

# Parentage assignment and extra-group paternity in a cooperative breeder: the Seychelles warbler (*Acrocephalus sechellensis*)

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## Abstract

We describe the development and initial application of a semiautomated parentage testing system in the Seychelles warbler (*Acrocephalus sechellensis*). This system used fluorescently labelled primers for 14 polymorphic microsatellite loci in two multiplex loading groups to genotype efficiently over 96% of the warbler population on Cousin island. When used in conjunction with the program cervus, this system provided sufficient power to assign maternity and paternity within the Seychelles warbler, despite the complications associated with its cooperative breeding system and a relatively low level of genetic variation. Parentage analyses showed that subordinate 'helper' females as well as the dominant 'primary' females laid eggs in communal nests, indicating that the Seychelles warbler has an intermediate level of female reproductive skew, in between the alternative extremes of helper-at-the-nest and joint nesting systems. Forty-four per cent of helpers bred successfully, accounting for 15% of all offspring. Forty per cent of young resulted from extra-group paternity.

**Keywords:** cooperative breeding, extra-pair paternity, likelihood analysis, microsatellite, parentage

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## Introduction

Precise measures of reproductive success are required before aspects of mate choice, sexual selection and fitness can be properly investigated. However, in breeding systems such as lekking, polygyny, polyandry and cooperative breeding it may be impossible to determine parentage from direct observations (Birkhead & Møller 1992; Andersson 1994; Cockburn 1998). Even within socially monogamous species, offspring are not always genetically related to their putative parents. Females may copulate with males other than their social mate, resulting in extra-pair paternity (reviewed in Gowaty 1996; Birkhead & Møller 1998). Moreover, females may lay eggs in the nests of other conspecific females (intraspecific brood parasitism; Yom-Tov 1980; Yamauchi 1993). Molecular methods, i.e. DNA profiling (Jeffreys *et al.* 1985; Burke 1989; Queller *et al.* 1993), are therefore required to determine parentage accurately.

The Seychelles warbler (*Acrocephalus sechellensis*) is a rare endemic of the Seychelles islands in the western Indian Ocean. By 1959, anthropogenic disturbance had pushed this species to the verge of extinction and only 29 individuals remained, confined to the 29-ha Cousin Island (Crook 1960). The population has since recovered, as a result of appropriate long-term management, and has been the focus of intensive study since 1985 (e.g. Komdeur 1992, 1994a,b; Komdeur *et al.* 1997).

Accurate parentage analysis of the Seychelles warbler is now required to address questions relating to the genetic benefits of mate choice, inbreeding avoidance and the evolution of cooperative breeding. Previous work has shown this species to have a cooperative breeding system in which offspring (usually female) from previous breeding seasons remain in the natal territory as helpers (Komdeur 1992; 1994a,b). The presence of individuals related to both the tested offspring and the putative parents complicates the parentage analysis and, in addition, incestuous matings may be difficult to detect (McRae & Amos 1999; Parker *et al.* 1999). Furthermore, the prolonged population bottleneck undergone by this species has reduced genetic

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variation within the population (Kappe 1998), and relatively low levels of allelic polymorphism are available for DNA profiling (see Richardson *et al.* 2000).

Recently, fluorescently labelled microsatellite markers and semiautomated systems have been developed that can efficiently type numerous separate loci simultaneously (Reed *et al.* 1994), while software packages such as CERVUS (Marshall *et al.* 1998; Slate *et al.* 2000) facilitate the accurate assignment of parentage based on a log-likelihood approach. With enough microsatellite markers, it should be possible to assign parentage accurately even in inbred species with complicated breeding systems (McRae & Amos 1999).

The purpose of this study was to develop a semiautomated multiplex system using a suite of microsatellite loci selected from 63 loci previously isolated in the Seychelles warbler (Richardson *et al.* 2000) to assign parentage in the Seychelles warbler. All the offspring produced on Cousin Island during the 1999 breeding season were sampled and used to test the power and applicability of the system and estimate the prevalence of extra-pair (and extra-group) paternity and intraspecific brood parasitism.

## Materials and methods

### *The study population*

The entire population of Seychelles warblers on Cousin Island was monitored continuously between May–September 1999 (the peak breeding period, Komdeur 1996). All suitable habitat on the island was occupied by 258 birds (> 6 months old) on 104 breeding territories. Almost all birds (247/258 = 96%) were individually colour-ringed and blood-sampled. Two adult breeding birds, four helpers and three floaters remained unringed and unsampled, and there was no blood sample for two adult birds ringed in the 1980s. Each territory was checked regularly for breeding activity at least once every two weeks by following the resident female for 30 min (Komdeur 1992). Nests were observed throughout the breeding cycle and behavioural observations were used (in conjunction with status data from previous years) to determine the status of all birds within each territory. In this study the ‘primary’ male and female were defined as the dominant, pair-bonded male and female in a territory, while the term ‘helper’ included all other birds (> 6 months old) resident in the territory. Molecular sexing using the polymerase chain reaction (PCR) method devised by Griffiths *et al.* (1998) was used to correctly determine the sex of each individual.

