in Gyrfalcon and Peregrine falcon [32] and one marker isolated from northern goshawk (*A. gentilis*) [38] proved to be effective for cross-amplification in *F. biarmicus* [39].

## Experimental design

We used the following experimental strategy: i) all thirty-two microsatellite loci were tested on two unrelated individuals of six *Falco* species (*F. biarmicus*, *F. cherrug*, *F. pelegrinoides*, *F. peregrinus*, *F. rusticolus*, *F. tinnunculus*), and amplification reactions were performed in singleplex to test for the right allelic range and specific amplification; ii) microsatellite loci giving positive PCR were tested in multiplex on additional four individuals for each species; iii) the set-up multiplexed microsatellite loci panel was used for genotyping 24 individuals for each pure falcon species and 19 F1 hybrid individuals from three species (five, four and ten specimens of *F. cherrug* x *F. peregrinus*, *F. peregrinus* x *F. rusticolus* and *F. cherrug* x *F. rusticolus*, respectively); eventually, 163 individuals were included in the study. We identified two sample sets to be used in the following analysis: sample set #1 (n=144) including individuals from pure species belonging to 46 parental groups tested for genetic variability and differentiation and parentage analysis; sample set #2 (n=91) enclosing individuals from pure species *F. cherrug*, *F. peregrinus* and *F. rusticolus* (n=72) and their hybrids (n=19) tested in the analysis of hybrid detection (Table 1).

## DNA extraction, amplification and fragment detection

DNA was isolated from feathers using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. After digestion in 180 µL ATL buffer, 20 µL proteinase K and 20 µL DTT and incubation overnight at 56 °C, the lysate was loaded in a QIAcube HT robotic station (Qiagen, Hilden, Germany) for further purification steps. DNA amplification was performed in a total volume of 8 µl with 20 ng of DNA as the template, 0.025 U of HotStarTaq® (Qiagen, Hilden, Germany), 0.8 µl of 10X PCR Buffer, 0.8 µl of 0.2% BSA, 0.48 µl of 25 mM $MgCl_2$, 0.4 µl of 2.5 mM dNTP mix, 0.1 µl of 10 µM of each primer. Each forward primer was labelled with fluorescent ABI dyes. Negative PCR controls were included in the amplification to monitor the performance of the process. DNA was amplified in a Veriti™ 96-Well Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA). According to melting temperature and reference bibliography, the following PCR thermal profile was used: initial denaturation at 94 °C for 15 min; 35 cycles at 94 °C for 90 s, annealing at 50 or 55 °C (Table 2) for 40 s, extension at 72 °C for 40 s; final extension at 72 °C for 10 min. Amplicons were separated through capillary electrophoresis in an ABI 3130xl genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA); alleles were scored in GeneMapper 5.0 using GeneScan 500 ROX size standard (Thermo Fisher Scientific, Waltham, MA, USA).

## Genetic variability and differentiation

Genetic variability and differentiation analyses were conducted on Sample Set #1 (Table 1). Allele number (NA), observed and unbiased expected heterozygosity (Ho, uHe), number of Private Alleles (PA) were computed using GenAlEx 6.41 [40]. Allelic Richness (AR) was estimated using FSTAT [41]. Species differentiation was estimated with pairwise Fst in Genetix 4.05 using Weir and Cockerham method and 1000 permutations [42]. The significance of differences in genetic diversity between species was tested using PAST software [43]. The distribution of genetic variation was quantified through Analysis of Molecular Variance (AMOVA) in GenAlEx 6.41 [40]; the significance of the variance components was based on 999 permutations.

The genetic structure was investigated using a Bayesian model-based clustering method implemented in the software Structure 2.3.4 [44]. The analysis was conducted using: a burn-in period at 10,000 followed by 100,000 replicates of the MCMC; five independent runs for each cluster (range 1-6 K); "admixture" model with "independent allele frequencies". Structure Harvester v0.6.92 [45] was used to determine the optimal number of genetic clusters according to the Evanno's delta-K method and the likelihood distribution. The software Clumpp 1.1.2 [46] was used to combine admixture values from multiple runs and results were visualized using Distruct v1.1 [47]. Factorial Correspondence Analysis (FCA) was carried out in Genetix 4.05 to check genetic structure and distances between species [42].

## Probability of identity and parentage analysis

Probability of Identity for unrelated (PID) and related samples (PID_sib, sibling) were computed using GenAlEx 6.41 [40]. Parentage assignment was performed using CERVUS 3.0 [48] and COLONY 2.0 [49]. In CERVUS, we simulated 10,000 offspring produced by 46 candidate fathers and 48

candidate mothers, with 100% parents sampled, 1% genotyping error rate and confidence levels assessed by Delta and LOD distribution (relaxed >80%, strict >95%). The minimum typed loci were set to ≥ 90% of the whole number of loci. In COLONY, we used non-inbreeding data, monogamy models and set the genotyping error rate value at 0.0001. All the analyses were conducted in Sample Set #1 (Table 1).

### Hybrid detection

Hybrid detection analyses were conducted on Sample Set #2 (Table 1). Each hybrid with relative pure species was investigated using a Bayesian method (range 1-3 K) and a factorial correspondence analysis as described above.

**Table 1.** Species and individuals included in the study. Sample set 1 (*F. biarmicus*, *F. cherrug*, *F. pelegrinoides*, *F. peregrinus*, *F. rusticolus*, *F. tinnunculus*): species and family groups tested for genetic variability, differentiation, and parentage analysis. Sample set 2 (*F. cherrug*, *F. peregrinus*, *F. rusticolus*, *F. cherrug x F. peregrinus, F. peregrinus x F. rusticolus, F. rusticolus x F. cherrug*): pure species and their hybrids used in the analysis of hybrid detection. *F. cherrug*, *F. peregrinus*, *F. rusticolus* are shared by both sample sets.

