Comparison between two molecular protocols for sex determination in birds, with implications for the management and conservation of the Eurasian Griffon vulture *Gyps fulvus*

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Abstract – Sex ratio is a parameter worth to be monitored in small animal populations, as it has wide implications for their conservation. Morphological sex identification in birds, especially in the Accipitridae, can be difficult if the animals are young or when there is no appreciable sexual dimorphism. Sex determination can be impossible when few and/or degraded biological material (e.g. feathers, blood traces, decomposed carcasses) is available. In this case, molecular markers represent the analytical method of choice. Two molecular Amplification Refractory Mutation System (ARMS) protocols, both based on the amplification of portions of the Chromo-Helicase DNA-binding protein (*CHD1*) gene, were tested on tissues from 6 Griffon vultures *Gyps fulvus* of known sex, and subsequently, on 9 shed feathers (*i.e.* degraded samples from individuals of unknown sex) collected on feeding sites. Protocol 1 consisted of one PCR reaction yielding two amplicons with larger sizes than those produced with Protocol 2, which, in contrast, consisted of two PCRs. Our results show that, overall, both molecular protocols are suitable for sex identification in the Griffon vulture. In particular, when good quality/quantity DNA is available (*e.g.* DNA from feathers of live animals, fresh or frozen blood and tissues), Protocol 1 was faster than Protocol 2, since one single PCR is performed. On the other hand, Protocol 2 can better suite to poor or degraded DNA (*e.g.* extracted from shed feathers or decomposed tissues), because the two amplifications produce smaller fragments. We selected Protocol 1 to analyse good quality DNA from feathers of 89 free-ranging Griffons, sampled during capture and ringing activities (years 2013-2015) by the Forest Service within the Monte Velino Reserve (central Apennines, Italy). The sex ratio obtained for this reintroduced population was 1: 2.07 (29 females and 60 males). We also explored the potential of applying this method in other 10 bird species: molecular and morphological identification of sex always yielded concordant results. This study shows that Protocol 1 can be used for sex identification in Griffons and other mainly monomorphic species for which population studies or monitoring programs are planned, or when sex could not be determined from few remains of otherwise dimorphic species.

Key-words: reintroduction, molecular sexing, monomorphic species, sex ratio, male-skewed population.

INTRODUCTION

The Griffon vulture *Gyps fulvus* is nowadays present in Italy in the eastern and western Alps, the Apennines as well as in Sicily, thanks to reintroduction programs, while the only remnant autochthonous population - though subjected to restocking - survives in north-western Sardinia (Fig. 1).

Within the last ten years the Griffon vulture has been showing a slightly increasing trend in the whole country, totalling 114 breeding pairs and 87 fledged chicks on 2014 (Vulture Conservation Foundation, 2015), although it is still regarded as critically endangered (Rondinini *et al.* 2013).

The species likely became extinct in the Apennines in the 17th century, and it has been reintroduced in the Monte Velino Nature Reserve (central Apennines, Abruzzo and Latium regions) by the Forest Service from 1994 to 2002 (Potena *et al.* 2009). The current population, which developed from 93 released individuals of Spanish origin, appears stable to slowly increasing in terms of nesting pairs and fledglings. The average number of recorded breeding pairs and fledglings for the last thirteen years (2003-2015)