Birds caught for the first time were ringed with a unique combination of three UV-resistant colour rings and a British Trust for Ornithology ring. Blood samples ( $\approx$  15  $\mu$ L) were collected by brachial venipuncture and then diluted

in 800  $\mu$ L of 100% ethanol in a screw-cap microfuge tube and stored at room temperature. Dead embryos were extracted from eggs that failed to hatch and were also stored in ethanol. We attempted to sample all offspring produced in the 1999 breeding season. The Seychelles warbler produces one clutch per season and this normally consists of just one egg (80%). However, on occasion two- or three-egg clutches may occur (Komdeur 1991). Fifty-nine offspring were sampled from 48 territories (one clutch per territory). Many nests could not be reached immediately after hatching and it is possible that mortality may have occurred in some nests. The offspring sampled may not therefore have included all the offspring that hatched. Eight offspring that died in the egg or nest are included in this analysis.

### *DNA extraction*

DNA was extracted from blood and embryonic tissue using a standard phenol extraction technique (following Bruford *et al.* 1998), or (for blood only), using a quicker salt extraction method as follows. One flake ( $\approx$  one-tenth) of the blood sample was removed from the ethanol and air-dried before being added to 250  $\mu$ L of proteinasing solution (0.2 mg/mL proteinase K, 50 mM Tris, 120 mM NaCl, 1% SDS, 20 mM EDTA, pH 8.0) and digested (with constant agitation) at 55 °C for 3 h. Then one volume of 4 M ammonium acetate was added and the solution was vortexed and left at room temperature for 15 min. The sample was centrifuged at 8000 g for 10 min and the supernatant decanted into a clean, labelled microfuge tube. To precipitate the DNA from the supernatant, two volumes of 100% ethanol was added and the sample was centrifuged at 8000 g for 10 min. The supernatant was decanted off and the pellet was rinsed in 1 mL of 70% ethanol and air-dried for 30 min. DNA samples were dissolved overnight in 250  $\mu$ L 10 mM Tris, 0.1 mM EDTA. The DNA concentration was then determined and, where necessary, samples were diluted to a concentration of between 10 and 50 ng/mL.

### *Microsatellite markers, PCR and multiplex loading*

Thirty of 63 microsatellite loci previously isolated from the Seychelles warbler were polymorphic (Richardson *et al.* 2000). Fifteen of these were selected, on the basis of polymorphism and product size, for use in the parentage study. Samples were PCR-amplified using the forward primer 5' end-labelled with a fluorescent phosphoramidite (6-FAM, HEX or NED) and the sequence GTTTCTT was added to the 5' end of the reverse primer to reduce noise from variable adenylation during the PCR (Brownstein *et al.* 1996). The 15 chosen loci are listed in Table 1. The loci were organized into two multiplex loading groups,

**Table 1** Fifteen microsatellite loci PCR amplified and multiplex loaded in two groups

Multiplex group	Locus	Allele sizes (bp)	Flourescent label	MgCl <sub>2</sub> conc. mM	T <sub>a</sub>	Dilution post PCR	H <sub>O</sub>	H <sub>E</sub>	Excl(1)	Excl(2)
1	<i>Ase9</i>	(130 + 131), 133, 138	Ned	2.0	58	1 : 5	0.42	0.44	0.09	0.21
	<i>Ae10</i>	122, 130, 143	6-Fam	2.0	58	1 : 4	0.42	0.42	0.09	0.21
	<i>Ase18</i>	184, 188, 196	6-Fam	2.0	58	1 : 4	0.49	0.57	0.12	0.21
	<i>Ase25</i>	173, 197, 202, 206, 209, 213	Hex	2.0	56	1 : 2	0.71	0.69	0.30	0.47
	<i>Ase27</i>	184, 209, 213, 217, 222, 225, 230	Ned	2.5	56	1 : 4	0.65	0.66	0.22	0.37
	<i>Ase37</i>	237, 241, 247	6-Fam	2.0	58	1 : 6	0.44	0.42	0.10	0.23
	<i>Ase42</i>	249, 253	Ned	2.0	58	1 : 6	0.29	0.29	0.04	0.11
	<i>Ase48</i>	272, (278 + 279), 284	6-Fam	2.0	56	1 : 4	0.64	0.53	0.15	0.24
	<i>Ase58</i>	283, 293, 299, 305, 310	Hex	2.0	58	1 : 2	0.73	0.67	0.31	0.49
2	<i>Ase4</i>	106, 108	6-Fam	2.0	61	1 : 5	0.40	0.42	0.08	0.16
	<i>Ase6</i>	117, 119, 125, 129	Ned	2.0	61	1 : 4	0.70	0.71	0.26	0.43
	<i>Ase13</i>	140, 147, 154	Hex	1.5	59	1 : 2	0.52	0.53	0.14	0.26
	<i>Ase35</i>	230, 232, 234	Ned	2.0	58	1 : 4	0.61	0.69	0.18	0.32
	<i>Ase46</i>	267, 273, 275	Hex	1.5	61	1 : 2	0.22*	0.45	—	—
	<i>Ase56</i>	299, 301, 305	6-Fam	2.0	58	1 : 5	0.42	0.44	0.08	0.19

Multiplex loading groups are run in separate lanes. Primer sequences and accession numbers are in Richardson *et al.* (2000). Alleles in brackets were lumped due to difficulties in resolving alleles with only one base pair difference.

