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Intra-specific brood parasitism revealed by DNA micro-satellite analyses in a sub-oscine bird, the vermilion flycatcher

Parasitismo intraespecífico revelado mediante análisis de microsatélites de ADN en un ave suboscina, el cardenalito o saca tu real

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ABSTRACT

Extra-pair reproduction is known to occur in many avian species. However, among passerines, the majority of studies on extra-pair reproduction have been carried out in oscine birds from temperate regions. Conversely, sub-oscines species, and particularly, species that inhabit tropical regions, have been studied to a much lesser extent. Given that a majority of avian species live in the tropics, it is important to study more tropical and sub-oscine species to have a more accurate picture of the rates of extra-pair reproduction among passerines, and a better understanding of the adaptive function of extra-pair reproduction in birds. Tropical species differ from temperate species in several ecological and life history traits, that may influence the occurrence of different modes of extra-pair reproduction and their prevalence. In this study we asked whether extra-pair reproduction occur in a sexually dimorphic and socially monogamous sub-oscine, the vermilion flycatcher (*Pyrocephalus rubinus*). We report cases of extra-pair paternity, extra-pair maternity and intra-specific brood parasitism, and discuss our results in the view of other studies with passerines.

Key words: extra-pair reproduction, vermilion flycatcher, *Pyrocephalus rubinus*, sub-oscine, intra-specific brood parasitism.

RESUMEN

Se sabe que la reproducción extrapareja ocurre en muchas especies de aves. Sin embargo, entre paserinos, la mayoría de los estudios se han llevado a cabo en aves oscinas de regiones templadas. Por el contrario, las especies suboscinas, y en particular las especies que habitan regiones tropicales, se han estudiado mucho menos. Por lo tanto, es importante estudiar más especies tropicales y suboscinas para tener una visión más acertada de las tasas de reproducción extrapareja en paserinos y un mejor entendimiento de la función adaptativa de la reproducción extrapareja en aves. Las especies tropicales difieren de las especies que habitan regiones templadas en diversos rasgos ecológicos y de historia de vida, que podrían influir en las tasas de ocurrencia de diferentes modos de reproducción extrapareja. En este estudio nos preguntamos si ocurre o no la reproducción extrapareja en el cardenalito o saca tu real (*Pyrocephalus rubinus*), un suboscino sexualmente dimórfico y socialmente monógamo. Reportamos casos de paternidad extrapareja, maternidad extrapareja y parasitismo intraespecífico, y discutimos nuestros resultados bajo la luz de otros estudios en paserinos.

Palabras clave: reproducción extrapareja, Saca tu real, *Pyrocephalus rubinus*, suboscino, parasitismo intra-específico.

INTRODUCTION

Although it is accepted that a majority of passerine birds are socially monogamous, there is increasing evidence that individuals seek extra-pair matings in the overwhelming majority of passerine species studied so far (reviewed in Griffith et al. 2002). Understanding the significance of the great inter-specific variation in the frequency of

extra-pair paternity (EPP; 0- > 70 % between species, Møller & Birkhead 1994) has become a paramount challenge for behavioural ecologists. Yet, after 20 years of studies and accumulating information on the topic, a general and convincing explanation of the observed inter-specific variation in rates of EPP remains elusive.

Among passerines, most studies on extra-pair reproduction (EPR) have been carried out in oscine birds from temperate regions, while sub-oscines and tropical species have been studied to a much lesser extent (Stutchbury & Morton 2001, Griffith et al. 2002). Sub-oscine species differ from their "sister group" (i.e., oscine or songbirds) in some life history and ecological traits that may influence the mechanism and occurrence of extra-pair reproduction: (1) Ontogeny of song production. Evidence indicates that while songbirds learn to sing, sub-oscines do not (i.e., song production in sub-oscines seem to be more determined by an "endogenous" mechanism; review in Kroodsma 1982). The process of learning to sing has been related to the evolution of complex and elaborate songs in oscines (Kroodsma 1982), and therefore relatively less variable and less complex songs could be expected in sub-oscines. Regarding oscines, different aspects of song structure (e.g., the presence of particular phrases in canaries *Serinu canaries*, Vallet & Kreuzer 1995, Vallet et al. 1998) have been related to mate preferences, and song repertoire size has been shown to play a key role in determining extra-pair reproductive success (Hasselquist et al. 1996); however, it is not known how, or even whether, any aspects of song structure in sub-oscines may influence the occurrence of EPR. (2) Geographic distribution. While sub-oscines occur mostly in their inferred geographical areas of origin, oscines have undergone extensive geographical dispersal from Australasia (Barker et al. 2004), resulting in a current worldwide distribution. This makes oscines, in terms of radiation, one of the most successful groups of birds. On the other hand, sub-oscines do not occur in Europe and are more widely distributed in tropical regions of the New World, Africa and Asia (Ericson et al. 2003, Moyle et al. 2006), accounting for more than 30 % of the world's richest avifauna, which occurs in the Neotropics (Chesser 2004). This is important because differences in ecological conditions between tropical and temperate regions (e.g., seasonality, Stutchbury & Morton 2001) may relate to different rates of EPP between species.

