

Microsatellite typing reveals mating patterns in the brood parasitic great spotted cuckoo (*Clamator glandarius*)

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Abstract

Despite the interest that avian brood parasites provoke due to their reproductive strategy, and the wealth of published studies, their mating system is one of the least known aspects of their natural history. This study describes the first attempt to use genetics to characterize mating patterns in a brood parasite, the great spotted cuckoo (*Clamator glandarius*). We developed a set of microsatellite markers for this species that allowed us to determine the parentage of 78% of the chicks sampled, with a high probability of certainty (false parental inclusion probability, $P_{FI} = 2 \times 10^{-4}$). Parentage analyses allowed us to determine mating patterns in the population, to show for the first time that there were several females laying in the same study site and sharing host nests, and that females used nests of two different host species in the same season. A total of 70% of the inferred mating relationships can be considered monogamous, and the rest were cases of genetic polygamy, although of unknown social structure. To ease parentage analyses, adult birds were successfully sexed using a combination of sex-specific PCR primers and SSCP techniques before parentage assignment.

Keywords: brood parasitism, *Clamator glandarius*, host selection, mating system, microsatellites, parentage

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Introduction

Mating systems in animals, defined as the way that individuals obtain mates, how many, the characteristics of pair bonds and patterns of parental care (Davies 1991), are thought to be determined by the spatial and temporal distribution of individuals of each sex (Emlen & Oring 1977), whether individuals of the opposite sex can be monopolized and to what degree (Birkhead & Møller 1992), and the patterns of parental care (Lack 1968; Clutton-Brock 1991). Parental care in particular is an important component of hypotheses concerning avian mating systems (Emlen & Oring 1977; Wittenberg & Tilson 1980). If one of the sexes (usually the male) is not essential for provisioning of the offspring, its contribution may end with mating, and the opportunity for new matings can in theory lead to polygamy (Emlen & Oring 1977).

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Parental care is absent in brood parasites, a group of bird species that lay their eggs in the nests of other species, the hosts, that take care of the parasitic offspring (Payne 1977; Rothstein 1990). In their case, no extra investment or reproductive effort is needed by either of the sexes after egg laying. This situation theoretically relieves brood parasites, both males and females, from the constraints of parental care, a fact that can be argued to facilitate the evolution of flexible mating systems in brood-parasitic species (Yokel 1986).

However, the mating system of avian brood parasites is one of the least known aspects of their natural history; most studies, with a few exceptions (Yokel 1986; Yokel & Rothstein 1991; Jones *et al.* 1997), have approached the problem from an indirect and anecdotal perspective, and using only behavioural data. It is now well established that conventional field observations of social interactions between males and females are not sufficient for determining mating systems and the reproductive success of individuals (Birkhead & Møller 1992; Avise 1996). The

advent of new genetic techniques such as DNA fingerprinting (Burke & Bruford 1987; Burke 1989) have enabled researchers to accurately determine the reproductive success of individuals and the 'real', genetic mating systems. Despite this, there is only one published study on the mating system of a brood parasite, the cuckoo *Cuculus canorus*, using genetic data (Jones *et al.* 1997).

The great spotted cuckoo (*Clamator glandarius*) is an African Cuculidae. Part of the population migrates to southern Europe to breed (Cramp 1985). Their main host in Europe is the magpie (*Pica pica*), although they occasionally use other corvid species, in particular the carrion crow (*Corvus corone*) (Cramp 1985; Soler 1990). Further details on the reproduction of great spotted cuckoos and their interactions with their hosts can be found elsewhere (Soler 1990; Soler *et al.* 1994; Soler *et al.* in press). The mating system of great spotted cuckoos has been described on the basis of behavioural data, but the information available is controversial: the species has been considered in different studies to be monogamous (Alvarez & Arias de Reyna 1974; Arias de Reyna *et al.* 1987), whereas other studies have suggested some form of polygamy or promiscuity (see references in Cramp 1985). However, all of these are based on observations of unmarked individuals and indirect evidence, such as the occurrence of parasitized hosts' nests with eggs apparently laid by different females (Cramp 1985).

In this study we present a set of microsatellite markers isolated in the study species, the great spotted cuckoo, and we use these markers to assign parentage and deduce mating patterns in a wild population of this species. Microsatellites (tandem repeats of sequence units normally less than 5 bp in length (Tautz 1989)) have become the marker type of choice for many studies of population genetics, parentage and individual identification. Microsatellites present many advantages, such as Mendelian inheritance, codominance, extensive polymorphism, and their amplification by PCR, allowing the use of small samples that may contain degraded DNA (Weber & May 1989; Bruford & Wayne 1993). The high variability and locus-specific genotypes provided by microsatellites make them a particularly well-suited genetic tool for solving the question of the mating systems of brood parasites, as in this case the database consists of groups of chicks scattered across different host nests, and a group of potential parents with no information relating these two groups.