T<sub>a</sub>, annealing temperature; H<sub>O</sub>, observed heterozygosity; H<sub>E</sub>, expected heterozygosity; —, no product detected.

\*presence of a null allele was detected, locus was excluded from further analysis.

Excl(1) = Exclusion probability of the locus for the first parent.

Excl(2) = Exclusion probability of the locus for the second parent (with first parent assigned).

containing nine and six loci, respectively. Within a loading group, 2–4 loci with nonoverlapping allele lengths were labelled with a single fluorescent dye, and in total three fluorescent dyes emitting different wavelengths were used.

PCR amplification reactions were performed in a final 10- $\mu$ L volume containing 10–50 ng of DNA, 1.0  $\mu$ M of each primer, 0.2 mM of each dNTP, 0.05 units of *Taq* DNA polymerase (Thermoprime Plus, Advanced Biotechnologies) and 1.0–2.0 mM MgCl<sub>2</sub> (Table 1) in the supplied reaction buffer [final concentration: 20 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 75 mM Tris-HCl, pH 9.0, 0.01% (w/v) Tween]. PCR amplification was performed using a Hybaid touchdown thermal cycler at one cycle of 95 °C for 3 min then 35 cycles of 94 °C for 1 min, 30 s at annealing temperature (Table 1), 72 °C for 45 s, followed by 72 °C for 5 min. For each individual bird, PCR products from each multiplex group were diluted (Table 1) and equal volumes combined. Two  $\mu$ L of the multiplexed mixture was combined with 2  $\mu$ L of a loading buffer containing 1.1  $\mu$ L deionized formamide, 0.45  $\mu$ L blue dextran loading dye and 0.45  $\mu$ L internal lane standard (ROX500, Applied Biosystems). These samples were then denatured by heating at 100 °C for 3 min and placed directly on ice before loading. One  $\mu$ L of each sample was electrophoresed on a 10% denaturing polyacrylamide gel at 3000 V, and the DNA fragments were detected using 2400 scans per hour (36 cm well-to-read) on an Applied Biotechnologies (ABI) 377 XL DNA sequencer. Fluores-

cently labelled DNA fragments were analysed using the Applied Biosystems GENESCAN (version 3.1) and GENOTYPER (version 2.5) DNA fragment analysis software.

#### Parentage analysis

Standard exclusion probabilities were calculated for each locus (Table 1) and for all loci combined. However, standard exclusion probabilities can be misleading due to the presence of close relatives in systems with cooperative breeding and/or high levels of philopatry (Double *et al.* 1997). In this study, parentage was determined by analysing the genotypic data using a likelihood-based approach in CERVUS (Marshall *et al.* 1998). The natural logarithm of the likelihood ratio is termed the LOD score. The simulation program within CERVUS was used to estimate the required critical difference in LOD scores between the first and second most likely candidate parent for assignment at a level of > 95% confidence and > 80% confidence. Parentage was assigned in a series of steps. First we attempted to assign maternity to a female within the natal group, then, if all group females were excluded, to an extra-group female. Once a female was assigned we attempted to assign paternity to a group male then, if all group males were excluded, to an extra-group male. Critical LOD values were calculated for the assignment of: (i) maternity to a group female — paternity not known; (ii) maternity to an extra-group female — paternity not known;

(iii) paternity to a group male — maternity assigned; and (iv) paternity to an extra-group male — maternity assigned. For the simulation of within-territory assignment (of either parent) the number of candidate parents was set at four — one greater than the maximum number of same-sex helpers in a group (same for both sexes in 1999) — and the proportion of sampled candidate parents was set at 75%. This simulates the chance that an unknown or nongroup individual may be the parent.

The existence in the candidate parent population of full sibs of the offspring whose parentage is being tested reduces our ability to assign the first parent, because siblings may share marker alleles obtained from the parents at each locus. In cases where neither parent is already known, nonexcluded full sibs are expected to have an equal or slightly higher LOD score than the true parent (Marshall *et al.* 1998). In the Seychelles warbler, where helper females are offspring from previous years, full sibs may include candidate females on the natal territory. In an attempt to avoid the incorrect assignment of a full sib, each case where both a helper female and the dominant female had LOD scores above zero was carefully examined. In such cases a summed LOD score was obtained for each combination of a candidate female plus the male subsequently assigned in conjunction with this female, and the summed LOD scores were compared. The pair giving the highest combined LOD score was assigned; however, the level of confidence could not be defined.