Because oscine species from temperate regions of the world have been extensively studied and tropical species have not, Stutchbury & Morton (2001) suggested that there is a "temperate zone bias" in our knowledge of avian mating systems. In fact, their book "Behavioral ecology of tropical birds" can be seen as a "call to arms" to focus our efforts on studying more tropical bird species. Because a larger diversity of bird species occur in the tropics than in temperate zones (Stutchbury & Morton 2001), what we may think as the "rule" that appears to be emerging in many passerine mating systems, that is, based on temperate species studies (i.e., socially, but not genetically, monogamous systems), may be the "exception". Clearly more studies in tropical species (and among them sub-oscines) are needed to verify if this is true or not.

Another form of EPR that may be related to different ecological conditions is intra-specific brood parasitism (ISBP, Reyer et al. 1997). This mode of EPR occurs when a female lays eggs in the nest of a conspecific host female, and the host female incubates and raises the young (Andersson & Åhlund 2001). Among the several forms of extra-pair reproduction, ISBP has been studied to a lesser extent (Birkhead et al. 1990), and has been considered to occur rarely in birds (Reyer et al. 1997). However, the rate of ISBP can greatly differ within and among avian species. For instance, the proportion of nest parasitized has been estimated from 5-46 % for some populations of starlings (*Sturnus vulgaris*) and up to more than 50 % for some species of ducks (review in Davis 1988). It is not known whether different rates of ISBP are related in some way to seasonality, but if ISBP is based in some degree on the probability of finding host nests, then we could expect higher rates of ISBP in temperate regions, where birds breed in a shorter period of time in comparison with tropical species, and therefore may have a higher probability of finding host nests during the breeding period.

Here, we studied the reproductive system of the vermilion flycatcher, *Pyrocephalus rubinus* (Tyrannidae, Boddaert, 1783), in a population of central Mexico. The vermilion flycatcher is a socially monogamous and sexually dichromatic sub-oscine. Sexual dimorphism is also related to the singing behaviour since females do not usually sing. Females build the nests and incubate the clutch of one to three eggs (usually three) and both parents feed the young (Díaz Ríos 2002, A. Ríos-Chelén personal observations). We investigated whether or not the reproductive system of the vermilion flycatcher includes the occurrences of EPR or, conversely, whether this species can be considered genetically monogamous.

MATERIAL AND METHODS

Study area

We studied a population of vermilion flycatchers (n = approximately 24 pairs) located in the forest of San Diego Metepec (19º17.97 N, 98º14.60 W), Tlaxcala, Mexico, where blood samples were collected in 2001 and 2003. The study site is a mixture of native *Pinus* spp. and introduced *Eucalyptus* spp. forest and open areas. Most vermilion flycatchers in this population are migrants, with the exception of two males that were year round residents. The reproductive season for this population starts around February, when most migrant males begin to establish territories, and finishes around late July or early August, by which time most males have departed for the non-breeding grounds.

DNA extraction and PCR products

Adults were captured with mist nests and baited spring traps and colour ringed. Chicks were collected at their nests when 8 days old and returned to their nest once their blood was obtained. Blood samples (70-240 µL) were taken by puncturing the brachial vein, and collecting the blood with capillary tubes. Samples were stored in lysis buffer at 4 ºC until DNA was extracted.

