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1 **Low evidence for extra-pair fertilizations in two reintroduced populations of Griffon**
2 **vulture (*Gyps fulvus*)**

3

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17

18

19 **Abstract**

20 The Griffon vulture (*Gyps fulvus*) is considered to be socially monogamous. However, extra-
21 pair fertilizations are suspected due to observations of extra-pair copulations in some
22 populations. We performed parentage studies based on ten polymorphic microsatellite
23 markers in two reintroduced colonies of Griffon vulture. Out of 40 genotyped chicks, we
24 found eight chicks whose genotypes mismatched those of their observed parents. Two could
25 be explained by the occurrence of a null allele at one locus. The six remaining mismatches
26 detected relied on mismatches at one locus, and they were not detect anymore when we

1 increased the potential genotyping error rate. We thus conclude that the Griffon vulture is
2 genetically monogamous, at least in low-density populations.

3

4 **Zusammenfassung**

5 **Geringe Anzeichen von Fremdvaterschaften in zwei wiedereingebürgerten Gänsegeier-** 6 **Populationen (*Gyps fulvus*)**

7

8 Es wird angenommen, dass Gänsegeier (*Gyps fulvus*) monogam leben. Da aber
9 außerpartnerschaftliche Kopulationen beobachtet wurden, ist anzunehmen, dass es auch
10 Fremdvaterschaften gibt. Wir untersuchten Verwandtschaft anhand von zehn polymorphen
11 Mikrosatellitenmarkern in zwei wiedereingebürgerten Gänsegeierkolonien. Von 40
12 genotypisch untersuchten Küken passten acht nicht zu ihren sozialen Eltern. Zwei dieser
13 Abweichungen ließen sich auf Nullallele an einem Locus zurückführen. Die sechs Übrigen
14 kamen durch eine Abweichung an einem Locus zustande und wurden nicht mehr entdeckt, als
15 wir den Fehlerspielraum des genetischen Typisierung erhöhten. Wir schließen daraus, dass
16 Gänsegeier zumindest in wenig dichten Kolonien genetisch monogam sind.

17

18

19

20 **Introduction**

21 Monogamy was long considered to be the dominant mating system in birds, but advances in
22 molecular techniques have revealed high levels of extra-pair fertilizations (EPFs) among
23 many avian species (Avisé 1996, Griffith et al. 2002). EPFs influence the variability of
24 reproductive success among individuals and thus individual fitness and effective population
25 size (Castro et al. 2004), which are important measures for conservation of endangered
26 species. The Griffon vulture (*Gyps fulvus*) was highly endangered across its range in the
27 middle of the 20th century, and local extinctions occurred in France (Terrasse 1983).

28 Conservation actions and successful reintroduction programs restored Griffon vulture

1 populations in southwestern Europe (Terrasse et al. 2004). The Griffon vulture is a long-lived
2 colonial scavenger, and it is considered to be sexually monomorphic and socially
3 monogamous (Roselaar 1979, Mendelssohn and Leshem 1983). The species typically has
4 long-term pair bonds, although some divorces had been observed (Sarrazin et al. 1996).
5 Several facts led researchers to suspect that EPFs could occur in this species despite a lack of
6 confirmation by genetic analysis. First, the Griffon vulture is a colonial nester, and pursuit of
7 extra-pair copulations (EPCs) by females has been suggested as a determinant of coloniality
8 evolution (Wagner 1993, Hoi and Hoi-Leitner 1997). Second, EPC has already been reported
9 in Old World vultures (Negro and Grande 2001) and recently for the studied species
10 (Xirouchakis and Mylonas 2007). On the contrary, due to the low fecundity rate in Griffon
11 vultures, *i.e.*, only one egg per year, intraspecific brood parasitism or “egg dumping” is
12 unlikely to occur (Arnold and Owens 2002). We studied whether EPFs occurred in two
13 reintroduced populations of Griffon vulture using parentage analysis on DNA microsatellite
14 fingerprinting.

15

16 **Methods**

17 *Studied populations*

18 The Griffon vulture was reintroduced to three areas in the French Alps from 1996 to 2004. In
19 Baronnies, 56 birds were released from 1996 to 2001. In Verdon and Vercors, respectively, 91
20 and about 60 vultures have been reintroduced since 1999. All released birds were marked
21 with an engraved Darvic ring that allows long-distance identification. Their first chicks
22 hatched in the wild in 1999 in Baronnies and in 2002 in Verdon. No successful reproduction
23 was observed in Vercors before 2008. In 2004, 45 pairs bred in Baronnies and 11 in Verdon.
24 During the studied period, 124 reproduction events have been observed in 59 nests in
25 Baronnies, and 28 reproduction events in 25 nests in Verdon. All chicks were marked at their