For extra-group assignment the number of candidate parents was taken as the number of adults of the appropriate sex known to be alive at any time during 1999 (126 males, 138 females). The combined number of males and females (264) was higher than the number of ringed adults seen during the field season (247) as we included any adults observed in previous visits to the island in 1999. We calculated that 96% of candidate parents were sampled, 99% of loci were typed and assumed a 1% rate of typing error.

#### *Statistical analysis*

All tests are two-tailed and corrected for continuity or tied ranks, as appropriate. Means are given  $\pm$  one standard deviation.

#### **Results**

The 15 loci were characterized using the 247 adult birds observed on Cousin Island during the breeding season (Table 1). A lower than expected frequency of heterozygotes led us to conclude that there was a high frequency of null alleles at locus *Ase46* and, consequently, this locus was excluded from the parentage analyses. No other locus contained an excess of homozygotes or deviated from Hardy–Weinberg equilibrium. The overall

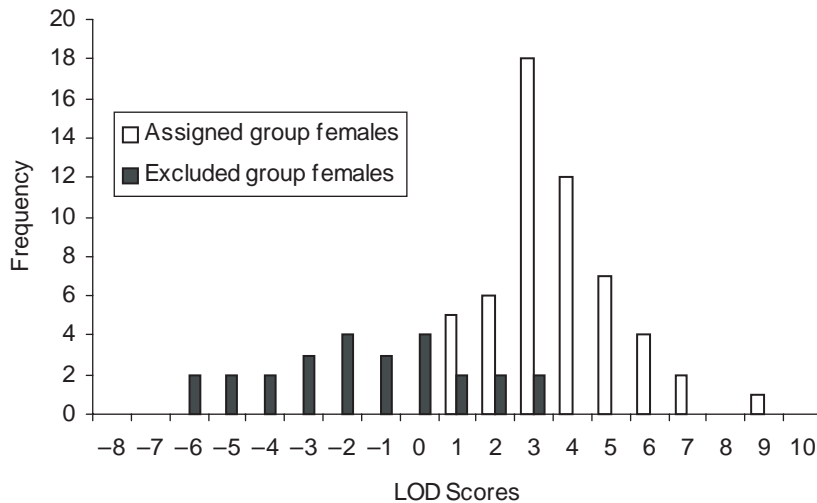
probability of exclusion for the set of 14 loci used in the parentage analyses was calculated in CERVUS using the actual allele frequency data for the 247 adult birds. The probability of exclusion was 0.911 for the first parent and 0.992 for the second parent (assuming the first parent was assigned correctly).

The critical LOD values calculated for assignment of parentage with > 80% and > 95% confidence, respectively, were as follows: (i) maternity to a group female with paternity unknown: > 0.00 and > 1.38; (ii) maternity to an extra-group female with paternity unknown: > 1.45 and > 2.47; (iii) paternity to a group male with maternity assigned: > 0.00 and > 0.58; and (iv) paternity to an extra-group male with maternity assigned: > 0.40 and > 1.70 (> 75% confidence threshold at 0.13). The assignment of maternity or paternity to within-group candidates at > 80% confidence required only a positive LOD. Therefore, where there was only one candidate parent within a group, that individual was assigned unless it had a negative LOD.

#### *Maternity*

Fifty-nine offspring from 48 clutches were used in the parentage analyses. We assigned 93% (55/59) of offspring to a territorial female, 46 with > 95% confidence and 55 with > 80% confidence. The distribution of the LOD scores of assigned and excluded group females can be seen in Fig. 1. Eighteen offspring were from territories with more than one group female. In 13/18 cases all but one group female was excluded on the basis of having both a negative LOD score and mismatches at two or more loci. For the other five offspring, at least one other group female (and in one case two) also had a positive LOD. For three of these offspring the female with the higher LOD score could be assigned with > 95% confidence and, in two cases, with > 80% confidence. However, in all these cases there were no marker mismatches between either of the adult females and the offspring. Of these five offspring, three were initially assigned to a helper female and two to the primary female based on the highest LOD scores. The summed LOD score for each candidate female plus the male subsequently assigned were then compared. In four cases the assigned female was confirmed. However, for one offspring, for which the difference between the candidate female LOD scores was the lowest, the primary female was assigned in place of the helper (helper vs. primary female; female LOD = 2.91 vs. 2.49; combined female + male LOD = 5.55 vs. 7.18).