DNA was obtained by phenol-chloroform extraction and ethanol precipitation (Sambrook et al. 1989). We used three micro-satellites markers (micro-satellites Py448, Py274, and Py390) developed for this species (see below for method). Approximately 3-10 ng of genomic DNA were amplified in 10 µL PCR reactions containing 1x buffer, 1.5 mM $MgCl_2$ (for micro-satellites Py274 and Py390) or 1.0 mM $MgCl_2$ (for micro-satellite Py448), 10 pM of each primer, 0.5 U of Taq and made up to volume with sterile distilled water. The reactions were denatured at 95 ºC for 4 min followed by 30 cycles of 95 ºC for 10 sec, primer-specific annealing temperatures (60, 54 or 56º C for micro-satellites Py448, Py390 and Py274 respectively) for 45 sec, 72 ºC for 45 seconds and final elongation at 72 ºC for 5 min. PCR products were separated on 6 % polyacrylamyde gels, visualized by silver staining, and allele lengths scored by visual inspection using a 10 base pair DNA ladder (Invitrogen) as a reference.

Development of micro-satellites

A microsatellite-enriched genomic library was developed using modifications to the protocol of Hamilton et al. (1999). Genomic DNA was extracted from whole blood, digested with Mbo I (Promega) and hybridized with a biotinilated $(CA)_{24}$ oligonucleotide (Operon) to capture single tandem repeats (STRs) of (CA)n. These fragments were cloned into a Bam HI site in pUC18 plasmid and used for transformation by heat shock of Epicurean Supercompetent *E. coli*. XL-1 (Stratagen). One hundred and 93 colonies were grown on a Nylon membrane (Electran+®, BDH) and their DNA was UV cross-linked. The membrane was hybridized with a P^{32} 5' end-labelled $(CA)_{22}$ oligonucleotide and used for autoradiography. From a total of 99 colonies, plasmids of 28 positive colonies were extracted using Perfectprep kit (Eppendorf) and their inserts were sequenced with universal -23M13 primers in an automated ABI Prism 377 sequencer.

For each sequence, primers were designed using Primer 3 program (Rozen & Skaletzky 2000). For each primer set, curves of Mg^{++} concentration and $T_{annealing}$ were performed to optimise amplification conditions (those yielding no extra-bands and sharp products; see Table 1 for characterization of micro-satellites).

TABLE 1

Characterization of *Pyrocephalus rubinus* micro-satellite loci. For locus Py448 there was no (100 %) clear motif (sequence added in GenBank). See "DNA extraction" for primer specific annealing temperatures and primer specific $MgCl_2$ concentration for PCR. Accession numbers are for the sequences, which have been deposited in GenBank

Caracterización de microsatélites de *Pyrocephalus rubinus*. Para el locus Py448 no hubo un motivo (100 %) claro (secuencia agregada en GenBank). Ver "DNA extraction" para información sobre las temperaturas y concentraciones de $MgCl_2$ específicas a cada primer, para realizar el PCR. Los números de acceso son para las secuencias depositadas en GenBank

Locus	Accession number	Repeat motif	Primer sequence (5'-3')	Alelle range (bp)
Py274	DQ834920	$(TG)_{10}N_{60}(CT)_2(GT)_7$	AGGCATGATGAGGAACTCCA CTCCCAAGGGAGGATGTCTA	245-450
Py390	DQ834919	$(CA)_3N_8(CA)_4GA$ $(CA)_4N_{12}(AC)_8$	CACACTCACACTCACGCTCA GTGTGTGCACGAACACCTG	192-205
Py448	DQ834918		CACTGTCACACAAAATCACACG GTCCCCTCTGTGCCTTGAG	250-332

DNA analysis

In 2001 six families were genotyped. From these families we obtained blood samples of all six males, three of the females and all but two of the 20 chicks. For this year we also obtained blood sample from five more adults in the population (two males, three females). In 2003, we sampled eight families, obtaining DNA from all eight males, five of the females and all 21 chicks. For this year we also obtained blood samples from eight more adults in the population (five males, three females). All adult individuals (14 in 2001 and 21 in 2003) were used to obtain allele frequencies (Table 2). We ran the PCR products using the three microsatellite markers from the putative father, mother and offspring in adjacent columns to facilitate the comparison of allele bands between family members. We considered a chick to be the result of EPR if at least one allele (band) was not shared between the nestling and either of the putative parents. The micro-satellites had relatively low numbers of alleles in this population (Table 2). This relatively low variability, plus the fact that not all adult birds were sampled, made it impossible for us to assign genetic parentage. However, we were able to identify chicks that had a different genetic father or mother from the adult on the nest. To lower the risk of typing errors and misreading some alleles, along with positive and negative controls we ran each gel with individuals that were formerly typed and used in other polyacrylamyde gels, and thus their bands served as a reference for new individuals.