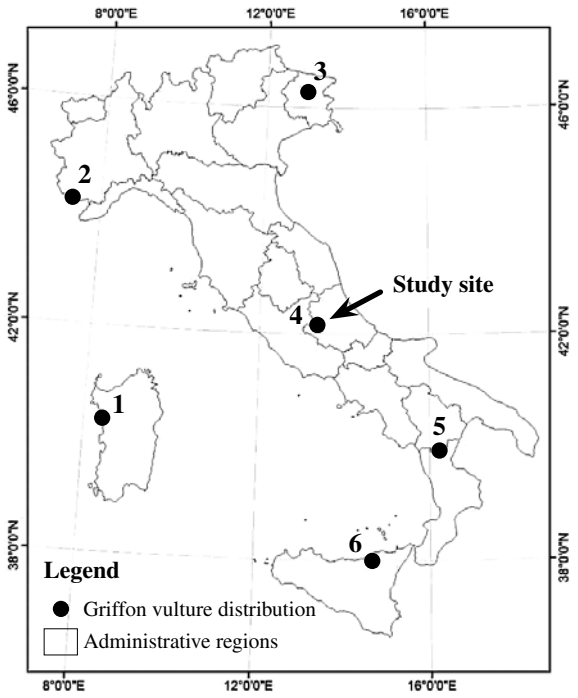

Figure 1. Distribution of *Gyps fulvus* in Italy (modified from Genero 2009). **1**) North-western Sardinia; **2**) Summering sites in the Alpi Marittime (Piedmont); **3**) Prealpi Friulane (Friuli Venezia Giulia); **4**) central Apennines (Abruzzo, Latium); **5**) Pollino National Park (Calabria); **6**) Nebrodi Regional Park (Sicily).

was 28 (± 5.7) and 21 (± 5.9), respectively, distributed in 6 colonies (Altea *et al.* in press). The monitoring of the supplemental feeding site and capture operations regularly show the spontaneous occurrence of Griffon vultures from Spain, France, Croatia and Portugal, while both some of the originally released and recently captured individuals are known to disperse to France and Spain (Forest Service, unpubl. records).

Sex ratio is one of the parameters to be monitored in small animal populations because it is important for their management and the success of reintroduction programs (Sarrazin & Legendre 2000). Morphological sex identification in birds, especially in the Accipitridae (order Falconiformes), is difficult to assess in young individuals or in species that lack a clear sexual dimorphism. Sex is hardly detectable from few and/or degraded biological material (e.g. feathers, blood traces, decomposed carcasses). In this case, molecular markers represent the analytical method of choice. The Chromo-Helicase DNA-binding protein gene (*CHD*), located on the sexual chromosomes Z and W of birds, is the target marker for molecular sex determination since early studies. Initially, some authors (Griffith *et al.* 1998) proposed a method based on different intron lengths of the *CHD1* gene, but it turned out to be scarcely reliable and not always applicable (Chang *et al*. 2008). Afterwards, the Amplification Refractory Mutation System (ARMS) methodology, based on the placement of the 3' primer terminus on a mutation conserved in the Falconiformes, proved effective for the identification of sex in this *taxon*.

We tested two ARMS protocols (Ito *et al.* 2003, Chang *et al.* 2008) developed to amplify portions of the *CHD1* gene in some species of Accipitridae but not in the Griffon. In birds, females are the heterogametic sex (ZW) while males are homogametic (ZZ). Both protocols amplify regions of the *CHD1* gene on the Z chromosome shared by males and females, as well as specific gene fragments located on the female chromosome W.

We applied these two protocols on Griffon samples to find the fastest and most reliable method for sex identification in free-living birds from the Apennine population of *G. fulvus*.

MATERIALS AND METHODS

The two protocols were first tested on 6 dead Griffon of known sex (identified during necropsy), used as positive controls, then on degraded samples using 9 shed Griffon feathers collected on feeding sites. To validate our method on field samples, feathers from 89 Griffon vultures, collected during capture and ringing activities (years 2013-2015) by the Forest Service and the Istituto Abruzzese Aree Protette in the Monte Velino Nature Reserve, were analysed for sex identification. Significant departure from a 1:1 sex ratio for the 89 captured vultures was tested by a goodness-of-fit test (Real *et al.* 2010). Finally, we explored the possibility to apply Protocol 1 in other bird species.

The DNA from 2-3 breast-plucked feathers for each Griffon individual was extracted using the QIAmp Mini-Kit (Qiagen, Hilden, Germany). Quill pens were digested over-night at 56°C with 180 µl Lysis Buffer ATL, 20 µl proteinase K (20 mg/ml) and 1mM DTT (Dithiothreitol). DNAs were quantified with the QuantiFluor® dsDNA System (Promega). The DNA of Griffon positive controls and additional 10 bird species belonging to 6 Families (see below) was isolated from approximately 25 mg of muscle using the QIAamp DNA Mini Kit. These tissues were preserved in the collection of the Forensic Genetic Laboratory (Centro di Referenza Nazionale per la Medicina Forense Veterinaria, Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana).