All sampled group females were excluded for four of the 59 offspring (7%). These offspring were then compared to all candidate females on the island. In each case, multiple females outside of the group had positive LOD scores and no single female could be assigned unambiguously as the



**Fig. 1** LOD scores for assigned and excluded group females.

Parentage	Parental status	<i>n</i>	%	95% confidence for percentages
<b>Maternity</b>				
Extra-group maternity ( <i>n</i> = 55)		0	0	0–6
Extra-group maternity clutches ( <i>n</i> = 45)		0	0	0–6
<b>Paternity</b>				
Extra-group paternity ( <i>n</i> = 55)		21	38	25–53
Extra-group paternity clutches ( <i>n</i> = 45)		18	40	26–55
<b>Status of mother</b>				
Within-group young (all 55 offspring)	Primary	47	85	77–98
	Helper	8	15	7–28
<b>Status of father</b>				
Within-group young	Primary	33	60	46–75
	Helper	1	2	0–11
Extra-group young	Primary	21	38	24–53
	Helper	0	0	0–6

**Table 2** Assignment of parentage in relation to sex and status

mother. We attempted to assign the paternity for these four offspring to candidate males on their natal territory, or within the entire population. In each case all territorial males were excluded and, due to the lower power of the parental assignment analysis for the first parent, no single male from outside the territory could be assigned as the father unambiguously. These offspring were excluded from all subsequent analyses.

The remaining 55 offspring from 45 territories were all assigned to within-group females (Table 2). Groups contained up to three females and up to three offspring. Overall, the primary female was assigned as the mother in 85% (47/55) of cases and helper females were assigned in 15% (8/55). The mean LOD for assigned offspring was  $3.18 \pm 1.58$ . There was no significant difference between the LOD scores of offspring assigned to primary females and

those assigned to helper females (primary vs. helpers:  $3.14 \pm 1.63$  vs.  $3.42 \pm 1.36$ ; *t*-test; *t* = 0.05, d.f. = 53, *P* = 0.62). Twelve multiple-female territories contained a total of 12 primary females and 16 helper females and produced 18 offspring. Of these offspring, eight (44%) were assigned to seven different helper females; therefore, 44% (7/16) of helper females gained maternity. In five multiple-female territories two or more offspring were sampled and mixed maternity occurred in 3/5 (60%) of these territories. In six broods with two or more chicks all offspring were assigned to the same female.

#### Paternity

We attempted to assign all 55 offspring to a male within the group. Thirty-four (62%) offspring could be assigned to a

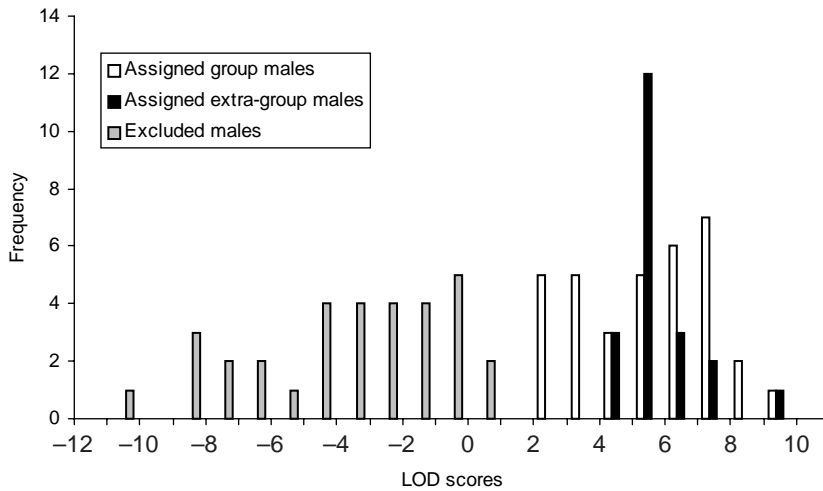


Fig. 2 LOD scores for assigned and excluded group males and for assigned extra-group males.

group male, all with > 95% confidence. Six offspring were from territories that contained multiple males (five territories with one, and one with two male helpers); however, in each case only one male in the territory had a positive LOD score. In all but one case the assigned male was the main primary male within the territory (Table 2). In the one case where a helper male was assigned as the father of the offspring, the primary male within the territory was also excluded as the father based on a negative LOD score. In 21 (38%) cases all group males were excluded, indicating that the offspring resulted from extra-group fertilizations. The bimodal distribution of the LOD scores, with no overlap between assigned and excluded group males (Fig. 2), suggests that we are able to assign paternity accurately to group males. The lowest LOD score achieved by a territorial male to which paternity was assigned was 1.05.

It is possible for a candidate male to match the offspring merely by chance and to be assigned falsely as the genetic father. We estimated the level of false assignment of paternity to group males by determining the frequency of sampled males which would have been incorrectly assigned as the father, on the basis of their LOD score, if resident in the natal territory. A minimum LOD of 1.00 (below the lowest LOD achieved when paternity was assigned to a group male) was used to determine which males matched offspring. Not including the assigned father, a mean of 5.9 (4.7%) males matched a given offspring with a LOD > 1.00. Each sampled territory contained an average of 1.16 males (52 candidate males on the 45 territories sampled). Therefore, the probability that a male on the territory other than the true father will match the offspring by chance,  $i = 5.4\%$  (mean number of group males  $\times$  percentage males matching by chance). The number of group males to which paternity has been assigned,  $A$ , will include a proportion assigned by chance:

$$A = t + i(n - t)$$

where  $t$  = the number of offspring fathered by territorial males and  $n$  = the total number of offspring. Here,  $n = 55$ ,  $A = 34$  and  $i = 0.054$ , giving  $t = 33.2$ . This equates to an estimated 0.77 territorial males being falsely assigned (= 2.3% of 34 offspring assignments to territorial males).