TABLE 2

Data on micro-satellite allele frequencies. Data from 14 and 21 adult individuals sampled in years 2001 and 2003 respectively. Note that in year 2003 our sample lacked allele 298 from locus Py448

Datos sobre frecuencias alélicas de microsatélites. Datos de 14 y 21 individuos adultos muestreados en los años 2001 y 2003 respectivamente. El alelo 298 del locus Py448 no se encontró en nuestra muestra del año 2003

Locus	Allele (bp)	Year 2001	Year 2003
Py448	250	0.2143	0.0238
	260	0.3214	0.5238
	298	0.0714	-
	318	0.1429	0.2143
	322	0.1429	0.1667
	330	0.0357	0.0476
	332	0.0714	0.0238
Py274	245	0.3571	0.4048
	255	0.3929	0.3810
	312	0.2143	0.0714
	450	0.0357	0.1429
Py390	202	0.8214	0.9524
	205	0.1786	0.0476

To obtain basic statistics such as observed and expected heterozygocity, and to verify if our loci were under Hardy-Weinberg equilibrium, we used Cervus software (Marshall et al. 1998) and Genepop software (Raymund & Rousset 1995), respectively.

RESULTS

Information on allele frequencies and individual genotypes are given in Table 2 and 4 respectively. Table 3 shows that locus Py274 was not under Hardy-Weinberg equilibrium, presenting a statistically significant heterozygote deficit both in 2001 and 2003.

Heterozygote deficit could be the result of several factors, for instance endogamy or presence of null alleles; this latter possibility is a potential source of bias in paternity analyses (Dakin & Avise 2004). Therefore, to take into account this possibility, we re-analysed our data using ML-Relate software (Kalinowski et al. 2006), for this software can take into account the presence of null alleles when estimating maximum-likelihood relationships between individuals. To this end, we tested in ML-Relate the hypothesis that putative parents had a Parent-Offspring relationship with their putative offspring (putative relationship) against the alternative relationship, that is, that these putative parents are unrelated to their putative offspring. For these analyses we used 1000 simulations and, in a first try, we set up a P value of < 0.05 as a criterion to decide if the putative relationship fits the data significantly better than the alternative relationship. These analyses showed that in 2001, 100 % families contained extra-pair offspring and 94 % chicks in the sample had extra-pair parentage. In 2003, 87 % families had extra-pair parentage and 80 % chicks resulted of extra-pair reproduction. It is very likely that given the sparseness of our data, the frequencies of EPR when using a $P < 0.05$ were overestimated. For this reason, we re-analysed our data, but this time using a more conservative approach, that is, considering a $P < 0.2$. In other words, this would give us at least an 80 % probability that the putative relationship fits the data better than the alternative relationship.

Because we are not certain that the observed heterozygote deficit is due the presence of null alleles, we still kept our original way of analysing our data, that is, by visual inspection of shared and non-shared bands. Hence, both ways of data analyses, can give us a range of incidence of extra-pair reproduction, encompassing both the possibility that the observed heterozygote deficit is or is not a by product of the presence of null alleles.

TABLE 3

Descriptive statistics of micro-satellite loci. H_O = observed heterozygocity, H_E = expected heterocygocity. Estimation of P values in HW (to test for Hardy Weinberg equilibrium) and Hdef (to test for heterozygote deficit) by the Markov chain method in Genepop software. Statistically significant P values are in bold. See "DNA extraction and PCR products" for primer specific annealing temperatures and primer specific $MgCl_2$ concentration for PCR

Estadística descriptiva de loci de microsatélites. H_O = heterogocidad observada, H_E = heterogocidad esperada. Se estimaron los valores de P en HW (para probar equilibrio de Hardy Weinberg) y Hdef (para probar si hay deficiencia de heterocigotos) por medio del método de cadena de Markov en el programa Genepop. Valores de P estadísticamente significativos se muestran en negrilla. Ver "DNA extraction and PCR products" para información sobre las temperaturas y concentraciones de $MgCl_2$ específicas a cada primer, para realizar el PCR