Materials and methods

Study area and field work

The study was carried out in the Hoya de Guadix (Granada, Spain), a cereal-producing high plateau (1000 m

above sea level) where great spotted cuckoos mainly parasitize magpies (Soler 1990; Soler *et al.* in press). The Hoya de Guadix is a patchy habitat, where vegetation is quite sparse, and treeless areas (both natural and cultivated areas) alternate with discontinuously distributed almond (*Prunus dulcis*) plantations and holm oak (*Quercus rotundifolia*) patches. Magpies breed mainly in the almond groves, and so aggregate in different plots within the area. We captured great spotted cuckoos in three of these plots, namely La Calahorra (3.01 km²), Fuente Alamo (0.82 km²), and Ferreira (1.16 km²), less than 15 km apart from each other; these plots show no differences in magpie nest density (Martinez *et al.* 1996) or kind of habitat (irrigated crops with almond groves near small villages).

Field work was carried out during the spring of 1993. We started to catch adult great spotted cuckoos as soon as they arrived in the area, at the beginning of March, using mist nets. We tried to catch them during the whole season, setting up the nets in adequate places, such as feeding or resting areas and trying to drive the birds to the nets. Birds were measured, marked and bled. Every bird was given a metal ring and a unique combination of colour rings on both tarsi. To facilitate identification each ring had attached a 4–5 cm streamer of the same colour. Approximately 200 µL of blood was extracted from the brachial vein and stored in 1 mL of 100% ethanol in a screw-cap microfuge tube.

During the breeding season, host (magpie and carrion crow) nests were checked at least once a week in order to find parasitized nests, and to record reproductive parameters such as laying date (the day the first egg was laid), clutch size, hatching date, number of chicks hatched and number of chicks fledged. The laying date for cuckoo eggs was estimated as 14 days (incubation period) before hatching; this is an estimate with some error as the beginning of incubation depends on host females, and so laying could have been a few days earlier. However, cuckoos usually lay in the middle of the host's incubation period (Soler *et al.* in press), and we can assume that most eggs are incubated as soon as they are laid. Cuckoo chicks were ringed and bled when 15-days old in a similar way as adult birds. We did not obtain blood samples from all the eggs laid because some nests were predated, some eggs did not hatch, and some chicks died in the nests before we could get the samples. At the end of the season we sampled a total of 73 chicks, 71% of the total number of eggs laid.

In total 21 adult birds were captured: seven in La Calahorra (numbers 1–7), six in Fuente Alamo (numbers 23–28) and eight in Ferreira (numbers 62–69). The number of parasitized nests was 63 (51.64%) and the mean number of cuckoo eggs per nest was 2.28 (SE = 0.18, *n* = 63). In all multiparasitized nests we estimated whether cuckoo eggs were laid by one or more females, using egg shape and egg colour as criteria. After parentage assignment we

compared the estimated number of females laying at each nest with the real number of females that laid the eggs.

Laboratory work

DNA extraction. DNA was isolated from blood using a standard phenol–chloroform extraction protocol (Bruford *et al.* 1992).

Microsatellite isolation and characterization. The microsatellite loci were isolated following the enrichment procedure described by Armour *et al.* (1994), with the exception that the DNA fragments were not PCR-amplified before hybridization enrichment (Gibbs *et al.* 1997). Size-selected (300–800 bp) *Mbo*I genomic DNA fragments were isolated from a Bluescript SK+ (Stratagene) library enriched for (CA)_n and (GA)_n sequences. A total of 166 clones cross-hybridized to α^{32} P-labelled poly-(dA–dC)×poly-(dG–dT) oligonucleotides, from which 49 were sequenced using DyeDeoxyterminators (Applied Biosystems) on a model 373A Applied Biosystems automated DNA sequencer. Twenty-two of these sequences were found to be appropriate for designing primers, of which we did so for 13 pure dinucleotide repeats (AC/GT), using the computer program PRIMER (Whitehead Institute for Biomedical Research). Microsatellite loci were amplified by PCR using these primer pairs; 10 of the 13 pairs gave PCR products.

Genotyping procedures. The allelic variation, and later the genotype of every individual, were determined by performing radioactive PCR and running the products on acrylamide gels. PCR was carried out in a 10 μ L reaction in a Perkin Elmer DNA thermal cycler model 480 for 30 cycles using 55 °C or 58 °C as the annealing temperature for 30 s. Primers were end-labelled with [γ^{33} P]-dCTP (2000 Ci/mmol) using T4 polynucleotide kinase (Pharmacia); the final PCR reaction contained 0.5 units of *Taq* polymerase (Advanced Biotechnologies), 1.5 μ M of each primer (one end-labelled, the other not), 0.6 mM MgCl₂, 0.2 mM dNTPs, in the manufacturer's buffer. Between 50 and 100 ng of DNA was included in each reaction. The products were electrophoresed in 6% denaturing polyacrylamide (Accugel sequencing grade, National Diagnostics) gels for around 3 h at 65 W, and the gels dried and exposed to X-ray film for 12–24 h (Fig. 1). Only seven loci had more than two alleles, and those seven were used in this study.

Samples from adult birds were run together to determine the number of alleles per locus. Autoradiographs were scored by giving each different band a different letter (A, B, etc.) and individuals with all different alleles detected were run in every gel to determine the genotype of the rest of the individuals (Fig. 1).

