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Short communication

Extra-pair paternity in the Lesser Kestrel *Falco naumanni*: a re-evaluation using microsatellite markers

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Modern molecular techniques based on DNA typing have revolutionized our view of avian mating systems. Birds were considered to be essentially monogamous only a few years ago (Lack 1968). In contrast, Griffith *et al.* (2002) have recently summarized how molecular techniques have revealed that birds are only rarely monogamous, with extra-pair offspring found in approximately 90% of the species studied to date. It has been known for some time that individuals in supposedly monogamous species often adopt a mixed reproductive strategy (Trivers 1972). Both males and females may seek extra-pair copulations (EPC), which could provide them with some genetic benefits (for a review of benefits and drawbacks of engaging in EPC, see Birkhead & Møller 1992; Wink & Dyrcz 1999).

Although there is great interest in the reasons for differences in extra-pair paternity (EPP) rates in birds, few studies have compared the results obtained by different molecular techniques. Among available markers, DNA fingerprinting in the 1990s and, more recently, microsatellites, are the most popular in elucidating genetic relationships in birds (Burke & Bruford 1987, Ellegren 1992), but, to our knowledge, comparisons of the performance of these two techniques using the same sample sets have not been reported.

In this study, we reappraised EPP in the Lesser Kestrel *Falco naumanni*. Our aim was to recalculate levels of EPP in this species using microsatellite markers instead of DNA fingerprinting, and to compare the results obtained with both techniques. Earlier studies of the copulatory behaviour of Lesser Kestrels (Negro *et al.* 1992) have already shown that some males pursue a mixed reproductive strategy, with a 6.7% incidence of EPC attempts, and DNA fingerprintings of 26 families revealed that 3.4% of the nestlings resulted from extra-pair fertilizations (Negro *et al.* 1996). We reanalysed 23 families from which we still had usable DNA from the fingerprinting study of Negro *et al.* (1996) together with DNA from a further eight new families from a different population sampled during the 2003 breeding season.

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Study species and sample collection

The Lesser Kestrel is a small migratory falcon which breeds in the Western Palearctic, from the Iberian Peninsula to China, and winters in Africa (Cramp & Simmons 1980). Lesser Kestrels typically breed in urban colonies of up to 100 pairs, usually in buildings (Cramp & Simmons 1980). They are socially monogamous, although polygynous males have been reported (Hiraldo *et al.* 1991, Tella *et al.* 1996).

Twenty-three Lesser Kestrel families were sampled at breeding colonies located in Los Monegros (Aragón, northeast Spain, 41°21′N, 0°11′W) during the 1993 breeding season. Eight families were sampled at two colonies in Huelva (Andalusia, southwest Spain, 37°10′N, 6°21′W) during the 2003 breeding season. Blood or feather samples were collected for both the putative parents and all nestlings. In both populations, the putative parents were captured at the nest when incubating or brooding small chicks to ensure that they were providing parental care and were not visitors unrelated to the nests. During their first week, young from selected nests were marked on the leg with a cloth strap. The purpose of this early banding was to detect cases of nest switching by nestlings and their subsequent adoption, a phenomenon frequently observed in the Los Monegros population (Tella *et al.* 1997). Early banding was not carried out in Andalusian colonies because movements between nests could be made only by flying. Adults and young were colour-banded. Banded adults were observed through spotting scopes to confirm that they were attending the nests where they were caught and were feeding their putative offspring.

In 1993, approximately 0.4 mL of blood from the brachial veins of both adults and nestlings was taken using 1-mL syringes and 30-gauge needles. Blood was preserved in lysis buffer consisting of 0.01 M NaCl, 0.01 M EDTA and 1% *n*-lauroylsarcosine, pH 7.5 (Seutin *et al.* 1991). Samples were stored at 4 °C until processing. In 2003, blood was collected from adults in the same way as in 1993. Blood was preserved in absolute ethanol and stored at 4 °C until processing. In the case of nestlings, one or two feathers were pulled from the back and preserved in a paper envelope. Feathers were stored at room temperature until processing.