Paternity was assigned to the 21 extra-group young using the set of 126 candidate males observed on the island in 1999. In all cases the offspring could be assigned to a male (7 > 95%, 18 > 80% and 21 > 75% confidence). For three offspring, paternity could only be assigned with > 75% confidence. Locus *Ase46* (containing null alleles) confirmed the correct paternity assignment of one of these three offspring; however, it did not help resolve the paternity of the other two offspring due to the presence of apparent homozygotes both in offspring and the potential fathers. As shown in Fig. 2, there was no significant difference between the mean LOD scores of offspring assigned to group males compared to those assigned to extra-group males (group males  $4.58 \pm 2.02$  vs. extra-group males  $4.94 \pm 1.12$ ;  $t$ -test for unequal variances;  $t = 0.86$ , d.f. = 53,  $P = 0.39$ ). However, the variance did differ significantly (group males vs. extra-group males = 4.14 vs. 1.34,  $F_{20,33} = 3.08$ ,  $P = 0.005$ ), which may suggest that some paternity assignments based on marginal LOD scores are incorrect, though the number of misassigned territorial males is expected to be very low (see above).

Extra-group males were always breeding males (Table 2) and often from adjacent territories (number of territories away from natal territory where 0 = adjacent territory: mean  $1.86 \pm 2.22$ , median = 1, mode = 0, range = 0–6; Fig. 3). There was no difference between the LODs of assigned extra-group males in adjacent compared with those from nonadjacent territories ( $5.32 \pm 1.30$  vs.  $4.49 \pm 0.95$ ,

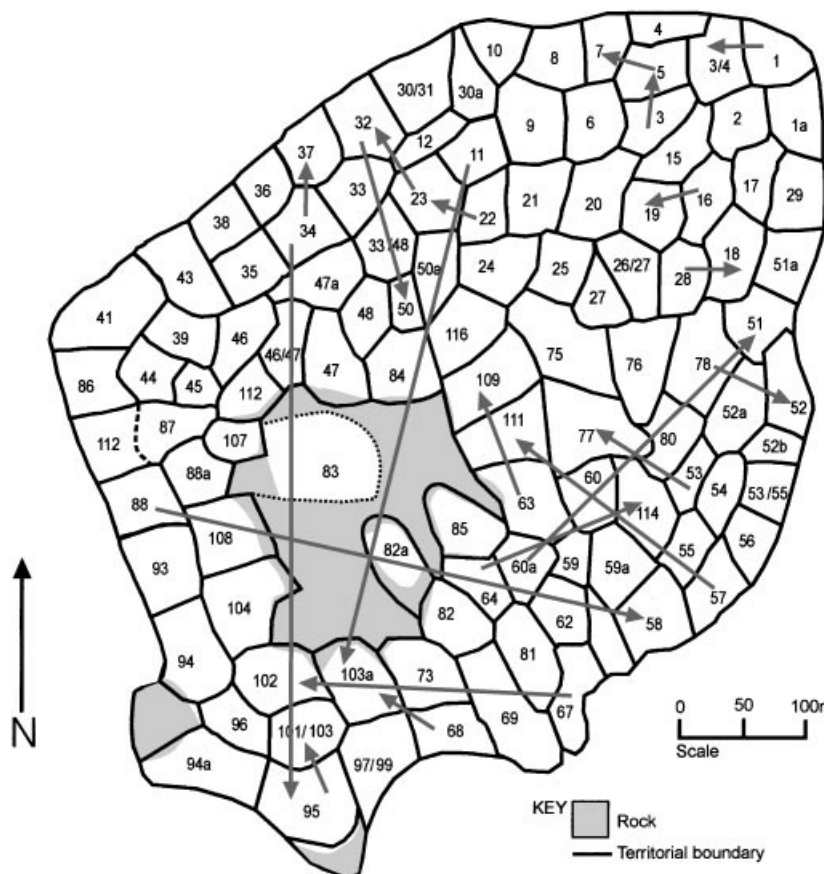


Fig. 3 Seychelles warbler territories on Cousin Island in July 1999. Arrows point from the territory of the assigned extra-group male to the natal territory of the extra-group offspring.

respectively;  $t$ -test;  $t = 1.69$ , d.f. = 19,  $P = 0.11$ ). Offspring from multiple-offspring nests were no more likely to be extra-group offspring (15/36) than offspring from single-offspring nests (4/9;  $\chi^2 = 0.08$ , d.f. = 1,  $P > 0.05$ ). However, this final test has very little power due to the small sample sizes involved.