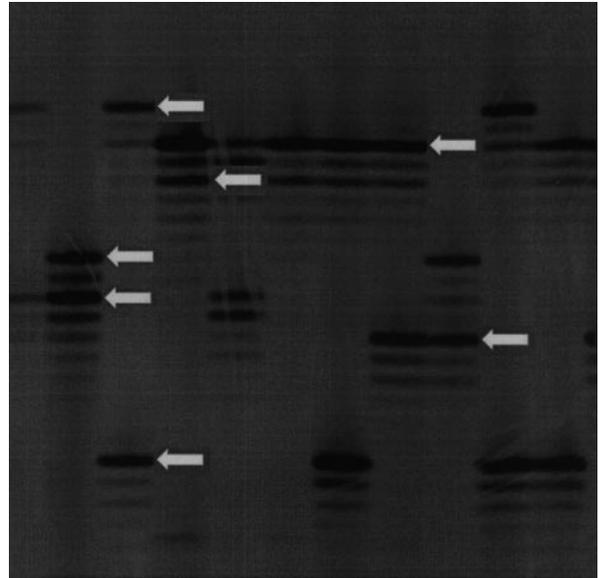


Fig. 1 Allelic variability at one of the microsatellite loci (Cgl 3) used in this study. Every arrow indicates a different allele.

Sexing procedures. Sexes are morphologically similar in the great spotted cuckoo (Cramp 1985). Therefore, in order to ease parentage analysis we determined the sex of every adult bird using sex-specific primers and single-stranded conformation polymorphism analysis (SSCP; Dracopoli *et al.* 1994). Approximately 50–100 ng of genomic DNA from each individual was used in a radioactive PCR reaction with similar conditions to those described above, but with an annealing temperature of 50 °C, 2 mM MgCl₂, and the inclusion of primers P2 and P3 from Griffiths & Tiwari (1995), P2 being end-labelled before the PCR reaction as described above. These primers amplify a 104 bp region in each of two related genes: C-W on the W chromosome and C-2 situated elsewhere in the genome (Griffiths & Tiwari 1995). Therefore, they produce two different products of the same size in females, and a single product in males (C-2, as they do not have a W chromosome). The SSCP method can resolve two DNA molecules differing by a single base and, in this case, differentiates between the C-2 and C-W PCR products, revealing a two-band pattern for females and only one band for males. The PCR products were rendered single stranded by heating in a denaturing buffer, then run on 8% nondenaturing polyacrylamide gels (10% glycerol) at 15 W overnight, and the gels dried and exposed for 12–24 h. We tested the accuracy of the method by running five individuals of known sex (one male, four females); the sexes of these were all correctly assigned.

Parentage determination

Every individual was genotyped for all loci, and the genotypes of adult birds were compared with those of the

nestlings. We considered an adult bird to be the possible parent of a chick when its genotype was compatible with that of the chick, i.e. when it could have contributed either allele at every locus in the nestling's genotype. After all potential parents had been identified, we investigated which pair of male and female individuals could have donated the allele combination in the chick, and that pair was considered the parents of that particular chick.

Statistical procedures

For each locus we calculated the observed (H_O) and expected (H_E) heterozygosities (Paetkau & Strobeck 1994) and the deviation from expected Hardy–Weinberg equilibrium using GENEPOP (version 3.1; Raymond & Rousset 1995). In particular, we tested whether there was significant heterozygote deficiency as an evidence for the occurrence of null alleles (Neumann & Wetton 1996). We also calculated the probability of identity (P_I , Paetkau & Strobeck 1994) and the false parental exclusion probability, i.e. the probability of detecting an incorrectly assigned parent (P_E , Bruford *et al.* 1992). The probability of false parental inclusion, defined as the probability of failing to detect an individual which has been incorrectly assigned as a parent, is $P_{FI} = 1 - P_E$ (Bruford *et al.* 1992). Assuming no linkage disequilibrium, the combined probabilities for

the seven loci can be calculated as the product of the single-locus probabilities. It is impossible to test for linkage, which is the most likely source of linkage disequilibrium, because of the absence of information on a large family of known father and mother. We therefore have to assume that there is no linkage disequilibrium, as in other paternity studies using microsatellites (Morin *et al.* 1994; Primmer *et al.* 1995; Neumann & Wetton 1996; Richard *et al.* 1996).

Results

Microsatellite loci and parentage assignment

We used seven loci to assess parentage. Expected and observed heterozygosity, number of alleles and probabilities of identity and false parentage exclusion are shown in Table 1. There was no heterozygote deficiency in any of the loci ($P > 0.05$ for all loci, score test for heterozygote deficiency, GENEPOP 3.1, Raymond & Rousset 1995), giving evidence for the absence or rarity of null alleles (Brookfield 1996; Neumann & Wetton 1996). The combined identity probability P_I was 4×10^{-7} , and the combined false parental inclusion probability P_{FI} was 2×10^{-4} . Eight (10.9%) nestlings were not attributed to any adult bird, 55 (75.3%) were assigned to one or two potential parents (a male, a female, or a male and a female with

Table 1. Microsatellite primers and variability details, indicating the type and number of repeats and the size in base pairs of the cloned alleles. N_a = number of alleles found in adult great spotted cuckoos (the total number found in all individuals in parentheses), observed (H_O) and expected (H_E) heterozygosity (Paetkau & Strobeck 1994), probability of identity (P_I , Bruford *et al.* 1992) and the probability of false parental exclusion (P_E , Bruford *et al.* 1992) for each locus. Probabilities were calculated using the allelic frequencies for the adult birds only