| Species | *Father* | *Mother* | *Offspring* |
|---|---|---|---|
| *F. biarmicus* | F_bi_1 | F_bi_2 | F_bi_3 |
| | F_bi_4 | F_bi_5 | F_bi_6 |
| | F_bi_7 | F_bi_8 | F_bi_9 |
| | F_bi_10 | F_bi_11 | F_bi_12 |
| | F_bi_13 | F_bi_14 | F_bi_15 |
| | F_bi_16 | F_bi_17 | F_bi_18 |
| | F_bi_19 | F_bi_20 | F_bi_21 |
| | F_bi_22 | F_bi_23 | F_bi_24 |
| *F. pelegrinoides* | F_pl_1 | F_pl_2 | F_pl_3 |
| | F_pl_1 | F_pl_2 | F_pl_4 |
| | F_pl_1 | F_pl_2 | F_pl_5 |
| | F_pl_6 | F_pl_7 | F_pl_8 |
| | F_pl_6 | F_pl_7 | F_pl_9 |
| | F_pl_10 | F_pl_11 | F_pl_12 |
| | F_pl_10 | F_pl_11 | F_pl_13 |
| | F_pl_15 | F_pl_16 | F_pl_18 |
| | F_pl_20 | F_pl_21 | F_pl_22 |
| | F_pl_1 | F_pl_23 | F_pl_24 |
| *F. tinnunculus* | F_ti_1 | F_ti_2 | F_ti_3 |
| | F_ti_4 | F_ti_5 | F_ti_6 |
| | F_ti_7 | F_ti_8 | F_ti_9 |
| | F_ti_10 | F_ti_11 | F_ti_12 |
| | F_ti_13 | F_ti_14 | F_ti_15 |
| | F_ti_16 | F_ti_17 | F_ti_18 |
| | F_ti_19 | F_ti_20 | F_ti_21 |
| | F_ti_22 | F_ti_23 | F_ti_24 |
| *F. cherrug* | F_ch_1 | F_ch_2 | F_ch_3 |
| | F_ch_4 | F_ch_5 | F_ch_6 |
| | F_ch_7 | F_ch_8 | F_ch_9 |
| | F_ch_10 | F_ch_11 | F_ch_12 |
| | F_ch_13 | F_ch_14 | F_ch_15 |
| | F_ch_16 | F_ch_17 | F_ch_18 |
| | F_ch_19 | F_ch_20 | F_ch_21 |
| | F_ch_22 | F_ch_23 | F_ch_24 |
| *F. peregrinus* | F_pr_1 | F_pr_2 | F_pr_3 |
| | F_pr_4 | F_pr_5 | F_pr_6 |
| | F_pr_7 | F_pr_8 | F_pr_9 |
| | F_pr_10 | F_pr_11 | F_pr_12 |
| | F_pr_13 | F_pr_14 | F_pr_15 |
| | F_pr_16 | F_pr_17 | F_pr_18 |
| | F_pr_19 | F_pr_20 | F_pr_21 |
| | F_pr_22 | F_pr_23 | F_pr_24 |
| *F. rusticolus* | F_ru_1 | F_ru_2 | F_ru_3 |
| | F_ru_4 | F_ru_5 | F_ru_6 |
| | F_ru_7 | F_ru_8 | F_ru_9 |
| | F_ru_10 | F_ru_11 | F_ru_12 |
| | F_ru_13 | F_ru_14 | F_ru_15 |
| | F_ru_16 | F_ru_17 | F_ru_18 |
| | F_ru_19 | F_ru_20 | F_ru_21 |
| | F_ru_22 | F_ru_23 | F_ru_24 |
| *F. cherrug x F. peregrinus* | | F_chxF_pr_1 | |
| | | F_chxF_pr_2 | |
| | | F_chxF_pr_3 | |
| | | F_chxF_pr_4 | |
| | | F_chxF_pr_5 | |
| *F. peregrinus x F. rusticolus* | | F_prxF_ru_1 | |
| | | F_prxF_ru_2 | |
| | | F_prxF_ru_3 | |
| | | F_prxF_ru_4 | |
| *F. rusticolus x F. cherrug* | | F_ruxF_ch_1 | |
| | | F_ruxF_ch_2 | |
| | | F_ruxF_ch_3 | |
| | | F_ruxF_ch_4 | |
| | | F_ruxF_ch_5 | |
| | | F_ruxF_ch_6 | |
| | | F_ruxF_ch_7 | |
| | | F_ruxF_ch_8 | |
| | | F_ruxF_ch_9 | |
| | | F_ruxF_ch_10 | |

## Results

### Screening of microsatellite loci

Thirty-two microsatellite markers (Fco001, Fco003, Fco005, Fco012, Fco014, Fco015, Fco016, Fpeu1, Fpeu26_1, Fpeu25_1, Fpeu33_1, Fpeu46_2, Fpeu56_1, Fpeu208_1, Fpeu248_1, Fpeu298_1, Fpeu342_1, Fpeu353_1, Fpeu98_2, Fpeu145_1, fp5, fp13, fp31, fp54, fp79_1, fp74_4, fp82_2, fp86_2, fp89, fp92_1, fp107, Age5) were chosen to evaluate their cross-amplification power? on six species of falcons (*F. biarmicus*, *F. cherrug*, *F. pelegrinoides*, *F. peregrinus*, *F. rusticolus*, *F. tinnunculus*). Data obtained revealed that twenty-six out of 32 markers gave amplicons in the two unrelated individuals from all the six tested species. The remaining 6 microsatellite loci (fp5, fp79_1, Fco005, Fco015, Fpeμ98_2, Fpeμ145_1) were discarded because they either did not give any amplification product or led to unreliable PCR amplicons. The twenty-six microsatellite markers selected in the first step were tested in multiplex PCRs in other four individuals for each pure species; five out of them (Fco001, Fco003, Fco012, Fco014, Fco016) were discarded because they lead to sub-optimal results or complete failure when multiplexed. One marker was selected (Fpeμ26_1) although not multiplexable because characterized by a high number of alleles. Eventually, a twenty-one multiplexed microsatellite loci panel was used for genotyping 163 individuals (Table 2).