## Discussion

### Parentage Assignment

Although the combined exclusionary power of the 14 loci used in this study was high, the relatively low genetic variability and the cooperative breeding system of the Seychelles warbler (Komdeur 1996b) meant that it was impossible to resolve directly the parentage of chicks without applying some biological logic to the parental assignment procedure. Maternity was assigned first, then within-group paternity, and finally we assigned extra-group males to offspring for which group males had been excluded. CERVUS was used to compute the likelihood of parentage at each stage. Therefore, we hoped to minimize the use of demographic rules to assist with the parental assignment (e.g. Double & Cockburn 2000).

Fifty-five offspring were assigned to a group female with a high level of confidence, but for four offspring all females on the natal territory were excluded. These unassigned offspring could also be the result of intraspecific brood parasitism, which has been shown to occur in other avian species (reviewed in Yom-Tov 1980; MacWhirter 1989; Yamauchi 1993). However, when we attempted to assign maternity to nongroup females, multiple females had positive LOD scores and no single female could be assigned unambiguously for these offspring. Furthermore, in three of the four cases there was an unringed adult female within the natal territory, which seems likely to explain the unassigned maternity. The remaining unassigned chick may merely be the result of a handling error that despite efforts to avoid such problems, is always a possibility. If brood parasitism had occurred one would expect offspring for which maternity could not be assigned to be present in nests containing multiple offspring, but this was never the case. However it is possible that the brood parasite could be displacing the original egg in the nest before laying her own (e.g. starlings Lombardo *et al.* 1989) though there is no evidence that this behaviour occurs here.

It is also possible that by first assigning maternity to females resident on the natal territory we may have falsely

assigned maternity to territorial females and overlooked cases of intraspecific brood parasitism. However, the chance that brood parasitism could occur was taken into account when calculating the critical LOD scores required for maternity assignment. Furthermore, it seems unlikely that brood parasitism occurs to any significant degree in the Seychelles warbler population. No behavioural evidence for brood parasitism has been observed (D. Richardson & J. Komdeur, personal observation), and the combination of being highly territorial, having high levels of nest guarding and having helpers who also defend the nest (Komdeur & Kats 1999) could effectively exclude extra-group females from getting to the nest.

The possible presence within the territory of helpers, who are full sibs of offspring, will reduce our ability to assign the first parent with a high degree of confidence (Marshall *et al.* 1998). However, we carefully examined all cases where each of two group females could not be excluded and used a combined assigned female + male LOD to resolve these cases (although we could not define a confidence level for these maternal assignments). New parentage assignment methods that calculate the likelihood and confidence of parentage for each combination of male and female within a population are required before this problem can be resolved in a more satisfactory manner.

We assigned paternity for the 55 offspring for which maternity had been resolved. Sixty-two per cent of offspring were assigned to a group male; however, some group males may match the offspring merely by chance and a degree of false paternal assignment is possible. We estimated that about one group male may have been falsely assigned paternity in this study. Allowing for this possibility does not substantially change the estimated level of extra-group paternity (from 38% to 40%).

Extra-group offspring were assigned to extra-group males with a high level of confidence (> 80%), except for three cases where the confidence of paternity assignment was only > 75%. Although this is probably sufficient when estimating levels of extra-group paternity in a population, it may be wise to exclude offspring for which paternity has been assigned only at the > 75% level from further analyses investigating inbreeding or fitness.

A previous study using CERVUS (Marshall *et al.* 1998) indicated that, even after assigning the mother, the presence of full sibs of the tested offspring might reduce the probability of resolving its paternity. As full sibs could occur among the Seychelles warbler candidate father population, this could account for some of the few cases where pairs of candidates could only be distinguished with relatively low confidence. However, none of the assigned males were offspring raised in previous years by the excluded group male (and therefore likely to be full sibs of the offspring tested); consequently we do not think that paternity has been falsely assigned to full sibs.

### Maternity

We have shown that both primary and helper females resident on the natal territory may reproduce. Overall, helper females produced 15% of offspring, however, most of the territories did not contain helpers. On multi-female territories 44% of helper females reproduced and accounted for 44% of the offspring. Mixed maternity was detected in 60% (3/5) of broods containing multiple offspring and multiple females, which enables us to reject the possibility that maternity was assigned to helpers through the incorrect classification of a female's status.

Previous work has shown that the Seychelles warbler has a cooperative breeding system with offspring remaining on the natal territory to become helpers but also suggested that joint nesting may be occurring occasionally (Komdeur 1992, 1994a,b). However, this has not been confirmed or quantified. The present study confirms that helpers do actually breed and shows that the cooperatively breeding system of the Seychelles warbler is a combination of joint nesting and helper-at-the-nest.