Locus	Primer sequences	Repeat type	Size	N_a	H_O	H_E	P_I	P_E
Cgl 1	Forward: AGCCAAATTCAGGGAGGTG Reverse: TGCAAGAGTGAGAGCCACC	(GT) ₂₆	146	3 (4)	0.43	0.45	0.35	0.38
Cgl 2	Forward: TTCITCACCTTTGTAGTCCGTG Reverse: TTATGAGTTGCTTTCCAGACA	(GT) ₁₂	219	5 (6)	0.67	0.72	0.13	0.66
Cgl 3	Forward: ATGCTGCAGCCTCTGACA Reverse: GACTCACTGGGAACCGTG	(CA) ₁₃	106	8 (9)	0.90	0.82	0.06	0.82
Cgl 4	Forward: TAGAAATCAGCCAAAGTGGACA Reverse: GTAGATGCAGAGCTTGTGGC	(CA) ₂₅	246	9 (9)	0.76	0.74	0.09	0.75
Cgl 5	Forward: ATGCTCATAGAGCAAGCAAGC Reverse: CCCACTTGCTGCTTTTCAGAT	(GT) ₂₂	141	5 (5)	0.62	0.56	0.24	0.50
Cgl 6	Forward: ATGCTCTGCCCTGCAAAC Reverse: GATACAGCCCTCCAGAGCAC	(GT) ₁₂	204	3 (3)	0.33	0.39	0.41	0.33
Cgl 7	Forward: GGGAAGCCATGATGATTATACC Reverse: GAAGTGTTGAGCAAGGCACA	(GT) ₄₉	179	14 (15)	0.95	0.90	0.02	0.93

compatible genotypes), and 10 (13.7%) were assigned to more than two adults or an incompatible pair of males and females (a pair that could be either the father or mother of the chick but could not account for the chick's genotype). For the chicks compatible with only one adult, we determined whether one or more mates were required to explain the allelic combination in all the offspring of that individual and reconstructed the putative genotype of the missing father/mother, giving it a specific name (a letter). We then tested these reconstructed genotypes against the genotypes of all the other chicks. After this final assignment of parentage was performed as follows: 51 nestlings were assigned to both male and female parents, and six to only one parent (a total of 57, or 78.1% of all the chicks), leaving 16 chicks unassigned.

When we assigned two or more chicks to the same female but two different males (or vice versa), the assignment of the different chicks to one male (or female) or the other was based on differences in at least three of the seven loci typed in the chicks.

Mating patterns

The different mating relationships inferred from the parentage data are represented in Fig. 2. Nine females were fertilized by one male and two females by two males, whereas 10 males fertilized only one female and three fertilized two. The difference between sexes is not

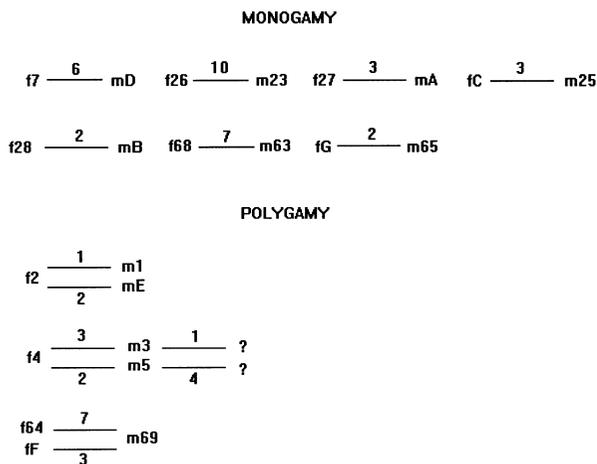


Fig. 2 Mating relationships deduced from parentage data. Lines join females (fN) and males (mN) that had common chicks. Numbers above and below the lines are the number of chicks produced by the pair joined by the line. When N is a number the figure refers to captured individuals, and when N is a letter the figure refers to uncaptured individuals. ? refers to uncaptured individuals with genotypes that could not be reconstructed. Monogamy refers to genetic monogamous relationships and Polygamy to relationships in which at least one of the individuals involved fertilized, or was fertilized by, more than one individual.

significant (Fisher's test, $P = 0.58$). To summarize, seven of the 10 different mating relationships can be considered monogamous, and 34 out of the 57 assigned chicks were the offspring of adults engaged in these arrangements, whereas the other 13 chicks were the offspring of couples in which at least one of the parents fertilized or was fertilized by two or more mates ('polygamous'), in three different mating arrangements (Fig. 2):

- Female 2 was assigned three chicks, two from an uncaptured male and one from male 1, in what could represent a polyandrous trio.
- Female 4 was fertilized by males 3 and 5, which in turn fertilized at least one or two more unknown females, whose genotype(s) could not be reconstructed.
- Male 69 had chicks with two different females, 64 and an uncaptured bird, which could apparently represent a case of polygyny.

Bird numbers 6, 24, and 66 were assigned no offspring, but as we did not sample all eggs we cannot be sure that they did not breed. The same applies to bird 62: it was the potential mother of three of the unassigned chicks, but we do not know whether it bred.