### Genetic variability and differentiation

All the 163 samples were successfully genotyped at 21 microsatellite loci. The mean Allele Number (NA) over species was 5.76 (± 0.21), with the highest values recorded respectively in *F. tinnunculus* (7.00 ± 0.63) and the lowest one in *F. pelegrinoides* (4.71 ± 0.36). Allelic richness (AR) ranged from 2.80 (± 0.27) and 5.49 (± 0.38) in *F. pelegrinoides* and *F. tinnunculus*, respectively. Mean observed Heterozygosity (Ho) ranged from 0.51 (± 0.06) in *F. rusticolus* and 0.64 (± 0.05) in *F. tinnunculus*, with an average value of 0.60 (± 0.02); while the unbiased expected heterozygosity ranged from 0.56 (± 0.06) to 0.75 (± 0.03) in *F. rusticolus* and *F. tinnunculus*, with an average value of 0.59 (± 0.02). Mean number of private alleles (PA) ranged from 0.38 (± 0.11) in *F. pelegrinoides* to 2.38 (± 0.51) in *F. tinnunculus* (Table 3). All analysed loci were polymorphic in all the tested species.

Estimates of genetic divergence using Fst for pairs of populations indicated significant genetic differentiation between all pairs of species (Table 4). Furthermore, the AMOVA showed that genetic variation among species was 25% while 68% was within individuals and 8% was partitioned among individuals (Table 5). Factorial Correspondence Analysis (FCA) revealed that *F. peregrinus* and *F. pelegrinoides* overlapped in the FCA graphic, both consistently diverging from other species. *F. cherrug and F. rusticolus* showed a low distance from each other and resulted not very distant from *F. biarmicus*. *F. tinnunculus* exhibited the greatest genetic distance from the other species. Evanno's method supported K=3 (Mean LnP(K)= -10020.58 ± 2.73 SD) as the optimal number of genetic clusters, clearly separating *F. tinnunculus*, *biarmicus F. cherrug*/*F. rusticolus* and *F. peregrinus*/*F. pelegrinoides* in the STRUCTURE output (Figure 1a). FCA conducted on the cluster *F. biarmicus* /*F. cherrug*/*F. rusticolus* showed a sub-structure in which the three species slightly diverged, and the result was confirmed by Bayesian analysis (K=3; Mean LnP(K)= -4340.98 ± 0.66) (Figure 1b).

## Individual identification and parentage analysis

The probability of identity resulted in different thresholds when estimated for unrelated or related individuals and depending on the species. A PID value lower than 0.001 was reached using an average of 5 markers, while the same value was obtained with PID_sib using an average of 11 loci (Figure 2). In parentage analysis performed by Colony the correct parent pair was assigned with the maximum probability value (1.000) but decreased in F_ru_6 (0.9999), F_ru_9 (0.9874) and F_ti_15 (0.9053); F_pl_24 was associated only with the correct mother (Table 6). In Cervus, all the individuals were associated with the correct parent pair. Both Delta and LOD distribution produced comparable results (Table 7).

## Hybrid detection

FCA carried out on hybrid falcons showed that *F. cherrug* x *F. peregrinus* and *F. peregrinus* x *F. rusticolus* were distributed in a mid-range position with respect to relative pure species; conversely, *F. rusticolus* x *F. cherrug* mainly placed in a mid-range position and partially overlapped to *F. cherrug*. Bayesian analysis supported K=2 (*F. cherrug* x *F. peregrinus* mean LnP(K)= -3303.92 ± 0.78 SD; *F. peregrinus* x *F. rusticolus* mean LnP(K)= - 2989.32 ± 1.15 SD;

**Table 2.** Microsatellite loci panel tested for the cross-amplification in falcon species.

| Multiplex | Locus | | Primer Sequence 5'-3' | Dye | Range (bp) | Ta (°C) | Reference |
|---|---|---|---|---|---|---|---|
| 1 | fp54 | F: | TGATTGCAGGAACTAAGAC | 6Fam | 80-132 | 55 | Nesje M, et al. [34] |
| | | R: | TACATTCGCCAAAGGACG | - | | | |
| | fp31 | F: | ATCACCTGCACATAGCTG | Hex | 117-167 | | Nesje M, et al. [34] |
| | | R: | TTTAGCTCCTCTCTCTCAC | - | | | |
| 2 | fp107 | F: | ACAGATTTGATTGCCAGG | 6Fam | 190-244 | 55 | Nesje M, et al. [34] |
| | | R: | TGCCATGTCACATTCATAC | - | | | |
| | fp86_2 | F: | GTAAATAAGCCTCCAAAAGG | 6Fam | 127-173 | | Nesje M, et al. [34] |
| | | R: | CATGCTTCCTGATTACTTC | - | | | |
| 3 | fp13 | F: | AGCTTGATTGAGGCTGTG | 6Fam | 84-106 | 55 | Nesje M, et al. [34] |
| | | R: | CCAAATTCCCTGCTGAAG | - | | | |
| | fp82_2 | F: | CTGCACGAGGAGATGATG | Hex | 123-163 | | Nesje M, et al. [34] |
| | | R: | CCAGATAGCTGTGAAATGG | - | | | |
| 4 | Age5 | F: | ACGTTACAGACACCGATTACTTCC | 6Fam | 127-171 | 55 | Topinka JR and May B [38] |
| | | R: | AGCCACGCGTCTGATACTTT | - | | | |
| | fp92_1 | F: | TTACTAGAAGGCTGCTCAG | Hex | 98-128 | | Nesje M, et al. [34] |
| | | R: | CGTATTCCAAACTTTATGGC | - | | | |
| 5 | fp89 | F: | CTCTGCCCTGAATACTTAC | Hex | 105-137 | 55 | Nesje M, et al. [34] |
| | | R: | GAATCTTGTTTGCATTGGAG | - | | | |
| | fp74_4 | F: | TGGCTTCTCTTATCAGTAAC | 6Fam | 115-183 | | Nesje M, et al. [34] |
| | | R: | GGCTGGGTGGAATTAAAG | - | | | |
| 6 | Fpeμ25_1 | F: | GGAGGGATTTGGACAACACC | Hex | 183-308 | 55 | Beasley J, et al. [35] |
| | | R: | AGGTCCAGGTCATAATGAAGGT | - | | | |
| | Fpeμ33_1 | F: | CGTGTGTGAGAGGCATTTGG | 6Fam | 206-286 | | Beasley J, et al. [35] |
| | | R: | GCTACAGGAGGAGGTGTACC | - | | | |
| 7 | Fpeμ46_2 | F: | GGTGGATGAGTATTGGCTTCC | Hex | 229-273 | 55 | Beasley J, et al. [35] |
| | | R: | GGCCACCACGTATGTTTTGA | - | | | |
| | Fpeμ298_1 | F: | CCCCAAGCATCTTTCTGTGG | 6Fam | 172-228 | | Beasley J, et al. [35] |
| | | R: | CCGATGCACAAGGTTCACAA | - | | | |
| 8 | Fpeμ56_1 | F: | TTGTGTAGGCAAGGCTAGGG | Hex | 198-274 | 55 | Beasley J, et al. [35] |
| | | R: | TTTGCAACACTACGTCCACC | - | | | |
| | Fpeμ208_1 | F: | TGACTCTGGCAGCGTATCAG | 6Fam | 200-265 | | Beasley J, et al. [35] |
| | | R: | TCCTTGTCCTTTGGCTGTCT | - | | | |
| 9 | Fpeμ342_1 | F: | CCCATCCTGTCCAAATGCAG | 6Fam | 210-286 | 55 | Beasley J, et al. [35] |
| | | R: | GGCGTTTCCTGGAGATAAGAG | - | | | |
| | Fpeμ353_1 | F: | CACGTAGCAGCAGTTGGATC | Hex | 171-221 | | Beasley J, et al. [35] |
| | | R: | TGGAAGATGCCTGTGGAAAA | - | | | |
| 10 | Fpeμ1 | F: | TGTAAGTGGTGTTAAAACAG | 6Fam | 144-212 | 50 | Beasley J, et al. [35] |
| | | R: | GATATTAATTCCAAAGTCCA | - | | | |
| | Fpeμ248_1 | F: | CAGCATGTTTTGGCCTGGAT | Hex | 190-262 | | Beasley J, et al. [35] |
| | | R: | GGTTGACCGGGAAGTAAAGTG | - | | | |
| - | Fpeμ26_1 | F: | CTGTTGCACTGGATCTAGCC | 6Fam | 148-244 | 50 | Beasley J, et al. [35] |
| | | R: | CCCCGCAAAACACAAGTGAT | - | | | |