Studies on systems with female helpers-at-the-nest show that the primary female normally monopolizes breeding (Rabenold *et al.* 1990; Haydock *et al.* 1996; Legge & Cockburn 2000). A few studies have shown that female helpers may, very rarely, gain maternity (Lundy *et al.* 1998; reviewed in Vehrencamp 2000); however, the present study indicates that related helpers can gain substantial levels of maternity. The Seychelles warbler, therefore, shows an intermediate level of female reproductive skew that may, along with two studies on communal breeding bird species (Bertram 1992; Jamieson 1997), challenge the dichotomous 'helper-at-the-nest vs. joint nesting system' classification of female reproductive skew in avian cooperative breeders. This dichotomy is, however, supported in a study by Vehrencamp (2000) that showed the distribution of female reproductive skew to be bimodal, and by other recent DNA fingerprinting studies (Lundy *et al.* 1998; Legge & Cockburn 2000). The present study does, however, show that data used in Vehrencamp's study overestimates female reproductive skew in the Seychelles warbler and indicate that estimates of reproductive skew based on behavioural observations are unreliable. Further parentage studies on supposed helper-at-the-nest species are needed to assess whether intermediate levels of female reproductive skew are really so rare in these systems.

Within many cooperative species with related helpers reproduction with other group members is likely to be constrained by inbreeding avoidance (but see Keane *et al.* 1996; McRae 1996; reviewed in Pusey & Wolf 1996; Koenig *et al.* 1999). Future work on the Seychelles warbler will focus on the paternity of the offspring produced by helper females.

### Paternity

In the Seychelles warbler the assignment of paternity to extra-group males cannot be explained by mate switching, as mate fidelity is extremely high (Komdeur 1991, 1992). Therefore, the results of this study indicate that, compared to other bird species (Birkhead & Møller 1992; Petrie & Kempenaers 1998), a high level of extra-pair (extra-group) paternity (38% of young) occurs in the Seychelles warbler. The majority of *Acrocephalus* warblers species studied have low levels of extra-pair paternity (Henderson reed warbler, *A. vaughani taiti* = 0%, Brooke & Hartley 1995; great reed warbler, *A. arundinaceus* = 3.1% Hasselquist *et al.* 1995; sedge warbler, *A. schoenobaenus* = 8.4%, Buchanan & Catchpole 2000; or 9.8% Leisler *et al.* 2000; marsh warbler, *A. palustris* = 3.1%, Leisler & Wink 2000). Only the aquatic warbler (*A. paludicola*), a species with an unusual promiscuous mating system, shows levels of extra-pair paternity similar to the Seychelles warbler (39.1%, Leisler & Wink 2000).

In a recent review on extra-pair and extra-group paternity in cooperatively breeding birds, Cockburn (1998) suggested that their frequency is, with notable exceptions, generally low. Extra-pair paternity is generally rare in non-passerines, irrespective of the breeding system (Westneat & Sherman 1997). If only passerine species are considered, high levels of extra-group paternity are still rare (reviewed in Cockburn 1998; see also Lundy *et al.* 1998), occurring only in the Australian fairy-wrens (Brooker *et al.* 1990; Mulder *et al.* 1994) and now the Seychelles warbler. No clear pattern of subordinate male paternity is obvious within the passerines.

Thirteen per cent of offspring were from territories containing male helpers but only one offspring (1.8% of offspring) had paternity assigned to a territorial male helper, which was therefore an extra-pair, but not an extra-group, male. However, this singular case may be the result of the incorrect status assignment of the two males in the territory. Larger sample sizes are needed to confirm if in Seychelles warblers, as in some other cooperative species (reviewed in Cockburn 1998), male helpers do occasionally gain paternity. All assigned extra-group males were breeding males; therefore these males appear better able to gain fertilizations, but whether this is because of male–male competition or female choice is, at present, unknown.

The Seychelles warbler shows a low degree of genetic diversity in comparison to related species (Kappe 1998), and so the observed high level of extra-group paternity does not support the prediction that the frequency of extra-pair paternity will be related to genetic diversity (Petrie & Lipsitch 1994; Petrie *et al.* 1998). It also contrasts with the general finding that insular populations are characterized by very low levels of extra-pair paternity (Griffith 2000). However, the Seychelles warbler has a complicated cooperative breeding system and cannot be directly compared with socially

monogamous species. Why female Seychelles warblers seek extra-group paternity is the focus of current investigation.

### Conclusions

This study shows that a semiautomated parentage testing system, combining 14 polymorphic microsatellite markers, provides sufficient power to resolve parentage within the Seychelles warbler, despite the complications associated with this species' cooperative breeding system. The parentage analyses indicate that both the dominant primary female and the subordinate helper females may lay eggs within a single nest. There was a high frequency of extra-group paternity (40%), paternity could be assigned for all extra-group offspring, and it was always adult breeding males, often from adjacent territories, that gained the extra-group paternity.

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This study is part of a project to investigate the fitness consequences of mate choice using the Seychelles warbler as a model species. David Richardson is the postdoctoral researcher on the project with primary responsibility for the fieldwork and data analysis, Francine Jury is the research technician who completed the genotyping, and Karen Blaakmeer participated in the fieldwork as a graduate student. The study is a collaboration between the laboratories of Terry Burke (Sheffield, UK), whose interests include sexual selection and population genetics, and Jan Komdeur (Groningen, the Netherlands), who has previously shown the Seychelles warbler to be an excellent model for tackling fundamental questions in evolutionary ecology.

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