Temporal distribution of egg laying

Although egg-laying date is not accurate, the estimates allow us to determine the approximate span of egg laying for individual females, bearing in mind that the estimate of egg-laying period is a lower limit (see the Materials and methods), and the existence of any temporal pattern for double-mated females. Figure 3 shows the egg-laying patterns for all females in each plot. There was considerable variability in the length of the egg-laying period, with a mean of 20.3 days ($SE = 4.23$). The longest period was found for female 26, with 44 days between the first and the last egg. Female 68 took 40 days to lay her eggs. We also had females that laid fewer eggs and then spent less time laying: females 2, C and G, probably took less than 6 days to lay, although we can not rule out that we missed some of their eggs, either because we could not sample them (see the Materials and methods) or because they moved to surrounding areas.

Finally, the temporal distribution of egg laying by 'polygamous' birds suggests an interchange of mates in the potentially polyandrous females (2 and 4), whereas the females paired with male 69 laid during approximately the same period of time (Fig. 3).

Confirmation of females sharing host nests

Parentage data revealed more than one female laying in the same plot, at least four in each of the three plots studied, and in some cases laying in the same host nests, what

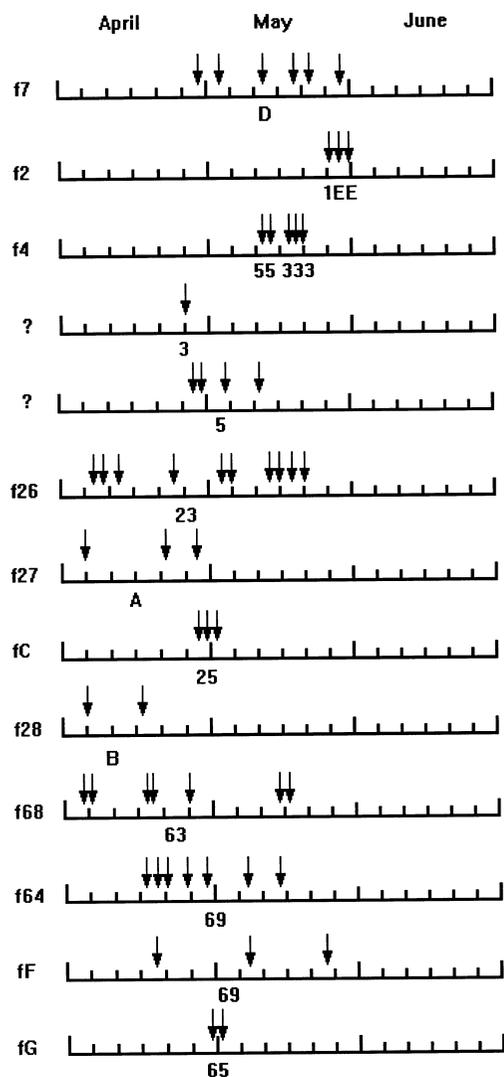


Fig. 3 Temporal pattern of egg laying for every female. Time is divided into 5-day periods. Arrows show the estimated laying day for each egg. The male that fathered each chick is specified below the line, once, if only one male, or below each arrow when two males fathered chicks with that female.

we have called 'sharing' host nests. The assignment of every chick to a female allowed us to calculate the actual number of females laying in the same nest, a parameter that was estimated *a priori* on the basis of egg morphology (see the Materials and methods). In this case we could only use those nests that were not abandoned or depredated after parasitism and in which we could sample all chicks. For this data set, the confirmed mean number of females laying per nest was 1.42 (SE = 0.11, $n = 21$), and for the 12 multiparasitized nests, there were no significant differences between the number of females laying in a nest as estimated from egg morphology and the number of females laying in that nest as determined from genetic

data (Wilcoxon matched pair test, $z = 1.60$, $P = 0.11$, $n = 12$). Only three out of 12 (25%) nests were mistakenly recorded as having eggs from two or more females when there was only one. The other nine nests all had eggs from more than one female (four out of four in La Calahorra, two out of five in Fuente Alamo and three out of three in Ferreira).

On the other hand, there were also several males breeding in the different plots. For multiparasitized nests in which we sampled all the chicks, in eight out of nine nests with eggs from more than one female the eggs were fertilized by more than one male (four out of four in La Calahorra, two out of two nests in Fuente Alamo, and two out of three in Ferreira).

Use of host species

In one of the three study plots (Fuente Alamo) two potential host species were available, magpies and carrion crows, and some carrion crow nests were parasitized by great spotted cuckoos. The eggs in three parasitized carrion crow nests were laid by the same female that parasitized most of the magpie nests in the area. This is the first study confirming that the same female cuckoo may parasitize different host species in the same season. The temporal distribution of egg laying for that female showed that eggs in carrion crow nests were laid either at the beginning or at the end of the laying period (Fig. 4), suggesting that crows were used as alternative hosts when no magpie nests were available.