*F. rusticolus* x *F. cherrug*, mean LnP(K) = -3368.26 ± 0.33 SD) as the optimal number of genetic clusters for all the three *Falco* hybrids (Figures 1c-1e).

# Discussion

In this study we proposed a new microsatellite loci panel for cross amplification in *Falco* species with a wide range of applications, from genetic variability and differentiation analysis to forensic investigation purposes. The effectiveness of such an implemented panel in individual identification, parentage analysis and hybrid detection could make it a practical tool to contrast illegal trafficking. Wildlife trade has been detected as a global threat to the sustainability of biodiversity [50], leading to destruction of habitats and overexploitation of many animal and plant species with consequent negative socio-economic effects at local and global scales, such as the extinction of species and the loss of biodiversity and ecosystem services [51,52]. In the past 30 years, demanding wildlife for commercial purposes has played negative effects on biodiversity [53] due to the removal of a large number of individuals from natural populations that, in turn, caused a loss of genetic variability in a short timescale [8,51-54]. Wildlife trade represents one of the major threats to the survival of bird populations [7]. One-fifth of wildlife trade records are found to be demanded for pets or entertainment purposes [55] and these requests are particularly high for birds used as pets or display, hunted for food and employed in sport [56]. Overexploitation affects over one-third of all birds [8] and it is the second most significant threat to migratory species [57,58]. Particularly, a major concern has been arisen about the international trade in falcons, a significant proportion of which involving Arabian countries where there is a strong falconry tradition. In the last decades, the trend in legal trade of falcons is strongly increased [12] and, even though all species of falcons have been included in the CITES Appendices, the illegal international trade has been continuing [59].

Forensics science has been successfully applied to investigation of wildlife crime supporting the implementation of wildlife protection programs and molecular analysis was proven as the most effective method for dealing with this issue [60]. Currently, microsatellite loci are amongst the most popular markers in genotyping assays being a rapid, informative, and low-cost approach to produce evidence of crime in forensic investigations. Microsatellites are easily analyzed and highly polymorphic; expertise in their use is widespread and their usefulness in individual identification, assignment of individuals to specific populations, and relatedness testing have been largely proven [27-30,61-63]. Given the high performances in the use of microsatellite-based approach to address questions related to forensic investigation in wildlife crimes, it could be advantageous to investing resources in the characterization of new and practical STR-panel for species particularly threatened by trade and illegal trafficking, such as *Falco spp*.

## Genetic variability and differentiation

The first aim of this study was to set up a unique panel of microsatellite loci for the most traded *Falco spp*. and assessing its reliability for both conservation and forensic purposes. Species-specific microsatellite loci have been identified in various *Falco* species and, in some cases, their cross-amplification power has been tested in a restricted number of related species; however, a single panel of microsatellite loci for different *Falco* species has not been validated yet.

Cross-amplification is a widely used time- and cost-effective approach reducing the need to design additional STR markers. Furthermore, it can significantly facilitate studies across related species, thus making the results directly comparable and the research more cost-effective. Cross-amplification is expected to work better for phylogenetically close species, even more, when target species are congeneric. Differences among species were described by genetic components at in microsatellite loci. The analysis revealed high level of allelic richness and private alleles were detected in at least six loci for each analyzed species. As confirmed by ANOVA using intralocus heterozygosity values for each population, observed heterozygosity did not differ statistically among species as well as unbiased expected heterozygosity (except for the pair *F. pelegrinoides*/*F. tinnunculus*). Despite the similar heterozygosities, the number of private alleles differed, suggesting an inter-population differentiation.

