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Received 19 January 1995, accepted 25 April 1995

The Auk 113(2):511-516, 1996

DNA Fingerprinting Reveals Monogamy in the Bushtit, a Cooperatively Breeding Species

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Cooperative breeding systems, which are characterized by individuals contributing parental care to offspring that are not their own direct descendants, have received much attention in the past three decades (Hamilton 1964, Brown 1978, 1987, Emlen 1982). The aid-givers may be nonbreeding adults, in which case they are usually called "helpers," or they may be cobreeders that share reproduction with the other group members of the same sex.

The Bushtit (*Psaltriparus minimus*) is one of the first species of cooperative breeders ever described (Skutch 1935). Bushtits breeding in the Chiricahua Mountains of Arizona display notable variation with respect to breeding-group composition. On average one-third of the nests have more than two attending adults (Sloane in press). The helpers are predominantly unmated males, or birds of both sexes that have failed in earlier breeding attempts (Sloane 1992).

In approximately 19% of nests, helpers have been observed to join prior to or during the egg-laying stage, thereby providing the opportunity for genetic contributions via extrapair fertilizations or intraspecific brood parasitism (Sloane in press). In addition, the relatively high incidence of double brooding in these birds (Sloane unpubl. data) may give additional reproductive options to helpers, if those joining a nest after the first clutch later become breeders or cobreeders for the second brood. Molecular-genetic studies are required to investigate the parentage contributions made by helpers.

The development of DNA probes (e.g. Jeffreys et al. 1985) that detect high levels of genetic variation has greatly simplified parentage determinations and also permitted the assignment of pairs of animals to relatedness categories (e.g. Wetton et al. 1987, Burke et al. 1989, Westneat 1990, Piper and Rabenold 1992, Quinn et al. 1994, Jamieson et al. 1994). Overall, studies

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have indicated that extrapair fertilizations, alternate mating systems, and intraspecific brood parasitism can be important sources of reproductive success (Quinn et al. 1987, Gibbs et al. 1990, Westneat 1990, Jamieson et al. 1994).

Here we use multilocus DNA fingerprinting to investigate parentage in a population of Bushtits in the Chiricahua Mountains. By describing the characteristics of the breeding groups and determining the parentage, we attempt to elucidate reproductive patterns in the Bushtit breeding system. In particular, we evaluate the possibility that double brooding provides reproductive opportunities to helper Bushtits.

Methods.—The fieldwork was conducted from March to July 1992 (by J.P.B.) in the Cave Creek basin of the Chiricahua Mountains in Arizona (31°51'N, 109°15'W) on a population of Bushtits studied since 1986 (Sloane 1992). The site is an open oak woodland at an elevation of 1,700 to 1,800 m (for details of study area, see Sloane 1992).

Breeding behavior was monitored through nest searches, which were performed twice weekly by walking transects and listening for Bushtit calls. We were confident that all nests on the study site were detected during regular encounters with the study birds during searches. Adults observed feeding nestlings were captured in mist nets and were banded with unique combinations of three plastic color leg bands and a U.S. Fish and Wildlife Service numbered aluminum band for identification.

Blood samples of 50 μl were collected by brachial venipuncture and stored in lysis buffer (4 M Urea; 0.2 M NaCl; 0.1 M Tris-HCl, pH 8.0; 0.5% n-lauroylsarcosine; 0.01 M CDTA) to a blood-to-buffer ratio of 1:40. Samples were obtained from nine complete families, consisting of 20 adults and 59 nestlings. Nestlings were sampled at 12 to 13 days posthatching (four to five days before fledging), color-banded, and immediately returned to the nest.

Genomic DNA was extracted from blood in buffer using standard protocols: incubation with proteinase K, phenol/chloroform extraction, and ethanol precipitation. DNA was dissolved in 0.2 to 0.6 ml of TNE_2 and quantified by fluorometry and agarose gel electrophoresis.