DNA extractions

DNA extracts from the samples collected in 1993 (Negro *et al.* 1996) were preserved in TE buffer (5 mM Tris/HCl, 0.1 mM EDTA, pH 7.4) and stored at –80 °C until

required. The extraction protocol we used for the additional samples collected in 2003 is a modification of that described by Gemmell and Akiyama (1996). Aliquots of blood and feather shafts were suspended in 1.5-mL microfuge tubes with 300 µL of digestion buffer (100 mM NaCl, 50 mM Tris/HCl, 1% SDS, 50 mM EDTA) and 3 units[a] of proteinase K. Digestion of the samples was carried out over a period of 2 h or more at 55 °C. Once the digestion was complete, an equal volume (300 µL) of 5 M LiCl was added to each tube. The sample was mixed thoroughly by inversion for 1 min and 600 µL of chloroform-isoamylic alcohol (24 : 1) was added. After vortexing, samples were spun for 15 min at 13 000 rev/min and the supernatant carefully removed to a new tube. Then, 1 mL of absolute ethanol was added until DNA precipitated. DNA was recovered by centrifuging at 13 000 rev/min for 15 min. The pellet was dried and washed twice with 70% ethanol, and later placed in 0.1–0.2 mL of TE buffer and stored at –20 °C.

Microsatellite genotyping

A microsatellite-based genotyping system was employed to enable the assignment of paternity to chicks (Ellegren 1992, Primmer *et al.* 1995). We used seven markers initially developed for the Peregrine Falcon *Falco peregrinus* by Nesje *et al.* (2000), which were also known to be polymorphic in Lesser Kestrels and other *Falco* species (Groombridge *et al.* 2000). The number of alleles, observed heterozygosity and parentage exclusion probability for first and second parents were estimated with Cervus 2.0 software (Marshall *et al.* 1998). The probability that any two individuals shared the same genotype was calculated with Identity 1.0 software (Wagner & Sefc 1999).

For each locus, the polymerase chain reaction (PCR) was carried out in a PTC-100 Programmable Thermal Controller (MJ Research Inc.) using the following PCR profile: 35 cycles of 40 s at 94 °C, 40 s at 55 °C, 40 s at 72 °C and finally 4 min at 72 °C. Each reaction was carried in 11 µL of a mix containing 0.2 units of Taq polymerase (Bioline), 1× PCR buffer (Bioline), 1.5 mM $MgCl_2$, 0.02% gelatin, 0.12 mM of each dNTP, 5 pmol of each primer and approximately 10 ng of genomic DNA. Primer sequences are available in the GenBank Database. F-primers were 5′-end labelled with HEX, TET or 6-FAM. Amplified fragments were resolved on an ABI Prism 310 Genetic Analyser (Applied Biosystems).

Parentage testing

All adults and chicks were genotyped. Mendelian inheritance was checked at every locus in every family. Nestlings sharing alleles from their putative mother and father at all loci were considered actual offspring of the couple. Those chicks sharing one allele from the mother in all loci but failing to match any of the two alleles of the putative father at some loci were considered the product of extra-pair fertilization. Nestlings that did not share alleles with either the putative mother or father were considered the result of intraspecific brood parasitism (IBP). Our sampling protocol (see above) precluded the possibility that these nestlings had moved from other nests.

Mendelian inheritance failed at locus fp107 in several families. As analysis with Cervus 2.0 software (Marshall *et al.* 1998) showed a significant ($P < 0.01$) heterozygote deficit in this locus, it was excluded for further analysis as the presence of null alleles was confirmed (see Pemberton *et al.* 1995).