PCR amplifications were carried out in a final volume of 25 µl, containing 50-100 ng template DNA. Pro-

tocol 1 (Ito *et al.* 2003) consisted of a single PCR reaction (with three primers P2/NP/MP), while Protocol 2 (Chang *et al.* 2008) involved two separate reactions (A: P2/CHD-ZW-common primer pairs; B: P2/CHD-W-specific primer pairs). The final concentrations of 25 µl PCR mixture for Protocol 1 were as follows: Applied Biosystems $MgCl_2$ solution (2.5 mM), Qiagen dNTPs (200 mM each), P2 primer (20 pmol), NP primer (10 pmol), MP primer (10 pmol), Applied Biosystems AmpliTaq Gold DNA Polymerase (1.25U). Primer sequences can be found in Table 1, while their position with respect to the *CHD1* gene (Z and W chromosomes) is reported in Fig. 2. Reaction conditions for the single amplification of Protocol 1 were: initial denaturation at 94°C for 5 min followed by 35 cycles composed of denaturation at 94°C for 30 sec, annealing at 50°C for 45 sec, extension at 72°C for 45 sec, and a final extension at 72°C for 5 min. PCR products (10 µl) were fractionated by electrophoresis, onto 1.5% agarose gel pre-stained with GelRed (Biotium). Gels were visualized under UV light. The final concentrations of 25 µl PCR mixture for Protocol 2 (reaction A) were as follows: Applied Biosystems $MgCl_2$ solution (2.5 mM), Qiagen dNTPs (200 mM each), P2 primer (10 pmol), CHD-ZW-common primer (10 pmol), Applied Biosystems AmpliTaq Gold DNA Polymerase (1U). The final concentrations of 25 µl PCR mixture for Protocol 2 (reaction B) were as follows: Applied Biosystems $MgCl_2$ solution (2.5 mM), Qiagen dNTPs (200 mM each), P2 primer (10 pmol), CHD-W-specific primer (10 pmol), Applied Biosystems AmpliTaq Gold DNA Polymerase (1U). Primer sequences can be found in Table 1, while their position with respect to the *CHD1* gene (Z and W chromosomes) is reported in Fig. 3. Amplification conditions for reactions A and B of Protocol 2 were: initial denaturation at 94°C for 5 min followed by 38 cycles composed of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, extension at 72°C for 20 sec, and a final extension at 72°C for 5 min. PCR products (10 µl) were fractionated by electrophoresis, onto 1.5% agarose gel pre-stained with GelRed (Biotium). Gels were visualized under UV light.

RESULTS

Both protocols were easy to use and reliable to determine the sex in *G. fulvus*: the gender of all control individuals was correctly assigned. The *CHD1* gene fragments amplified with Protocol 1 (378 and 290 bp) were larger than those obtained with Protocol 2 (258 and 148 bp). The first protocol resulted faster than the second, because it required a single amplification reaction and electrophoresis run (Fig. 4a), compared to two PCRs and electrophoretic runs of the latter (Fig. 4b). For Protocol 2, we tried to set up a single reaction, combining the three primers simultaneously, but no different banding patterns between males and females were observed (Fig. 4c). Consequently, two separate PCRs were maintained. When tested on degraded samples, we found that Protocol 1 (Fig. 5a) was less efficient than Protocol 2 (Fig. 5b) in determining the sex in 3 out of 4 individuals with the lowest DNA content (Fig. 5a, 5b, lanes 4, 5 and 8).

We selected Protocol 1 for the analysis of field samples from the Apennine Griffons because feathers from living birds represent a good-quality DNA source. The sex ratio obtained for this reintroduced population was 1:2.07 (29 females and 60 males), which significantly deviated from the expected 1:1 ratio ($\chi^2_1 = 10.8$, $p = 0.001$).

Protocol 1 was then applied to both male and female individuals of *G. fulvus*, *Gyps rueppellii, Buteo buteo, Psittacus erithacus* and *Asio otus*, checking the electrophoretic pattern in each sex (Tab. 2).