1 nests. During chick ringing, parents left the nest before the ringer reached the nest. During the
2 breeding period (from December to August), breeding birds were identified on nests using a
3 spotting scope ($\times 60$). Distance to nest varied from 300 to 800m. Duration of observation of
4 each nest varied from 1 to 5 hours depending on the activity level of the breeders.
5 Observations of one adult taking over nest duty from another adult allowed us to confirm their
6 breeding status. Frequently, breeders' rings could not be read during the 5 hours of
7 observation when breeders were inactive. Some birds could not be identified due to ring
8 losses. Therefore, despite regular monitoring of pairs throughout the breeding season,
9 identification relied on few records. Basic information on rings (at least presence/absence)
10 were available for 192 out of the 248 breeders in Baronnies and 50 out of the 56 breeders in
11 Verdon. 144 breeders, of which 65 were successful breeders, were identified in Baronnies
12 whereas in Verdon 42 breeders, of which 30 successful ones, were identified. These
13 identifications were used to build "observed" genealogies of chicks hatched from 2001 to
14 2004 in Baronnies and from 2002 to 2004 in Verdon.

15 *Molecular and parentage analysis*

16 Blood or feathers were taken from most of the released individuals. Growing feathers of
17 hatched chicks were collected during ringing and stored in 70% ethanol. All sampled
18 individuals were sexed with molecular techniques (Bosé et al. 2007). This molecular sexing
19 allowed us to discard observations of two individuals of the same sex breeding with each
20 other. For instance, two females were observed breeding in Baronnies. We excluded this pair
21 from the analysis of EPFs. Using ten microsatellite markers (GF3F3, GF3H3, GF8G1, GF9C1,
22 and GF11A4 from Mira et al. 2002 ; GVBV11, GVBV12, GVBV13, GVBV17, and GVBV20
23 from Gautschi et al. 2000), we genotyped all sampled individuals (for protocol, see Le Gouar
24 et al. 2008). DNA extracted with the CTAB method (Le Gouar et al. 2008) from blood and
25 growing feathers were highly concentrated. Some individuals were genotyped three to four

1 times to determine how many amplifications were needed to obtain a reliable genotype. Both
2 heterozygous and homozygous genotypes were reliable with one amplification since we found
3 the same genotype for all the amplifications. However, in some rare cases, some loci did not
4 amplify. We thus considered individuals with fewer than four missing loci (only 4 individuals
5 were discarded). Among them, we selected potential parents in the Baronnies and Verdon
6 colonies as those individuals believed to have resided in the colonies during breeding season
7 (*i.e.*, those that were neither dead nor seen in another colony). We checked for identical
8 genotypes in the data set.

9 We computed allele frequencies, mean number of alleles by locus, as well as expected and
10 observed heterozygosity rates using GENETIX 4.02 software (Belkhir et al. 1996). Several
11 technical and biological hurdles independent from EPF, such as null alleles, linkage
12 disequilibrium, mutations and scoring errors, could lead to microsatellite genotyping
13 discrepancies (Hoffman and Amos 2005). Because null alleles, which result from the
14 amplification failure of one allele, lead to scoring a heterozygote individual as homozygous,
15 their occurrence could be suspected if an excess of homozygotes, *i.e.*, Hardy-Weinberg
16 disequilibrium, is detected. Departure from Hardy-Weinberg equilibrium (HWE) for each
17 locus and genotypic disequilibrium were tested using a Markov chain as implemented in
18 GENEPOP 3.4 software (Raymond and Rousset 1995). Additionally, we computed for each
19 locus the average non-exclusion probability for the identities of two unrelated individuals
20 (PID) and of two siblings (PIDsib) and the estimated null allele frequency using the program
21 Cervus 3.0 (Kalinowski et al. 2007).

22 For parentage analysis, we used the categorical allocation method implemented in the
23 FAMOZ software (Gerber et al. 2003) to construct the “genetic” genealogy. This method
24 assigned offspring to parents and to pairs on likelihood score calculations (LOD score)
25 derived from genotypes and allele frequencies (Jones and Ardren 2003). To define statistical

1 LOD score thresholds for parents and pairs, we computed simulations of 10,000 offspring for
2 each population. As mutations and scoring errors always occurred in the data sets, we first
3 introduced a proportion of 0.001 typing error in both the LOD score calculation and the
4 simulations. We then increased this rate to 0.1 to span the error rate variation reported in
5 (Hoffman and Amos 2005). Parents and pairs with LOD scores above threshold were
6 considered to be true genetic parents (Gerber et al. 2003). We then compared the “genetic”
7 genealogy and the “observed” genealogy to test whether EPF occurred. When mismatches
8 between observed and genetic genealogy were revealed, genotypes were confirmed by
9 additional independent tests.