RESULTS

We genotyped 96 nestlings and 62 adults from 31 broods. We found 72 different alleles, with an observed heterozygosity of 0.65. The combined probability of exclusion for the marker set was greater than 0.99. The likelihood of two individuals carrying an identical genotype was estimated at 3.13×10^{-6}. Seven of the 96 (7.25%) chicks mismatched with the 'social' male in several loci. All of them were considered to be extra-pair offspring.

Overall, extra-pair young appeared in three out of 31 nests sampled (9.67%). The level of EPP using microsatellites was low for the two populations that we studied: six nestlings out of 72 (8.3%) in Los Monegros vs. one nestling out of 24 (4.2%) in Huelva. The difference between the two populations was not significant (Yates corrected chi-squared = 0.45, $P = 0.19$). We detected two nests in the Los Monegros population where the attendant males could not be assigned as the actual fathers. Therefore, their respective broods of three nestlings each arose from extra-pair fertilizations as microsatellite analysis confirmed the females as the actual mothers. The remaining extra-pair chick came from a brood in the Huelva population and had three nest-mates sired by the male attending the nest. This single extra-pair nestling shared the alleles of the attending mother but not those of the putative father at some loci. This was interpreted as a case of mixed fertilization, the first ever detected in the Lesser Kestrel. In addition, two nestlings in two other broods from Los Monegros shared alleles from neither the attending male nor the attending female at several loci. They were considered the result of IBP, as suggested by Negro *et al.* (1996).

In the Los Monegros subset of families, all previous results from our DNA fingerprinting study (Negro *et al.* 1996) matched those obtained using microsatellites, except in one family. In this case, we detected unambiguously that the male attending the nest was not the genetic father of the nestlings, contrary to our previous DNA fingerprinting results.

DISCUSSION

This microsatellite analysis showed a slightly higher incidence of EPP than that reported in our previous study

based on multilocus radioactive probes (7.25% vs. 3.4%). The difference is unrelated to the inclusion of samples from a different population but it is due to a misinterpretation of the fingerprinting band pattern in just one of the families. The microsatellite analysis for this family has been repeated in triplicate, and always yielded the same results. Despite the fact that a mean (± sd) of 10.9 ± 2.6 scorable bands was analysed in the fingerprintings of the families (see Negro *et al*. 1996), the re-examination of the band profile in the controversial family raised some doubts about its interpretation. In fact, the number of diagnostic bands was very low in this family, and some bands taken as identical by descent could actually be different given that a further re-examination revealed that some of them could not be exactly the same band in size, and their intensity on the Southern hybridization was different. The band patterns obtained with multilocus radioactive probes can be misleading and difficult to interpret in some cases, especially when the potential fathers are relatives, the population is highly inbred or when additional bands appear in the offspring due to mutation (see for instance Lubjuhn *et al*. 2002). However, working with microsatellite markers provides better resolution and a better background for detecting locus polymorphism, thus reducing the chance of human error.

The level of EPP reported in this study (9.67%), although higher than previously estimated (3.8%), still remains relatively low. It seems to be typical of raptors that they show low rates of EPP compared with other birds, and particularly with short-lived passerines (Birkhead & Møller 1992, Griffith *et al*. 2002); low rates of EPP have been reported in close relatives of the Lesser Kestrel, including the solitary-breeding Eurasian Kestrel *Falco tinnunculus* (1.9%, Korpimäki *et al*. 1996) and the American Kestrel *Falco sparverius* (11.2%, Villaroel *et al*. 1998).

To conclude, our reappraisal of EPP in Lesser Kestrels confirms a low incidence in this species, and identifies microsatellite markers rather than multilocus DNA fingerprinting as a better choice for this kind of study. This is because paternity assignments are straightforward and there is no need to consider, as is necessary with DNA fingerprinting, whether two bands of the same molecular weight correspond to the same allele.

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ENDNOTE

[a]Enzyme activities are usually measured in terms of the activity unit (U), which is defined as the amount of enzyme that will catalyse the transformation of 1 μmol of the substrate per minute under standard conditions.

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