We digested 10 μg of DNA for 4 to 5 h with *Hae*III ethanol-precipitated and redissolved in 20 μl of TNE_2. We combined 4 μg of sample DNA with 3 ng of a DNA cocktail of lambda digested with (1) *Bst*EII, (2) *Hin*DIII/*Eco*RI, and (3) *Hin*DIII as a control for possible differential mobility between samples (Galbraith et al. 1991), and loaded in a 0.8% agarose gel. Electrophoresis was performed at 1.2 to 1.5 V/cm for approximately 45 h. DNA was then transferred by Southern blotting to a membrane (Immobilon-N), which was air dried and then baked at 80°C for 1 to 2 h.

Blots were probed overnight at 65°C with radiolabelled Jeffreys probe 33.6 or 33.15 (Jeffreys et al. 1985) or the mouse probe pSP2.5RI (PER; homologous to *Drosophila* periodic locus; Georges et al. 1988), washed, and placed with film and a single intensifying screen at −70°C for 1 to 14 days. Following sequential probing with fingerprint probes, the membranes were probed with lambda, to provide molecular size markers.

The banding patterns of offspring were compared with those of the putative parents. Bands were considered to be the same if their relative intensities were similar (within 2× the intensity as determined by eye) and if the position of the band centers was within 1 mm. Band positions were assessed by measuring the distance to the nearest internal size marker (Galbraith et al. 1991).

After scoring, the band-sharing coefficient,

$$D = 2\,N_{AB}/(N_A + N_B), \qquad (1)$$

was calculated, where N_{AB} is the number of bands shared by both individuals, and N_A and N_B are the number of bands scored in lane A and B, respectively (see Wetton et al. 1987). Band-sharing coefficients range from zero, when no bands are shared, to one, when all bands are shared.

Results.—Six of the nine families studied were attended only by one pair of adults, a male and a female. In group P2, two males and one female were observed feeding nestlings. Since this nest was discovered during chick-rearing, the stage during which the helper joined, his identity is unknown. Nests 204 and 207 consisted of single pairs through incubation of the first clutch. The males at these nests disappeared soon after banding and did not feed nestlings. Two other males were observed following the female at nest 204 while she fed the nestlings, but neither male was observed bringing food to the nest. The female at this nest was successful at raising the young on her own, and left the nest after doing so. Nestlings were fed by the female and a helper male at nest 207 for the first clutch;

TABLE 1. Band-sharing coefficients ($\bar{x} \pm$ SD) of dyads of different relationships across three probes, along with the mean.

Relationship	n (dyads)	PER	33.15	33.6	Mean
Unrelated	268	0.172 ± 0.102	0.155 ± 0.082	0.219 ± 0.115	0.182 ± 0.073
Half-sibs	21	0.494 ± 0.095	0.450 ± 0.050	0.605 ± 0.050	0.516 ± 0.049
Full-sibs	145	0.621 ± 0.137	0.622 ± 0.110	0.690 ± 0.103	0.644 ± 0.082
Parent-offspring	123	0.589 ± 0.122	0.616 ± 0.188	0.646 ± 0.122	0.617 ± 0.096

this male then assisted the female with a second clutch.

The three probes used revealed highly variable banding patterns. The DNA fragment size ranges were: (PER) 2.2–13 kb; (33.15) 2.2–20 kb; and (33.6) 2.2–16 kb. The extent of duplicated detection of fragments between probes was as follows: 0.19 for PER vs. 33.15; 0.19 for PER vs. 33.6; and 0.21 for 33.15 vs. 33.6. The mean number (±SD) of scored fragments detected were: (PER) 18.7 ± 4.9; (33.15) 16.1 ± 3.9; and (33.6) 13.0 ± 4.8. Some fragments detected in individual offspring were not found in either parent ($n = 7$). No more than two were found in a single individual. These fragments, presumed to arise from mutations, occurred at a rate of 0.002 per fragment. The average band-sharing coefficient among unrelated adults was 0.172 for PER, 0.155 for 33.15, and 0.219 for 33.6.