10

11 **Results**

12 The proportion of identified breeders decreased in Baronnies from 100% in 1999 to 21% in
13 2004 and was exhaustive each year in Verdon. Some individuals released in Vercors and in
14 Verdon were observed breeding in Baronnies. Among identified parents, 35 (54%) were
15 genotyped in Baronnies (20 males, 14 females and one unsexed parent) and 15 (50% of
16 identified parents) in Verdon (9 males and 8 females). We genotyped 30 chicks hatched from
17 2001 to 2004 in Baronnies, and we selected 48 potential genotyped parents. In Verdon, the
18 number of genotyped chicks hatched from 2002 to 2004 was 10, and 24 potential genotyped
19 parents were considered. All microsatellite markers were polymorphic, and the mean number
20 of alleles by locus was 6.1 for Baronnies and 5.4 for Verdon. No identical genotypes were
21 found in the data set. The cumulative probability of identity of unrelated individuals was
22 <0.0001 , and that of siblings was 0.0009. We found no linkage disequilibrium between loci.
23 Observed and expected heterozygosity rates were, respectively, 0.589 (± 0.244) and 0.573
24 (± 0.238) in Baronnies and 0.532 (± 0.285) and 0.540 (± 0.255) in Verdon. Global tests for
25 deficits of heterozygotes from HWE were not significant in both populations. However,

1 departure from HWE was detected at locus GF11A4 in both populations. Null allele
2 frequency at this locus was estimated to be 0.07.
3 For both populations, mismatches between observed and genetic genealogy were detected
4 with error rates of 0.001. These mismatches (confirmed with two repeated amplifications)
5 involved one father and two mothers in Baronnies and one father in Verdon (table 1). In
6 Baronnies, one extra-pair paternity event was suspected because of a mismatch between the
7 heterozygous genotypes of a father and two putative chicks born in 2002 and 2005 at locus
8 GVBV12 (table 1). One female with a homozygous genotype at GF3H3 (146/146) was
9 excluded as a potential parent of a chick with a heterozygous genotype at this locus (136/144).
10 In Verdon, one father was excluded because of a mismatch between its heterozygous
11 genotype and the genotypes of three putative chicks born in 2002, 2003 and 2004,
12 respectively, at locus GF9C1 (table 1). Those discrepancies were not significant when
13 adopting an error rate of 0.1. By contrast, with both error rates of 0.001 and 0.1, one female in
14 Baronnies was excluded as a possible mother of two offspring because of a mismatch between
15 their genotypes at locus GF11A4. Both offspring were homozygous at this locus with
16 different alleles from the heterozygous genotype of the putative mother.

17

18 **Discussion**

19 Our results showed little evidence of EPF in Griffon vultures. Concerning evidence of extra-
20 pair paternity, two males were observed during several consecutive years breeding with the
21 same female, which supports accurate identification of these males. Consistent genotypes of
22 offspring indicated that they have the same father, which suggest that the extra-pair male was
23 the same each year. However, when we increased the rate of potential genotyping errors, all
24 of the extra-pair paternities and half of the intraspecific brood parasitism were not detected.
25 Although independent re-tests were performed, microsatellite genotyping error could still

1 occur because of allele dropout, mutation or null allele. Checking for allele dropout using the
2 whole Griffon vulture genotype dataset ($n=796$), we found low percentages (<5%) of allele
3 dropout for all loci. Mutations in microsatellite markers often involved single- or two-step
4 mutations (one or two repeat units) and occurred more often for markers of larger sizes
5 (Ellegren 2000). Mismatches due to one or two repeat differences at the GF9C1 and GVBV12
6 loci, two large-sized markers, led to detection of EPF, questioning the occurrence of
7 mutations at those loci. Null allele was suspected for one locus (GF11A4) because of a high
8 proportion of observed homozygotes compared to expectations under HWE. Consequently,
9 EPF revealed because of a mismatch at this locus has to be considered with caution,
10 especially if it involves a homozygous genotype, as was the case for one female in Baronnies.
11 Therefore, intraspecific brood parasitism evidence could be discarded due to the potential for
12 a null allele at the locus of mismatch. Concerning EPFs, only sequencing of the relevant
13 regions would allow to discard technical problems as source of mismatches rather than
14 biological ones.