The results of FCA and Bayesian analysis reflect the current knowledge about taxonomy and systematics of Falconidae family. Falcons can be broadly divided into three major monophyletic groups [64] large and mid-sized falcons consisting of the *F. peregrinus* and the subgenus *Hierofalco*; the Old-World kestrels, including *F. tinnunculus*; and finally, the hobbies (subgenus Hypotriorchis, not represented in this study). Coherently with this

**Table 3.** Genetic variability indices: Allele Number (NA), Private Alleles (PA), Allelic Richness (AR), observed Heterozygosity (Ho), unbiased expected Heterozygosity (uHe). Data are shown as Mean with Standard Error reported in brackets.

| | NA | PA | AR | Ho | uHe |
|---|---|---|---|---|---|
| *F. biarmicus* | 5.905 (0.45) | 0.810 (0.16) | 4.603 (0.29) | 0.551 (0.05) | 0.687 (0.03) |
| *F. cherrug* | 6.143 (0.60) | 0.476 (0.16) | 4.783 (0.44) | 0.621 (0.05) | 0.658 (0.05) |
| *F. pelegrinoides* | 4.714 (0.36) | 0.381 (0.11) | 2.800 (0.27) | 0.578 (0.04) | 0.600 (0.04) |
| *F. peregrinus* | 5.429 (0.44) | 0.571 (0.18) | 4.233 (0.31) | 0.617 (0.04) | 0.631 (0.04) |
| *F. rusticolus* | 5.381 (0.58) | 0.619 (0.25) | 4.027 (0.41) | 0.513 (0.06) | 0.561 (0.06) |
| *F. tinnunculus* | 7.000 (0.63) | 2.381 (0.51) | 5.486 (0.38) | 0.645 (0.05) | 0.746 (0.03) |

**Table 4.** Pairwise $F_{st}$ value among species calculated in Genetix. All the values are statistically significant.

| | *F. cherrug* | *F. pelegrinoides* | *F. peregrinus* | *F. rusticolus* | *F. tinnunculus* |
|---|---|---|---|---|---|
| *F. biarmicus* | 0.136 | 0.295 | 0.258 | 0.232 | 0.210 |
| *F. cherrug* | - | 0.275 | 0.247 | 0.125 | 0.237 |
| *F. pelegrinoides* | - | - | 0.087 | 0.340 | 0.288 |
| *F. peregrinus* | - | - | - | 0.317 | 0.271 |
| *F. rusticolus* | - | - | - | - | 0.287 |

**Table 5.** Analysis of Molecular Variance (AMOVA) in six species of falcons: Degrees of freedom (Df), Sum of Square (SS), Mean of Square (MS), Variance Component (VC), % of Variance Component (%VC).

| Source | Df | SS | MS | VC | %VC |
|---|---|---|---|---|---|
| Among Species | 5 | 573.49 | 114.69 | 2.23 | 25% |
| Among Individuals | 138 | 1033.90 | 7.49 | 0.69 | 8% |
| Within Individuals | 144 | 88.50 | 6.12 | 6.12 | 68% |
| **Total** | 287 | 2488.85 | - | 9.04 | 100% |

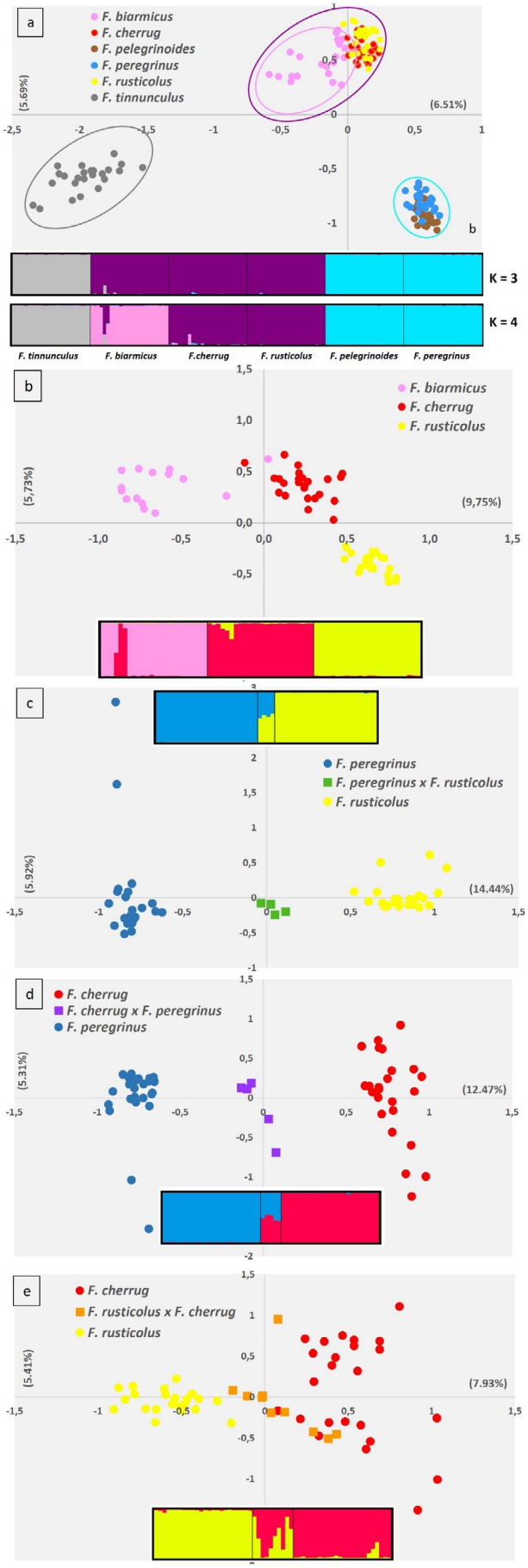


**Figure 1.** Factorial Correspondence Analysis (FCA) plot obtained using Genetix and visualized in Excel, and Structure clustering results. Each colour represents an estimated population group (cluster, K). Pure *Falco* species (a), detail of hierofalcon species (b), *F. peregrinus x F. rusticolus* (c), *F. cherrug x F. peregrinus* (d), *F. rusticolus x F. cherrug* (e).