Locus	2001 (n = 14)				2003 (n = 21)			
	H_O	H_E	HW	Hdef	H_O	H_E	HW	Hdef
Py274	0.429	0.696	**0.004**	**0.007**	0.429	0.682	**0.017**	**0.0007**
Py390	0.357	0.304	1.000	1.000	0.095	0.093	1.000	1.000
Py448	1.000	0.828	0.764	1.000	0.762	0.664	0.391	0.656

TABLE 4

Genotypes of individuals organized by families (putative parents and chicks), and different types of extra-pair reproduction (EPR) as suggested by non-sharing bands between individuals and analyses in ML-Relate. Analysis of three different micro-satellite suggest the occurrence of extra-pair paternity (EPP), extra-pair maternity (EPM) and intra-specific brood parasitism (ISBP) in the vermilion flycatcher breeding system. Numbers in parenthesis show the number of loci that were involved in mismatched bands that occurred between nestling and putative parents. Bold numbers represent alleles that were not shared between nestling and adults. In 2003, allele 250 from locus Py274 was only found in Chicks 1 and 2 from Lic Family (Family 4). "Type of EPR I" refers to cases of EPR as suggested by visual inspection of non-sharing band(s) between individuals. "Type of EPR II" refers to cases of EPR as suggested by analyses performed in ML-Relate software taking into account null allele (s) in locus Py274. In 2001, Family 1 (Bos) laid five eggs; two of them in a first brood, the remaining three in a second brood. In this same year (2001), Family 5 (Lic) laid five eggs, two of them fledged (Chicks 1 and 2) in a first brood, three of them (Chicks 3, 4 and 5) in a second brood. Therefore, although we had six families in 2001, we had eight broods in this year. From family 5 in 2001, we could obtain DNA from Chicks 1, 3 and 5 only

Genotipos de individuos organizados por familias (padres putativos y pollos) y diferentes tipos de reproducción extrapareja (EPR) sugeridos por bandas no compartidas entre individuos y análisis en ML-Relate. El análisis de tres diferentes microsatélites sugiere la ocurrencia de paternidad extrapareja (EPP), maternidad extrapareja (EPM) y parasitismo intraespecífico (ISPB) en el sistema reproductivo del cardenalito. Los números en paréntesis muestran el número de loci que estaban involucrados en bandas no compartidas entre padres putativos y pollos. Los números en negrita representan alelos que no fueron compartidos entre pollos y adultos. En el año 2003, el alelo 250 del locus Py274 se encontró solamente en los pollos 1 y 2 de la Familia Lic (Familia # 4). "Type of EPR I" se refiere a casos de EPR sugeridos por inspección visual de bandas no compartidas entre individuos. "Type of EPR II" se refiere a casos de EPR sugeridos por análisis hechos en el programa ML-Relate tomando en cuenta alelos nulos en el locus Py274. En el año 2001, la Familia 1 (Bos) puso cinco huevos; dos de ellos en una primera nidada, los tres restantes en una segunda nidada. En este mismo año (2001), la Familia 5 (Lic) puso cinco huevos, dos de ellos eclosionaron (pollos 1 y 2) en una primera nidada, tres de ellos (Pollos 3, 4 y 5) en una segunda nidada. Por esto, aunque en el año 2001 teníamos seis familias, tuvimos ocho nidadas en total. De la Familia 5 en el 2001, pudimos obtener ADN solo de los pollos 1, 3 y 5.

Year												
2001							2003					
Family	Individual	Alleles in micro-satellite			Type pf EPR I	Type of EPR II	Family	Individual	Alleles in micro-satellite			Type of EPR I	Type of EPR II
		Py448	Py274	Py390					Py448	Py274	Py390		
1 Bos	Male	260/332	245/255	202/205			1 Res1'	Male	260/318	245/450	202/202		
	Female	260/322	245/450	202/202				Female	318/318	245/255	202/202		
	Chick1	260/260	245/245	202/205		EPM		Chick1	260/318	245/245	202/202		
	Chick2	322/332	245/450	202/205				Chick2	260/318	245/245	202/202		
	Chick3	260/322	245/450	202/202		EPP		Chick3	260/318	245/245	202/202		
	Chick4	322/260	245/450	202/202		EPP							
	Chick5	260/260	245/450	202/202		EPP	2 IntLi	Male	260/330	255/255	202/202		
								Female	260/318	245/245	202/202		
2 Res1	Male	322/318	245/245	202/202				Chick1	260/260	**245**/245	202/202	EPP (1)	EPP
	Female	298/250	245/255	202/202				Chick2	260/330	**245**/245	202/202	EPP (1)	EPM