In all 10 broods, nestlings were the offspring of a sexually monogamous pair. The band-sharing coefficients among full-sibling and parent-offspring dyads are in the range expected for first-order relatives (Table 1). Based on band-sharing coefficients, adults of breeding groups were unrelated. The coefficient between the two males at nest P2 was 0.277, between the female and her mate was 0.172, and between the female and the helper was 0.142. At nest 207, the band-sharing coefficient between the males was 0.259, between the female and the first male was 0.281, and between the female and the second male was 0.278. The probability that parentage was misassigned due to an undetected extrapair fertilization was 8.1×10^{-11}, or due to an undetected intraspecific brood-parasitism event was 3.9×10^{-27}.

Despite the presence of more than one male at nests P2 and 204, mixed paternity did not occur. The mean band-sharing coefficient, across the probes, between the assigned father (male 2) and the seven nestlings of nest P2 were 0.543, 0.511, 0.513, 0.614, 0.565, 0.517 and 0.502 compared with those between male 1 (helper) and the nestlings, which were 0.258, 0.245, 0.151, 0.256, 0.260, 0.293 and 0.180. Based on the numbers of novel bands and band-sharing coefficients, male 1 was excluded as a father.

The fingerprints of group 207 verify that the female at this nest was serially monogamous (Fig. 1). This helping male, or stepfather in this case, was excluded as the father of the first brood as the mean number of novel bands, across the three probes, per nestling was 19.1 ± 4.2. The stepfather male, which was mist-netted at the nest while feeding the first brood, fathered the second brood of three nestlings. The three nestlings of this brood had a mean band-sharing coefficient of 0.701 ± 0.045 with the second male. Nestlings of the first brood (N1–N7) are maternal half-siblings to the three nestlings (N8–N10) of the second. The 21 dyads (7 × 3) of half-siblings in Table 1 were derived from this family.

Discussion.—The DNA results show that in this breeding season the Bushtits studied were sexually monogamous and that no egg-dumping behavior (intraspecific brood parasitism) or extrapair fertilizations occurred. DNA fingerprinting has revealed sexual monogamy in other species: Fulmar (*Fulmarus glacialis*, Hunter et al. 1992); Willow Warbler (*Phylloscopus sibilatrix*, Gyllensten et al. 1990); and the cooperatively breeding Florida Scrub-Jay (*Aphelocoma coerulescens*, Quinn unpubl. data).

Sloane (in press) suspected that nests with more than one attending male could be genetically polyandrous due to mating opportunities arising from both the timing of joining (i.e. before egg laying) and a lack of mate-guarding behavior on the part of either male. In addition, there was evidence that females occasionally laid eggs in others' nests. These conditions are common to many plural cooperative breeders and may set the stage for alternative reproductive strategies (Curry 1988). Davies (1992) de-