For additional 6 species only one gender was available: *Aquila chrysaetos* (male), *Gallus gallus* (female), *Chloebia gouldiae* (male), *Lonchura striata* (female), *Nycticorax nycticorax* (male), and *Athene noctua* (male). *Buteo buteo* and *Nycticorax nycticorax* were tested with the same protocol by Lee *et al.* (2008). For all samples, molecular identification of sex was consistent with morphological assignment, as determined during the necropsy. Standard banding patterns were always obtained (*i.e.* one band in males and two in females), without observing non-specific bands.

Table 1. Primer setails.

Primer name	Protocol	Sequence (5' to 3?)	References
NP	Protocol 1	GAGAAACTGTGCAAAACAG	Ito *et al.* 2003
MP	Protocol 1	AGTCACTATCAGATCCGGAA	Ito *et al.* 2003
P2	Protocol 1 and 2	TCTGCATCGCTAAATCCTTT	Griffith *et al.* 1998
CHD-ZW-common	Protocol 2	GATCAGCTTTAATGGAAGTGAAG	Chang *et al.* 2008
CHD-W-specific	Protocol 2	GGTTTTCACACATGGCACA	Chang *et al.* 2008

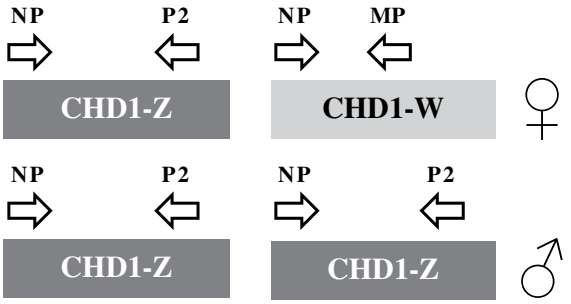

Figure 2. Position of primer pairs in Protocol 1.

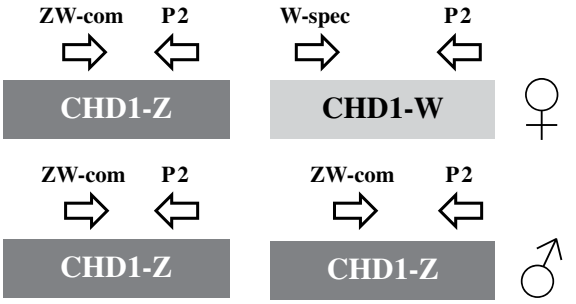

Figure 3. Position of primer pairs in Protocol 2.

DISCUSSION

Overall, both protocols can be used for molecular sex identification in the Griffon vulture. When good quality/quantity DNA is available (*e.g.* DNA from feathers of live animals, fresh or frozen blood and tissues), then Protocol 1 was faster than Protocol 2, since one single PCR is performed. Nevertheless, Protocol 2 can better suite to poor or degraded DNA (*e.g.* extracted from shed feathers or decomposed tissues), because the two amplifications produce smaller fragments.

Within the Apennine population of *G. fulvus* examined in this study, the number of males is twice that of females. An excess of males in different populations of monogamous bird species was already found in the literature (Breitwisch 1989, Nebel *et al.* 2004), although a ratio not significantly different from 1:1 has been reported for the Griffon vulture in France (Bosé *et al.* 2007). A marked imbalance toward one sex, like that obtained for the Griffons here investigated, may sound as an alarm bell for a small population of a species which is considered in critical danger of extinction in Italy (CR category - A2b, Red List of Italian vertebrates).