	Chick1	298/**260**	245/245	202/**205**	EPP (2)	EPP		Chick3	260/318	**312**/**312**	202/202	ISBP (1)	ISBP
	Chick2	298/**260**	245/245	202/202	EPP (1)	EPP							
	Chick3	298/**330**	245/245	202/202	EPP (1)	EPP	3 JuEs1	Male	260/322	245/255	202/202		
								Female	260/322	312/255	202/202		
3 Kind	Male	260/250	312/312	202/202				Chick1	322/322	245/255	202/202		
	Female	332/322	312/312	202/202				Chick2	260/322	245/255	202/202		
	Chick1	332/260	312/312	202/202				Chick3	260/322	245/255	202/202		
	Chick2	**250**/250	**245**/312	**205**/202	EPM (3)	EPM							
4 Esq2	Male	260/250	255/255	202/205			4 Lic	Male	260/322	245/245	202/202		
	Chick1	318/250	450/255	202/205				Female	260/322	450/450	202/202		
	Chick2	318/250	450/255	202/202		EPP		Chick1	322/322	**250**/**250**	202/202	ISBP (1)	EPP
								Chick2	322/322	**250**/**250**	202/202	ISBP (1)	EPP
5 Lic	Male	322/260	245/245	202/202				Chick3	322/322	**450**/450	202/202	EPP (1)	EPP
	Chick1	322/250	450/245	202/202									
	Chick3	260/260	245/245	202/202			5 Res1	Male	318/318	450/255	202/202		
	Chick5	322/260	245/245	202/202				Female	260/330	312/312	202/202		
								Chick1	260/**332**	**255**/255	202/202	ISBP (2)	ISBP
6 Mor	Male	260/250	245/255	202/202				Chick2	318/330	**450**/450	202/202	EPM (1)	
	Chick1	260/322	255/255	202/202		EPP							
	Chick2	260/322	255/255	202/202		EPP	6 Bos	Male	260/332	245/255	202/205		
	Chick3	260/260	255/255	202/202				Chick1	250/260	245/255	202/205		
								Chick2	260/260	245/255	202/205		
							7 Zan	Male	260/260	450/450	202/202		
								Chick1	260/322	450/245	202/202		
								Chick2	260/322	450/245	202/202		
							8 Mor	Male	250/260	245/255	202/202		
								Chick1	260/260	245/450	202/202		EPP
								Chick2	260/260	245/245	202/202		EPP
								Chick3	260/260	245/245	202/202		EPP

ML-Relate results (taking into account the possibility of null alleles)

In 2001, six out of eight broods (75.0 %) contained extra-pair offspring. Among these eight broods, EPP was found in four (50.0 %), and extra-pair maternity (EPM) in two (25.0 %). In terms of all chicks in the sample (n = 18), nine (50.0 %) were probably the result of EPP, and two (11.1 %) the result of EPM. Overall, 11 chicks (61.1 %) were extra-pair offspring. In 2003, four out of eight broods (50.0 %) contained extra-pair offspring. From these eight broods, three (37.5 %) contained EPP chicks, one (12.5 %) contained an EPM chick, and two (25.0 %) contained a chick that probably resulted from ISBP. In terms of chicks in the whole sample (n = 21), seven (33.3 %) were the result of EPP, one (4.7 %) was the result of EPM, and two (9.5 %) the result of ISBP. Hence, 10 chicks (47.6 %) had extra-pair parentage.