Discussion

Microsatellite data and parentage assignment

The system consisting of the seven loci used in this study has proven to be sufficiently powerful to resolve genetic relationships between individuals. The variability at these loci was, with the exception of two loci, very high. The probability of identity (4.0×10^{-7}) is similar to or better than that obtained in other microsatellite systems (2.0×10^{-5} and 4.6×10^{-2} , Paetkau & Strobeck 1994; 1.3×10^{-8} , Ellegren *et al.* 1995; 1.8×10^{-6} , Richard *et al.* 1996). Hence, we were able to assign only one father and mother to most nestlings with a small probability of false parental inclusion ($P_{FI} = 2 \times 10^{-4}$). The number of nestlings for which it was impossible to determine the identity of the parents was small, given that the most probable cause for this is that we could not catch all breeding birds (at the end of the season we were sure of the presence of several unmarked birds in the area).

The assignment of nestlings to the same male but different female, or vice versa, (as happened to female 2 and males 1 and E) because of differences in genotypes between chicks may be interpreted as a misassignment

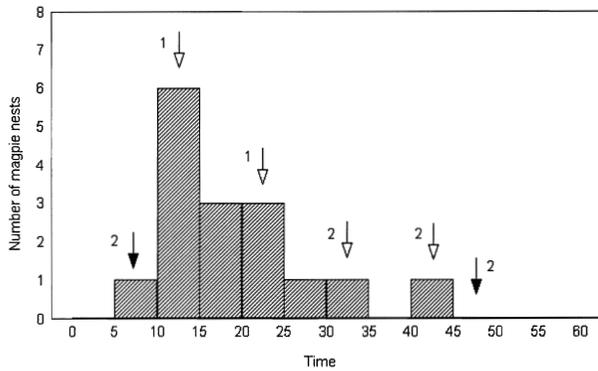


Fig. 4 Temporal pattern of egg laying by the great spotted cuckoo female that parasitized magpies and carrion crows. Bars represent the number of magpie nests available in the plot. Day 1 = 1st April. The number of eggs laid in a 5-day period is indicated by an arrow (black arrows for eggs laid in crow nests, white arrows for eggs in magpie nests).

due to mutation at a particular locus or to real differences in paternity or maternity. Mutation rates have been reported to be low at microsatellite loci, making them a relatively stable system adequate for parentage analyses (usually below 10^{-4} , Queller *et al.* 1993; Weber & Wong 1993), and apparently it is only high in a few microsatellites with a large number of repeats (Primmer *et al.* 1996), which is not the case in our study. Therefore, we conclude that the results cannot have been due to mutation because the assignment of two chicks to the same female but different male (or vice versa) was due to different genotypes in at least three loci between the chicks.

Mating patterns

The genetic mating system for individual great spotted cuckoos in the Hoya de Guadix has been determined from molecular data. The study clearly supports a flexible mating system in the species, as it has revealed four different mating relationships, although it is difficult to characterize them in terms of social relationships such as monogamy, polygamy or promiscuity, because of the absence of a detailed observational study.

Genetically monogamous pairs were predominant. Although we did not sample all eggs laid, we believe that 71% of eggs sampled is a proportion high enough to consider the results to be accurate. On the other hand, even though we did not sample some eggs/chicks, it seems unlikely that we could miss all the eggs from one of the females paired with a polygynous male, thereby misinterpreting the relationship as monogamous (or vice versa, missing all the eggs from a female fertilized by a male but not the ones fertilized by another male). Apart from this, we do not know whether some of our individuals moved to areas that we did not sample and mated with other

individuals. So, the frequency of genetic monogamy must be interpreted as an upper limit.

The alternative arrangements reported here cannot be satisfactorily classified as social polygyny, polyandry or promiscuity in the absence of behavioural data. For example, female 4 was fertilized by two different males, and the egg-laying pattern showed that eggs fertilized by male 5 were laid before the eggs from male 3 (Fig. 3). This cannot, however, be interpreted as sequential polyandry (Davies 1991) without data on copulation behaviour because the sperm from different mates may not necessarily have been used sequentially. In the same way, male 69 had offspring with two different females, 64 and F, whose laying periods overlapped (Fig. 3). The male could have been polygynous and remain mated with both females throughout the season, or have maintained a bond with only one, the other being an extrapair relationship.

The genetic data on parentage available for the cuckoo *Cuculus canorus*, although based on a small sample size, also indicates alternative mating relationships, monogamy for females and polygyny for males (Jones *et al.* 1997). The same applies to the cowbirds, which are mainly monogamous but occasionally polygynous (Yokel 1986; Yokel & Rothstein 1991). In the case of the great spotted cuckoo there are several situations that could result both in genetic monogamy and multiple mating.

(i) Males and females are supposed to collaborate when searching for host nests and during egg laying (Alvarez & Arias de Reyna 1974) and they have been assumed to maintain a pair-bond throughout the breeding season (Mountford & Ferguson-Lees 1961; Valverde 1971; Arias de Reyna *et al.* 1987), although all of these studies were carried out with unmarked individuals. The laying period of a female may last between 1 and 1.5 months, as confirmed in this study (females 26 and 68). Therefore, if the collaboration of male and female is necessary to lay the eggs successfully in the host's nests, there is a reason for the maintenance of a seasonal pair-bond that could result in genetic monogamy. However, polygyny is still possible if a male can help two females at the same time.