15 The low occurrence of EPF could be due to the low breeding densities of the studied
16 populations. Several studies have found that breeder density and extra-pair paternity rate are
17 positively correlated among populations of the same species (Møller and Ninni 1998), but
18 they failed to determine if this relationship was causal or due to other covariate factors
19 (Griffith et al. 2002). Nonetheless, our results agree with those of other studies on socially
20 monogamous species of raptors that revealed low to null rates of EPFs (Rudnick et al. 2005,
21 Saladin et al. 2007). Our results suggest that Griffon vultures are genetically monogamous.
22 Indeed, strict monogamy in vultures has been debated because EPCs have been observed.
23 Their frequencies are usually low in wild-born populations (3.3% in Griffon vulture,
24 Xirouchakis and Mylonas 2007; 0.5% in Cape Griffon, *Gyps coprotheres*, Mundy et al. 1992 ;
25 0.52% in Bearded vulture, *Gypaetus barbatus*, Bertran and Margalida 1999; 2.6% in Egyptian

1 vulture, *Nephron percnopterus*, Donázar et al. 1994), but Mee et al. (2004) observed an EPC
2 frequency of 23% in a reintroduced population of California condor, *Gymnogyps*
3 *californianus*. They argued that EPCs may be enhanced in reintroduced populations because
4 of increasing social interactions due to concentration of food at few feeding stations, limited
5 mate choice and a high degree of inbreeding among breeders (Mee et al. 2004). If we assume
6 that EPCs led to EPFs, our results contrast with Mee et al.'s observations. However, a high
7 frequency of EPCs with a low level of EPFs could exist, as in the monogamous *Lanyus scops*
8 owl (Hsu et al. 2006). Limitation of EPFs could be also due to a high copulation rate between
9 partners of the same breeding pair. Indeed, high copulation rates are common in raptors
10 (Mougeot et al. 2002) and are supposed to be an effective behaviour to ensure paternity for
11 the male and male fidelity and investment in chick rearing for the female (Hunter et al. 1993,
12 Birkhead and Møller 1998). Moreover, the high investment of each parent in chick rearing
13 restrains them from seeking EPC (Saladin et al. 2007).
14 The low to null EPF rate observed here, which must be confirmed in larger populations,
15 makes it possible to directly estimate the effective population size from a breeding pair census.

16

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3

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19

1 Table 1: Genetic discrepancies in genealogies revealed with 0.001 genotyping error in Baronnies and Verdon colonies. For each locus,
 2 percent of missing data, null allele frequency, average probability that the genotypes at a single locus do not differ between two unrelated
 3 individuals (PID) and average probability that the genotypes at a single locus do not differ between two full siblings (PIDsib) are presented.
 4 Mismatches are highlighted in grey. Year of birth of chick are indicated. - : no amplification.

		GF11A4	GVBV17	GF9C1	GF3H3	GF3F3	GVBV20	GVBV13	GVBV12	GVBV11	GF8G1
% of missing data		0%	28%	2.3%	4.6%	0.6%	1.7%	1.1%	1.7%	1.1%	21%
Null allele frequency		0.075	0.033	0.011	0.003	0.043	0.0004	0.028	0.012	0.027	0.016
PID		0.05	0.41	0.04	0.09	0.49	0.22	0.60	0.03	0.18	0.45
PIDsib		0.35	0.63	0.33	0.39	0.71	0.51	0.78	0.32	0.49	0.67
Baronnies	observed father	130-140	-	254-254	144-146	176-178	137-137	176-176	242-268	164-170	-
	observed mother	130-135	184-184	264-268	136-146	176-176	139-139	176-176	238-254	162-162	278-278
	Nest A										
	<i>chick2002</i>	135-140	184-186	254-268	136-146	176-176	137-139	176-176	238-266	162-164	278-278
	<i>chick2005</i>	130-130	184-184	254-268	136-144	176-178	137-139	176-176	238-266	162-164	-
	observed father	130-135	184-184	254-266	136-144	176-176	137-137	176-178	220-240	162-164	278-278
	Nest B										
	observed mother	135-150	-	256-260	146-146	176-176	137-139	176-178	236-240	158-162	276-278
	<i>chick2001</i>	130-135	184-186	254-260	136-144	176-176	137-139	178-178	236-240	162-164	276-278
Nest C											
observed mother	125-155	184-184	262-262	142-146	176-178	139-139	176-176	238-238	162-162	278-278	

	<i>chick2003</i>	130-130	184-184	260-262	144-146	176-178	137-139	176-176	236-238	162-162	278-278
	<i>chick2004</i>	135-135	184-184	-	144-146	176-176	137-139	176-176	-	-	-
Verdon	observed father	135-140	-	260-266	136-146	176-176	137-137	176-176	236-240	158-164	278-278
	observed mother	140-140	184-184	254-254	138-142	176-176	139-141	176-176	240-240	158-162	278-278
Nest D	<i>chick2002</i>	135-140	184-186	254-256	138-146	176-176	137-139	176-176	240-240	158-164	-
	<i>chick2003</i>	140-140	184-186	254-256	136-142	176-176	-	176-176	236-240	158-162	-
	<i>chick2004</i>	140-140	184-184	254-256	142-146	176-176	137-139	176-176	240-240	162-164	278-278

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