Visual inspection results

In 2001 two out of eight broods (25.0 %) contained at least one chick that resulted from extra-pair reproduction. In one (12.5 %) of those broods, all chicks (n = 3) presumably resulted from EPP (in all of them, one allele from the father could not be matched to the male on the nest). In the other brood (12.5 %), there was EPM. In this brood one chick was not the offspring of the female on the nest, but was probably the offspring of the male (i.e., we had no evidence to state that it was not: the genotype of the nestling shared at least one allele with its social father at all three loci). In terms of all nestlings in the sample (n = 18), three chicks (16.6 %) resulted from EPP, and one (5.5 %) from EPM. Overall, four out of 18 chicks (22.2 %) probably resulted from extra-pair reproduction (Table 5). In 2003, three out of eight broods (37.5 %) were found to have extra-pair offspring. Among these eight nests, EPP was found in two of them (25.0 %), EPM in one of them (12.5 %), and ISBP in three of them (37.5 %). In terms of number of chicks in the whole sample (n = 21), three (14.2 %) were not the offspring of the male on the nest (i.e., extra-pair paternity), and four (19.0 %) were the result of ISBP. As in 2001, and considering all 21 chicks in the sample, we found one chick (4.7 %) whose mother was not the female on the nest, but whose father was probably the male at the nest (i.e., EPM or quasi-parasitism, Yezerinac 1995). Hence, eight chicks out of 21 (38.0 %) had extra-pair parentage.

Table 5 summarizes both the "visual inspection" and the "ML-Relate" results.

TABLE 5

Percentage of broods and chicks that were the result of different modes of extra-pair reproduction in 2001 and 2003. EPP = extra-pair paternity, EPM = extra-pair maternity, ISBP = intra-specific brood parasitism, EPR = extra-pair reproduction. Results from visual inspection and from MR-Relate are shown for comparison

Porcentaje de nidadas y pollos que fueron resultado de diferentes modos de reproducción extrapareja en los años 2001 y 2003. EPP = paternidad extrapareja, EPM = maternidad extrapareja, ISBP = parasitismo intraespecífico, EPR = reproducción extrapareja. Se muestran resultados de inspección visual y de EM-Relate para comparar

Variable	Mode	Year			
		2001		2003	
		Visual inspection	ML-Relate	Visual inspection	ML-Relate
Broods (%)	EPP	12.5	50.0	25.0	37.5
	EPM	12.5	25.0	12.5	12.5
	ISBP	0.0	0.0	37.5	25.0
	EPR	25.0	75.0	37.5	50.0
Chicks (%)	EPP	16.6	50.0	14.2	33.3
	EPM	5.5	11.1	4.7	4.7
	ISBP	0.0	0.0	19.0	9.5
	EPR	22.2	61.1	38.0	47.6

DISCUSSION

Despite our small sample size, we found evidence that the mating system of the vermilion flycatcher is far more complicated than simple monogamy. Our results point to cases of extra-pair paternity, extra-pair maternity (quasi-parasitism) and intra-specific brood parasitism.

The most striking possible effect of null alleles in our data might have been an underestimation of EPP and EPM in 2001, and an overestimation of ISBP combined with an underestimation of EPP in 2003 (see Table 5). However, since null alleles are not the only possible source of heterozygote deficit, it may be better to take those results as an upper boundary of extra-pair reproduction in this species, rather than accepting that the possible effects of null alleles provide an accurate picture of extra pair parentage (see below). Besides null alleles, other potential sources of bias in our results would be a high mutation rate, and having misread some alleles in our gels. Based on studies made with *Drosophila melanogaster* and humans, mutation rates in micro-satellites have been considered to be relatively low (i.e., 10^{-2}-10^{-4}; DeWoody & Avise 2000, Bailey et al. 2007). This, with the relatively low numbers of alleles reported for each micro-satellite in this study combined with the relatively large number of chicks presenting an extra band does not point to mutation as a likely source of bias in our results. It is also unlikely that we might have misread some alleles. This is because the use of a positive control (i.e., from a plasmid) aided us in determining the gel area where we should expect to find our micro-satellite bands. The use of other individuals as positive controls (i.e., alleles coming from individuals that had already been run in previous gels) also provided a reference for new individual's alleles. Moreover, mismatched bands were very different in size (3-72 bp, and 5-200 bp for years 2001 and 2003 respectively; Table 2) which made identification of mismatched bands a relatively easy task. Nevertheless, the rates of EPR found in this study should be viewed with caution since, in our visual inspection we found that only two out of four (50.0 % for 2001) and one out of eight (12.5 % for 2003) extra-pair chicks were found to have a mismatch at more than one locus (Table 4).