(ii) Genetic monogamy could arise if genes are the only or main contribution of males to offspring (good genes hypothesis, Hamilton & Zuk 1982; Weatherhead 1984), which is probably the case in brood parasites. Females should exert mate choice and copulate with high-quality, preferred males and avoid poor-quality males. This would result in genetic monogamy for most females although not necessarily pair bonds, and in attempts by the females paired to low-quality males to obtain extrapair fertilizations (Yokel & Rothstein 1991).

(iii) The existence and defence of breeding territories by either males or females (or both) could result in different mating arrangements, including genetic monogamy.

If males defend a breeding territory against other males as a way to monopolize resources (host nests in this case) and attract females, we could expect both monogamy and polygamy to originate, depending on the characteristics of territories (Davies 1991). On the other hand, once territories are established, female–female aggression can limit the mating system to monogamy (Wittenberger & Tilson 1980). Although a breeding territory may exist in the great spotted cuckoo (Arias de Reyna *et al.* 1987), our data do not support the existence of territories in the Hoya de Guadix, as several males and females bred in the same plot and shared host nests, and most nests with eggs from two different females also had chicks from two males. This fact seems to invalidate the exclusivity of male breeding territories as well as the female–female aggression hypothesis. However, the limited number of multiparasitized nests for which we have data on parentage for all chicks only allows us inconclusively to suggest non-territoriality or a complex territorial system in our area.

Use of host nests by great spotted cuckoos

The study has confirmed that several females share host nests, a fact only indirectly shown, but widely accepted for this species (Cramp 1985; Arias de Reyna *et al.* 1987; Soler 1990; Soler *et al.* in press). Our data provide good evidence for a frequent occurrence of this behaviour, despite the small sample size of multiparasitized nests where we could sample all the eggs, because estimations on the number of cuckoo females laying per nest based on egg shape and colour were very accurate (75% of cases correct). We can then assume that the estimated number of females laying per nest in this and other studies is accurate. However, our mistakes estimating the number of females per nest were always in the same direction: reporting eggs from one female as laid by two. This means that intraclutch variability in cuckoo females might be large enough to bias in some degree the estimates based on egg shape and colour.

Finally, we have also confirmed for the first time in this species that females can use nests from two different host species. Host specificity is one of the most interesting topics in the ecology of brood parasitism, because of its implications for the coevolutionary processes between parasites and hosts (Rothstein 1990). In the European cuckoo, females are very specific in host use, resulting in 'gentes' of cuckoos having eggs that mimic those of particular host species (Brooke & Davies 1988). This subject has not been well studied in the great spotted cuckoo. Although magpies are their main host in Europe (Cramp 1985), carrion crows and other corvids are also parasitized (Soler 1990). Our data suggest that carrion crows are used as alternative hosts when no magpie nests are available (Fig. 4), which fits with the fact that the breeding success

of cuckoos is lower in carrion crow nests than in magpie nests (Soler 1990). Therefore, cuckoos laying in crow nests may be making 'the best of a bad job' when no other preferred options (magpie nests) are available.

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References

- Alvarez F, Arias de Reyna L (1974) Mecanismos de parasitacion por *Clamator glandarius* y defensa por *Pica pica*. *Doñana Acta Vertebrata*, **1**, 43–65.
- Arias de Reyna L, Recuerda P, Trujillo J, Corvillo M, Cruz A (1987) Territory in the Great Spotted Cuckoo (*Clamator glandarius*). *Journal für Ornithologie*, **128**, 231–239.
- Armour JAL, Neumann R, Gobert S, Jeffreys AJ (1994) Isolation of human simple repeat loci by hybridization selection. *Human Molecular Genetics*, **3**, 599–605.
- Avise JC (1996) Three fundamental contributions of molecular genetics to avian ecology and evolution. *Ibis*, **138**, 16–25.
- Birkhead TR, Møller AP (1992) *Sperm Competition in Birds*. Academic Press, London.
- Brooke M de L, Davies NB (1988) Egg mimicry by cuckoos *Cuculus canorus* in relation to discrimination by hosts. *Nature*, **335**, 630–632.
- Brookfield JFY (1996) A simple new method for estimating null allele frequency from heterozygote deficiency. *Molecular Ecology*, **5**, 453–455.
- Bruford MW, Hanotte O, Brookfield JFY, Burke T (1992) Single-locus and multilocus DNA fingerprinting. In: *Molecular Genetic Analysis of Populations* (ed. Hoelzel AR), pp. 225–269. IRL Press, Oxford.
- Bruford MW, Wayne RK (1993) Microsatellites and their application to population genetic studies. *Current Opinion in Genetics and Development*, **3**, 939–943.
- Burke T (1989) DNA fingerprinting and other methods for the study of mating success. *Trends in Ecology and Evolution*, **4**, 139–144.
- Burke T, Bruford MW (1987) DNA fingerprinting in birds. *Nature*, **327**, 149–152.
- Clutton-Brock TH (1991) *The Evolution of Parental Care*. Princeton University Press, Princeton, New Jersey.
- Cramp S (1985) *The Birds of Western Palearctic*, Vol. IV. Oxford University Press, Oxford.
- Davies NB (1991) Mating systems In: *Behavioural Ecology*, 3rd edn (eds Krebs JR, and Davies NB), pp. 263–294. Blackwell Scientific Publications, Oxford.