classification, the common kestrel *F. tinnunculus* showed the greatest genetic distance and clustered separately from the other studied species. Bayesian analysis clearly identified another cluster (both using K=3 and K=4) including Peregrine falcon (*F. peregrinus*) and the barbery falcon (*F. pelegrinoides*), which graphically overlapped in FCA too. Indeed, controversy exists around the placement of these two species. The Peregrine falcon is differentiated into various subspecies, which are only slightly distinct, and therefore Barbery falcon is sometimes listed as a subspecies of Peregrine falcon (*F. peregrinus pelegrinoides*) [65] despite molecular data suggested it could be recognized as an independent species [64]. The hierofalcon group, a term commonly used for the so-called "desert falcons", consists of four ecologically and morphologically similar species - the Saker falcon (*F. cherrug*), the Gyrfalcon (*F. rusticolus*), the Lanner falcon (*F. biarmicus)* and the Laggar falcon (*F. jugger*) [66,67] that constituted another cluster in our Bayesian analysis. Evanno's method supported the inclusion of *F. cherrug*, *F. biarmicus* and *F. rusticolus* in the same cluster (K=3). Indeed, divergence time within hierofalcons is extremely recent and the group members are closely related to each other [11]. However, in FCA analysis *F. cherrug* and *F. rusticolus* overlapped and slightly diverged from *F. biarmicus* showing the presence of a sub-structure (K=4), in which the latter formed a separated cluster.

Conversely, FCA conducted on hierofalcon species only showed that *F. cherrug* and *F. rusticolus* were separated and were split into two clusters, according to the results previously obtained by principal component analysis [66], although divergence between Saker, Lanner and Gyrfalcon remains very small. Genetic structure analyses were consistent with Fst values confirming that the analysed species are not genetically homogeneous but show various differentiation levels reflecting the pattern of the known phylogenetic relationships. Furthermore, AMOVA revealed a high genetic differentiation among the studied species, and this could confirm the expected low gene flow among the populations observed in the present study since individuals are captive-bred specimens.

## Individual identification and paternity test

Our results permitted to define a unique panel of 21 highly polymorphic microsatellite loci, able to identify individuals and family groups in six species belonging to genus *Falco*. The results indicate that the tested microsatellite panel ensures a high power to discriminate individuals. Five loci were enough to distinguish with high confidence between unrelated individuals, whereas eleven loci allowed differentiating among siblings, reaching a PID and a PID_ sib lower than 0.001 which should be sufficiently low for forensic application [68]. The power of microsatellite-based assignment techniques is dependent on several factors, including usefulness of markers, genotyping errors, and test procedure itself [69]. In Colony, all offspring but one was associated with the correct parent pair, and the maximum probability value was reached in almost all of offspring individuals. Instead, the totality of family groups was correctly identified in Cervus with a confidence level higher than 95%. Such results revealed that the presented STR panel was highly informative in parentage and sibship inference, identifying the correct parent pair in all the tested species.

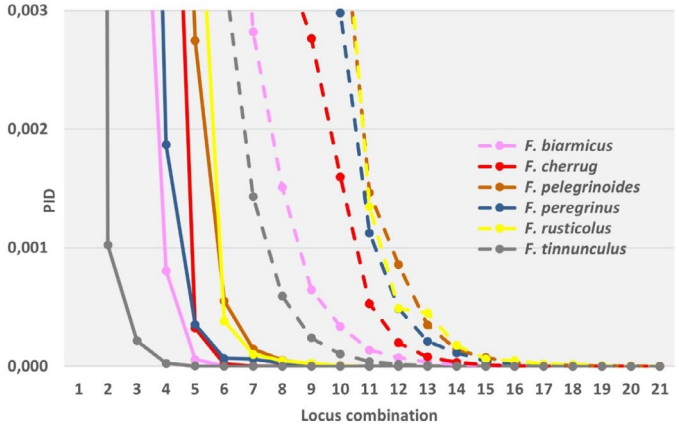


**Figure 2.** Graph of PID (continuous line) and PID_sib (dashed line) trend in species.

**Table 6.** Parental assignment values from Colony. *Falco biarmicus* (F_bi), *Falco cherrug* (F_ch), *Falco pelegrinoides* (F_pl), *Falco peregrinus* (F_pr), *Falco rusticolus* (F_ru), *Falco tinnunculus* (F_ti).

| | Inferred father | Inferred mother | Probability |
|---|---|---|---|
| F_bi_3 | F_bi_1 | F_bi_2 | 1.0000 |
| F_bi_6 | F_bi_4 | F_bi_5 | 1.0000 |
| F_bi_9 | F_bi_7 | F_bi_8 | 1.0000 |
| F_bi_12 | F_bi_10 | F_bi_11 | 1.0000 |
| F_bi_15 | F_bi_13 | F_bi_14 | 1.0000 |
| F_bi_18 | F_bi_16 | F_bi_17 | 1.0000 |
| F_bi_21 | F_bi_19 | F_bi_20 | 1.0000 |
| F_bi_24 | F_bi_22 | F_bi_23 | 1.0000 |
| F_ch_3 | F_ch_1 | F_ch_2 | 1.0000 |
| F_ch_6 | F_ch_4 | F_ch_5 | 1.0000 |
| F_ch_9 | F_ch_7 | F_ch_8 | 1.0000 |
| F_ch_12 | F_ch_10 | F_ch_11 | 1.0000 |
| F_ch_15 | F_ch_13 | F_ch_14 | 1.0000 |
| F_ch_18 | F_ch_16 | F_ch_17 | 1.0000 |
| F_ch_21 | F_ch_19 | F_ch_20 | 1.0000 |
| F_ch_24 | F_ch_22 | F_ch_23 | 1.0000 |
| F_pl_3 | F_pl_1 | F_pl_2 | 1.0000 |
| F_pl_4 | F_pl_1 | F_pl_2 | 1.0000 |
| F_pl_5 | F_pl_1 | F_pl_2 | 1.0000 |
| F_pl_8 | F_pl_6 | F_pl_7 | 1.0000 |
| F_pl_9 | F_pl_6 | F_pl_7 | 1.0000 |
| F_pl_12 | F_pl_10 | F_pl_11 | 1.0000 |
| F_pl_13 | F_pl_10 | F_pl_11 | 1.0000 |
| F_pl_18 | F_pl_15 | F_pl_16 | 1.0000 |
| F_pl_22 | F_pl_20 | F_pl_21 | 1.0000 |
| F_pl_24 | * | F_pl_23 | 1.0000 |
| F_pr_3 | F_pr_1 | F_pr_2 | 1.0000 |
| F_pr_6 | F_pr_4 | F_pr_5 | 1.0000 |
| F_pr_9 | F_pr_7 | F_pr_8 | 1.0000 |
| F_pr_12 | F_pr_11 | F_pr_10 | 1.0000 |
| F_pr_15 | F_pr_13 | F_pr_14 | 1.0000 |
| F_pr_18 | F_pr_16 | F_pr_17 | 1.0000 |
| F_pr_21 | F_pr_19 | F_pr_20 | 1.0000 |
| F_pr_24 | F_pr_22 | F_pr_23 | 1.0000 |
| F_ru_3 | F_ru_1 | F_ru_2 | 1.0000 |
| F_ru_6 | F_ru_4 | F_ru_5 | 0.9999 |
| F_ru_9 | F_ru_7 | F_ru_8 | 0.9874 |
| F_ru_12 | F_ru_10 | F_ru_11 | 1.0000 |
| F_ru_15 | F_ru_13 | F_ru_14 | 1.0000 |
| F_ru_18 | F_ru_16 | F_ru_17 | 1.0000 |
| F_ru_21 | F_ru_19 | F_ru_20 | 1.0000 |
| F_ru_24 | F_ru_22 | F_ru_23 | 1.0000 |
| F_ti_3 | F_ti_1 | F_ti_2 | 1.0000 |
| F_ti_6 | F_ti_4 | F_ti_5 | 1.0000 |
| F_ti_9 | F_ti_7 | F_ti_8 | 1.0000 |
| F_ti_12 | F_ti_10 | F_ti_11 | 1.0000 |
| F_ti_15 | F_ti_13 | F_ti_14 | 0.9053 |
| F_ti_18 | F_ti_16 | F_ti_17 | 1.0000 |
| F_ti_21 | F_ti_19 | F_ti_20 | 1.0000 |
| F_ti_24 | F_ti_22 | F_ti_23 | 1.0000 |