Depending on the method used (visual inspection or ML-Relate), extra-pair paternity ranged from 16.6 % to 50.0 % chicks in 2001 and from 14.2 % to 33.3 % chicks in 2003. Other studies in socially monogamous species have found that on average 11.1 % of offspring and 18.7 % of broods result from extra-pair paternity (Griffiths et al. 2002). However, given the small sample sizes in our study, our results may not be representative of the population as a whole, and thus should serve more to describe the occurrence of EPR in the vermilion flycatcher, rather than to compare it with other avian populations. Some studies on oscine birds have shown a relation between male phenotype (i.e., song repertoire size) and EPP, whereby females may seek genetic benefits for their offspring (Hasselquist et al. 1996). Extra-pair paternity has been shown to occur at relatively high levels in a few suboscines. For instance, Woolfenden et al. (2005) reported, for the acadian flycatcher (*Empidonax virescens*), that 58 % of nests contained extra-pair offspring and 40 % of nestlings were extra-pair young, while Tarof (2001) reported up to 61.9 % of broods having extra-pair offspring in the least flycatcher (*Empidonax minimus*). This relatively high incidence of extra-pair offspring in the least flycatcher may be explained in part by the "open mating system" characteristic of this species, whereby clusters of individuals can be visited by individuals from other sites trough out the breeding season (Kasumovic et al. 2003). On the other hand, Dolan et al. (2007), studying the suboscine eastern kingbird (*Tyrannus tyrannus*), found that those males singing early in the dawn chorus were more successful in increasing their reproductive success via extra-pair paternity, which reached a level of 61 % of nests and 47 % of offspring. It is still not known what aspects of male phenotype might promote extra-pair paternity in the vermilion flycatcher.

Since we could not assign parentage, we cannot discard the possibility that cases where we found EPP or EPM are in fact instances of ISBP. Because of this, we focus the remainder of our discussion on the occurrence of this mode of extra-pair reproduction.

This is the first time that ISBP has been reported in the Vermilion Flycatcher. ISBP has been reported in other species (Birkhead et al. 1990, Petrie & Møller 1991, Jackson 1993, McRae & Burke 1996, Lyon 2003), with

relatively low frequencies of occurrence in some (e.g., zebra finch, *Taeniopygia guttata*: 10.9 % of offspring, Birkhead et al. 1990; the sand martin, *Riparia riparia*: 1.8 % of offspring, Alves & Bryant 1998) and relatively high in others (e.g., nests parasitized: up to 46 % in starlings *Sturnus vulgaris*, up to 24 % in cliff swallows *Hirundo pyrrhonota*, up to 31 % in swallows *H. rustica*; review in Davies 1988, and perhaps up to 39 % of offspring in the eastern kingbird, McKitrick 1990). However, in this latter case, it could not be discerned whether these extra-pair reproduction events where result of ISBP, quasi-parasitism or both. Several hypotheses have been brought forward to explain the occurrence of ISBP. One possibility is that neither the male nor the female are aware of other female laying egg(s) on their nest. In this hypothesis the social partner gain no benefit from ISBP. In the case of the vermilion flycatcher, this hypothesis seems likely because only females incubate eggs and thus the nest may occasionally be left unattended for relative long periods of time. On the other hand, it is also possible that one or both pair members may obtain some benefits by allowing the parasitic female to lay eggs in the host nest (McRae & Burke 1996). For example, males may cooperate with parasitic females by letting her lay eggs in their nests in exchange for copulations. If this is the case, we should expect this male to sire some offspring in the parasitic female's own nest or in his own nest (i.e., quasi-parasitism or extra-pair maternity).

We found that in 2003 between 9.5 % and 19.0 % of chicks in our sample was probably the result of ISBP. Regardless of the method used (visual inspection or ML-Relate), we found that in one of the nests where ISBP presumably occurred also occurred a case of extra-pair maternity. While we could not determine whether the mother of this quasi-parasitic offspring was the same female that laid the ISBP egg in this nest, this result points to the "in exchange of copulations" hypothesis. However, for the time being this interpretation is speculative and a more detailed study, where paternity and maternity can be assigned, is needed to address this hypothesis.

To conclude, we found evidence that the vermilion flycatcher, although socially monogamous, incur in different modes of extra-pair reproduction. This expands our knowledge on the mating system of a suboscine species, a group of passerines that deserves more studies. The observed frequency of EPR in this study underlines the need to conduct further studies to assess which individual attributes (if any), either morphological (e.g., body mass and size, plumage colouration) or behavioural (e.g., song and flight display: Smith 1967, 1970, Ríos-Chelén & Macías 2004, Ríos Chelén et al. 2005), promote extra-pair reproduction in this sub-oscine species.

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