- Drapcopoli NC *et al.* (1994) *Current Protocols in Human Genetics*. John Wiley and Sons, Inc.
- Ellegren H, Lifjeld JT, Slagsvold T, Primmer CR (1995) Handicapped males and extrapair paternity in pied flycatchers: a study using microsatellite markers. *Molecular Ecology*, **4**, 739–744.
- Emlen ST, Oring LW (1977) Ecology, sexual selection, and the evolution of mating systems. *Science*, **197**, 215–223.
- Gibbs M, Dawson D, McCamley C, Wardle AF, Armour JAL, Burke T (1997) Chicken microsatellite markers isolated from libraries enriched for simple tandem repeats. *Animal Genetics*, **28**, 401–417.
- Griffiths R, Tiwari B (1995) Sex of the last wild Spix's macaw. *Nature*, **375**, 454.
- Hamilton WD, Zuk M (1982) Heritable true fitness and bright birds: a role for parasites? *Science*, **218**, 384–387.
- Jones DA, Gibbs HL, Matsuda T, de Brooke M, L, Uchida H, Bayliss MJ (1997) The use of DNA fingerprinting to determine the possible mating system of an obligate brood parasitic bird, the Cuckoo *Cuculus canorus*. *Ibis*, **139**, 560–572.
- Lack D (1968) *Ecological Adaptations for Breeding in Birds*. Chapman and Hall, London.
- Martinez JG, Soler M, Soler JJ (1996) The effect of magpie breeding density and synchrony on brood parasitism by great spotted cuckoos. *Condor*, **98**, 272–278.
- Morin PA, Wallis J, Moore JJ, Woodruff DS (1994) Paternity exclusion in a community of wild chimpanzees using hypervariable simple sequence repeats. *Molecular Ecology*, **3**, 469–478.
- Mountford G, Ferguson-Lees I (1961) The birds of the Coto Doñana. *Ibis*, **103a**, 86–109.
- Neumann K, Wetton JH (1996) Highly polymorphic microsatellites in the house sparrow *Passer domesticus*. *Molecular Ecology*, **5**, 307–309.
- Paetkau D, Strobeck C (1994) Microsatellite analysis of genetic variation in black bear populations. *Molecular Ecology*, **3**, 489–495.
- Payne RB (1977) The ecology of brood parasitism in birds. *Annual Review of Ecology and Systematics*, **8**, 1–28.
- Primmer CR, Ellegren H, Saino N, Møller AP (1996) Directional evolution in germline microsatellite mutations. *Nature Genetics*, **13**, 391–393.
- Primmer CR, Møller AP, Ellegren H (1995) Resolving genetic relationships with microsatellite markers: a parentage testing system for the swallow *Hirundo rustica*. *Molecular Ecology*, **4**, 493–498.
- Queller DC, Strassmann JE, Hughes CR (1993) Microsatellites and kinship. *Trends in Ecology and Evolution*, **8**, 285–288.
- Raymond M, Rousset F (1995) GENEPOP (version 3.0) – population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.
- Richard KR, Whitehead H, Wright JM (1996) Polymorphic microsatellites from sperm whales and their use in the genetic identification of individuals from naturally sloughed pieces of skin. *Molecular Ecology*, **5**, 313–315.
- Rothstein SI (1990) A model system for the study of coevolution: avian brood parasitism. *Annual Review of Ecology and Systematics*, **21**, 481–508.
- Soler M (1990) Relationships between the great spotted cuckoo and its corvid hosts in a recently colonized area. *Ornis Scandinavica*, **21**, 212–223.
- Soler M, Soler JJ, Martinez JG (1997) Sympatry and coevolution between the great spotted cuckoo (*Clamator glandarius*) and its magpie (*Pica pica*) host. In: *Parasitic Birds and their Hosts*. (Rothstein SI and Robinson S eds) Oxford University Press, in press.
- Soler M, Soler JJ, Martinez JG, Møller AP (1994) Micro-evolutionary change in host response to a brood parasite. *Behavioral Ecology and Sociobiology*, **35**, 295–301.
- Tautz D (1989) Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Research*, **17**, 6463–6471.
- Valverde JA (1971) Notas sobre la biología de reproducción del crialo *Clamator glandarius*. *Ardeola Volumen especial*, 590–629.
- Weatherhead PJ (1984) Mate choice, in avian polygyny: why females prefer older males. *American Naturalist*, **123**, 873–875.
- Weber JL, May PE (1989) Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *American Journal of Human Genetics*, **44**, 388–396.
- Weber JL, Wong C (1993) Mutation of short tandem repeats. *Human Molecular Genetics*, **2**, 1123–1128.
- Wittenberger JL, Tilson RL (1980) The evolution of monogamy: hypotheses and evidence. *Annual Review of Ecology and Systematics*, **11**, 197–232.
- Yokel DA (1986) Monogamy and brood parasitism: an unlikely pair. *Animal Behaviour*, **34**, 1348–1358.
- Yokel DA, Rothstein SI (1991) The basis for female choice in an avian brood parasite. *Behavioral Ecology and Sociobiology*, **29**, 39–45.

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