## Hybridization

The third aim of this study was to test the use of the proposed panel for the identification of *Falco* hybrids. Hybridization among falcon species is rare in nature, although some observations of natural hybridization among hierofalcons exist [66-70]. These falcons can easily hybridize in captivity and can also hybridize with the only slightly more divergent Peregrine falcon. Indeed, captive-bred hybrids of Saker and Gyrfalcons are amongst the most traded raptor species, representing a remarkable percentage of falconry birds. Falcon hybrids possess phenotypic and behavioural characteristics desirable by falconers, such as agility, aesthetical appeal, and robustness [70,71]. For instance, *F. cherrug* and *F. rusticolus* were crossed to unify the strength of Saker with the robust physique of Gyrfalcon, obtaining a specimen more tolerant to heat and disease; *F. peregrinus* was crossed for its speed and endurance, generating hybrids with superior hunting skills.

The set-up microsatellite panel allowed us to discriminate F1 hybrids from pure parental species. Due to the increasing volume in Falcon hybrids trade,

**Table 7.** Parentage analysis in Cervus using LOD and Delta computation. The different confidence values are indicated by the following codes: *= confidence level ≥ 95%; + = confidence level ≥ 80%. *Falco biarmicus* (F_bi), *Falco cherrug* (F_ch), *Falco pelegrinoides* (F_pl), *Falco peregrinus* (F_pr), *Falco rusticolus* (F_ru), *Falco tinnunculus* (F_ti).