In monogamous species where cooperation among in-

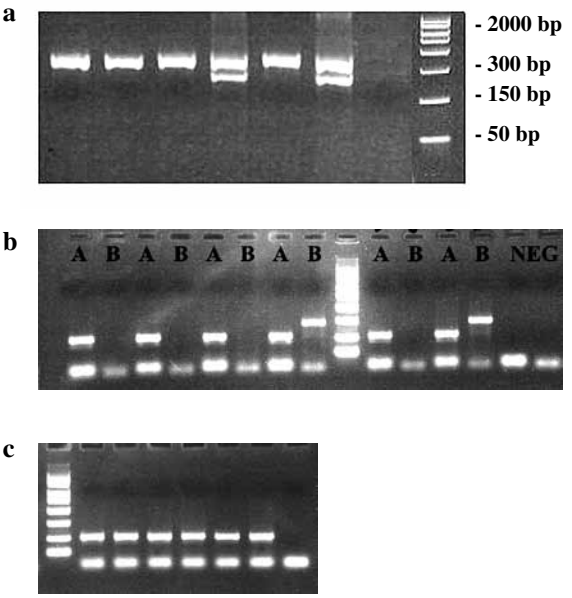

Figure 4. Electrophoretic banding pattern in 6 control samples of Griffon obtained with a) Protocol 1 (one single PCR); b) Protocol 2 (two PCRs); A = P2/CHD-ZW-common primer pair; B = P2/CHD-W-specific primer pair. c) Protocol 2 (one single PCR). See text for details.

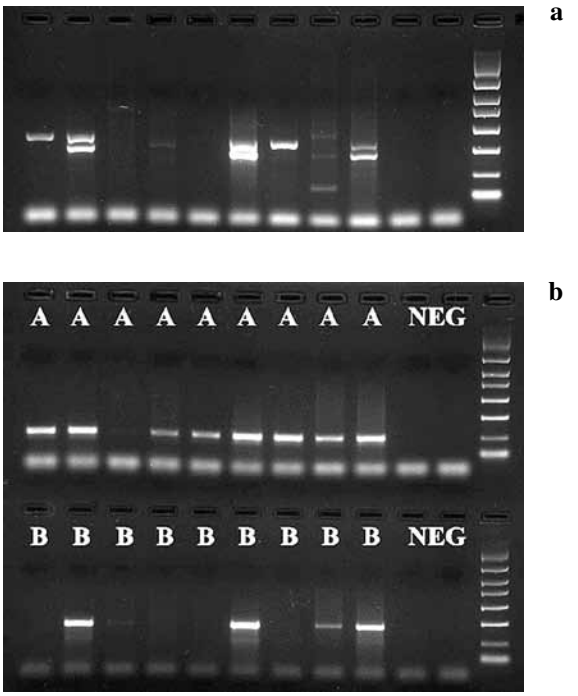

Figure 5. Comparison between Protocol 1 (a) and Protocol 2 (b) in 9 degraded samples of Griffon (less than 20 ng of template DNA were loaded in PCRs). A = P2/CHD-ZW-common primer pair; B = P2/CHD-W-specific primer pair.

Table 2. Molecular sex identification in males and females of *G. fulvus* and other four bird species, based on Protocol 1.

Species	Family	Morphological identification	Molecular identification
Gyps fulvus	Accipitridae	F	F
Gyps fulvus	Accipitridae	M	M
Gyps rueppelli	Accipitridae	F	F
Gyps rueppelli	Accipitridae	M	M
Buteo buteo	Accipitridae	F	F
Buteo buteo	Accipitridae	M	M
Asio otus	Strigidae	F	F
Asio otus	Strigidae	M	M
Psittacus erithacus	Psittacidae	F	F
Psittacus erithacus	Psittacidae	M	M

dividuals enhances resources exploitation, we expect that a balanced sex ratio within breeders contributes to an increase in population growth rate, while maximizing reintroduction success at the same time (Legendre 2004). Preliminary identification of sex in individuals targeted to act as population founders in monogamous species is critical, especially when the number of released animals is very small (ca. 10 birds/year in the study area). No information on sex ratio was available for the Griffon vultures which have been released in our study area, so we could not support the hypothesis that nowadays imbalanced sex ratio still depends on a sex-biased stock of founders. Preliminary data show that in our sample sex ratio is skewed toward males in young and immature vultures too, which is clearly not depending on the sex of released birds. Additionally, we do not expect a sex-biased survival for Griffon vulture (Bosé *et al*. 2007) and we showed that ranging behaviour was similar in males and females (Altea *et al.* 2013). Therefore, it would be beneficial to conduct a DNA-based study (using nuclear markers, such as microsatellites) in the Griffon population of central Apennines to estimate genetic variability and the effective number of breeders. The reasons for such a biased sex ratio, its consequences on population growth and, ultimately, on reintroduction success should also be concurrently investigated. Sex identification of birds cannot be disregarded in *ex situ* conservation breeding programmes, and remains fundamental to population, behavioural, evolutionary and forensic studies.

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