| | LOD | | | | | Delta | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Offspring ID | Candidate Mother | Pair Confidence | Candidate Father | Pair Confidence | Trio Confidence | Candidate Mother | Pair Confidence | Candidate Father | Pair Confidence | Trio Confidence |
| F_bi_3 | F_bi_2 | * | F_bi_1 | - | * | F_bi_2 | * | F_bi_1 | - | + |
| F_bi_6 | F_bi_5 | * | F_bi_4 | * | * | F_bi_5 | * | F_bi_4 | * | * |
| F_bi_9 | F_bi_8 | * | F_bi_7 | * | * | F_bi_8 | * | F_bi_7 | * | * |
| F_bi_12 | F_bi_11 | * | F_bi_10 | * | * | F_bi_11 | * | F_bi_10 | * | * |
| F_bi_15 | F_bi_14 | * | F_bi_13 | * | * | F_bi_14 | * | F_bi_13 | * | * |
| F_bi_18 | F_bi_17 | * | F_bi_16 | * | * | F_bi_17 | * | F_bi_16 | * | * |
| F_bi_21 | F_bi_20 | * | F_bi_19 | * | * | F_bi_20 | * | F_bi_19 | * | * |
| F_bi_24 | F_bi_23 | * | F_bi_22 | * | * | F_bi_23 | * | F_bi_22 | * | * |
| F_ch_3 | F_ch_2 | * | F_ch_1 | * | * | F_ch_2 | * | F_ch_1 | * | * |
| F_ch_6 | F_ch_5 | * | F_ch_4 | * | * | F_ch_5 | * | F_ch_4 | * | * |
| F_ch_9 | F_ch_8 | * | F_ch_7 | * | * | F_ch_8 | * | F_ch_7 | * | * |
| F_ch_12 | F_ch_11 | * | F_ch_10 | * | * | F_ch_11 | * | F_ch_10 | * | * |
| F_ch_15 | F_ch_14 | * | F_ch_13 | * | * | F_ch_14 | * | F_ch_13 | * | * |
| F_ch_18 | F_ch_17 | * | F_ch_16 | * | * | F_ch_17 | * | F_ch_16 | * | * |
| F_ch_21 | F_ch_20 | * | F_ch_19 | * | * | F_ch_20 | * | F_ch_19 | * | * |
| F_ch_24 | F_ch_23 | * | F_ch_22 | * | * | F_ch_23 | * | F_ch_22 | * | * |
| F_pl_3 | F_pl_2 | | F_pl_1 | * | * | F_pl_2 | | F_pl_1 | * | * |
| F_pl_4 | F_pl_2 | * | F_pl_1 | * | * | F_pl_2 | * | F_pl_1 | * | * |
| F_pl_5 | F_pl_2 | | F_pl_1 | * | * | F_pl_2 | | F_pl_1 | * | * |
| F_pl_8 | F_pl_7 | * | F_pl_6 | * | * | F_pl_7 | * | F_pl_6 | * | * |
| F_pl_9 | F_pl_7 | * | F_pl_6 | * | * | F_pl_7 | * | F_pl_6 | * | * |
| F_pl_12 | F_pl_11 | * | F_pl_10 | * | * | F_pl_11 | * | F_pl_10 | * | * |
| F_pl_13 | F_pl_11 | * | F_pl_10 | * | * | F_pl_11 | * | F_pl_10 | * | * |
| F_pl_18 | F_pl_16 | * | F_pl_15 | * | * | F_pl_16 | * | F_pl_15 | * | * |
| F_pl_22 | F_pl_21 | * | F_pl_1 | * | * | F_pl_21 | * | F_pl_1 | * | * |
| F_pl_24 | F_pl_23 | * | F_pl_1 | * | * | F_pl_23 | * | F_pl_1 | * | * |
| F_pr_3 | F_pr_2 | * | F_pr_1 | * | * | F_pr_2 | * | F_pr_1 | * | * |
| F_pr_6 | F_pr_5 | * | F_pr_4 | * | * | F_pr_5 | * | F_pr_4 | * | * |
| F_pr_9 | F_pr_8 | * | F_pr_7 | * | * | F_pr_8 | * | F_pr_7 | * | * |
| F_pr_12 | F_pr_10 | * | F_pr_11 | * | * | F_pr_10 | * | F_pr_11 | * | * |
| F_pr_15 | F_pr_14 | * | F_pr_13 | * | * | F_pr_14 | * | F_pr_13 | * | * |
| F_pr_18 | F_pr_17 | * | F_pr_16 | * | * | F_pr_17 | * | F_pr_16 | * | * |
| F_pr_21 | F_pr_20 | * | F_pr_19 | * | * | F_pr_20 | * | F_pr_19 | * | * |
| F_pr_24 | F_pr_23 | * | F_pr_22 | * | * | F_pr_23 | * | F_pr_22 | * | * |
| F_ru_3 | F_ru_2 | * | F_ru_1 | * | * | F_ru_2 | * | F_ru_1 | * | * |
| F_ru_6 | F_ru_5 | * | F_ru_4 | | * | F_ru_5 | * | F_ru_4 | | * |
| F_ru_9 | F_ru_8 | * | F_ru_7 | * | * | F_ru_8 | * | F_ru_7 | * | * |
| F_ru_12 | F_ru_11 | * | F_ru_10 | * | * | F_ru_11 | * | F_ru_10 | * | * |
| F_ru_15 | F_ru_14 | * | F_ru_13 | * | * | F_ru_14 | * | F_ru_13 | * | * |
| F_ru_18 | F_ru_17 | * | F_ru_16 | * | * | F_ru_17 | * | F_ru_16 | * | * |
| F_ru_21 | F_ru_20 | * | F_ru_19 | * | * | F_ru_20 | * | F_ru_19 | * | * |
| F_ru_24 | F_ru_23 | * | F_ru_22 | * | * | F_ru_23 | * | F_ru_22 | * | * |
| F_ti_3 | F_ti_2 | * | F_ti_1 | * | * | F_ti_2 | * | F_ti_1 | * | * |
| F_ti_6 | F_ti_5 | * | F_ti_4 | * | * | F_ti_5 | * | F_ti_4 | * | * |
| F_ti_9 | F_ti_8 | * | F_ti_7 | * | * | F_ti_8 | * | F_ti_7 | * | * |
| F_ti_12 | F_ti_11 | * | F_ti_10 | * | * | F_ti_11 | * | F_ti_10 | * | * |
| F_ti_15 | F_ti_14 | * | F_ti_13 | * | * | F_ti_14 | * | F_ti_13 | * | * |
| F_ti_18 | F_ti_17 | * | F_ti_16 | * | * | F_ti_17 | * | F_ti_16 | * | * |
| F_ti_21 | F_ti_20 | | F_ti_19 | * | * | F_ti_20 | | F_ti_19 | * | * |
| F_ti_24 | F_ti_23 | * | F_ti_22 | * | * | F_ti_23 | * | F_ti_22 | * | * |

having a tool to genetically identify hybrid species would be helpful. Hybrid specimens of Peregrine falcon showed clear signs of admixture with respective parental species, whereas a variable level of admixture has been found in the hierofalcon hybrids. This latter pattern could be explained by low genetic divergence or by shared ancestral polymorphisms among species belonging to this group. Anyway, Bayesian analysis revealed that all parental and F1 individuals could be correctly identified using the proposed microsatellite loci panel. The Saker, the Gyrfalcon and their hybrids could be unambiguously distinguished, although closely related, thereby highlighting the reliability of these markers to identify hybridization events.

Hybridization could have direct conservational risk. In Europe and in the Middle East every year several specimens of hybrid falcons escape from their

owner or are released into the wild [10]. These hybrids may interbreed with wild individuals, as it occasionally happened in Scandinavia and Germany between *F. peregrinus* and *F. rusticolus* x *F. peregrinus* hybrids [72]. While hierofalcons and Peregrine Falcon generate viable offsprings but rarely capable to sire because females are often sterile, hierofalcon hybrids preserve their reproductive potential for several generations [70]. Indeed, long-term hybrid lineages of hierofalcon hybrids represent the standard stocks of falcons commonly bred in the United Arab Emirates. Therefore, hybrid specimens might represent a potential threat for natural populations due to genetic introgression and gene pool erosion.

## Conclusion

The proposed microsatellite loci panel may be of high utility, being suitable for a range of applications requiring polymorphic markers. According to its high cross-species amplification potential, it could be useful in research projects on wild populations to describe genetic diversity, genetic structure, mating system and gene flow. Moreover, it may provide a tool in forensic genetics for individual identification and parentage analysis to investigate parental claims, illegal transfer or suspected smuggling in *Falco* species and their hybrids.

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## Conflict of Interest

The authors declare no conflict of interest.

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