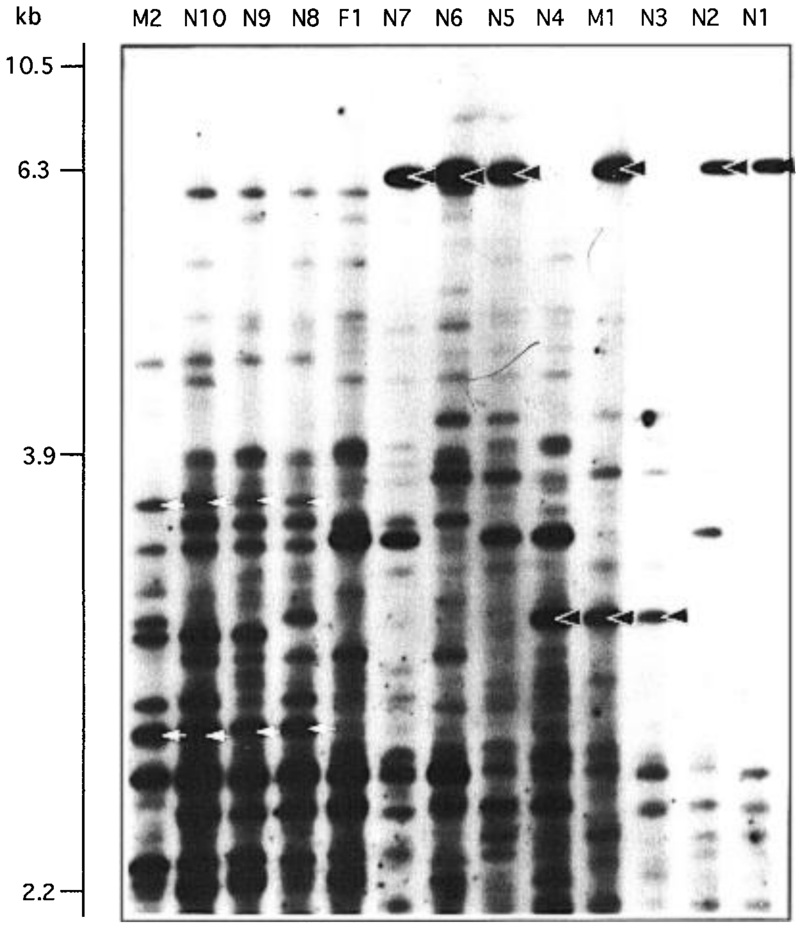

Fig. 1. DNA fingerprints of *Hae*III digested DNA from double-brooded nest 207 probed with PER. Molecular-size markers indicate approximate fragment size. Arrowheads indicate bands inherited by nestlings from father (white = male 1, black = male 2).

scribed a communal-mating system in Dunnocks (*Prunella modularis*) where paternity in trios is correlated with the percentage of exclusive mating access by one of the two males. Studies of other cooperative breeders have revealed two potential reproductive consequences of helpers. Rabenold et al. (1990) reported that "helper" Stripe-backed Wrens (*Campylorhynchus nuchalis*) are commonly cobreeders. Mulder et al. (1994) suggested that helper Fairy Wrens (*Malurus cyaneus*) have emmancipated female breeders, leading to high levels of paternity by non-group members. Although we cannot dismiss such reproductive consequences of Bushtit helpers, we found no DNA evidence in support of them.

Unlike the helper system of the Florida Scrub-Jay in which helpers are usually the offspring of one or both breeders (Woolfenden and Fitzpatrick 1984), Bushtit helpers examined here appeared to be unrelated to the group breeders. Between 1986 and 1990, one-third of the nests observed had multibird groups attending them. DNA evidence from our study indicates that helpers are not closely related ($r < 0.5$) to the rest of the breeding group; as such, kin selection is an unlikely factor in the evolution of the behavior in Bushtits.

Serial monogamy in the double-brooded nest suggests that an additional reproductive strategy may underlie the "helping" behavior. The "stepfather" might have been described as purely altruistic. However, reproduction was realized by this individual, inviting speculation into the role of breeder replacement in the evolution of helping behavior in the Bushtit. Similar reproductive tactics have been observed in secondary (unrelated) helpers of Pied Kingfishers (*Ceryle rubis*); these secondary helpers frequently assume primary breeding status by replacement and, thus, achieve the direct fitness benefits (Reyer 1991).

The response of replacement mates to unrelated offspring can be either full adoption, indifference, or infanticide. The probability of adoption increases significantly when the replacement takes place early in the breeding season and when a second brood is a possibility (Rohwer 1986). Given that there is a male-biased sex ratio and double brooding occurs in this population of Bushtits (Sloane unpubl. data), we might expect full adoption by males when they replace early in the season. Therefore, the second brood may provide a major source of direct fitness to helpers; helpers may be "waiting in the wings" while adopting the first brood. Double brooding can add another important dimension to the evolution of aid-giving behavior when indirect fitness benefits associated with helping kin are not involved.

Our study raises questions concerning the importance of double brooding in the evolution of cooperative breeding. Our data, which were obtained during a year of relatively low nesting density, suggest that significant reproductive opportunities may be realized by helpers when a second brood is possible. In years when nesting density is higher, the incidence of multibird nests would be more prevalent and the selective importance of alternative reproductive tactics enhanced. Under such conditions, reproductive advantages to helpers would increase.

Acknowledgments.—We would like to thank the Natural Sciences and Engineering Research Council for financial support through operating grants to B.N.W. and J.S.Q. Peter Dunn and Walter Piper made valuable suggestions on a previous version of this manuscript. We also thank E. Harrison and N. Staus for excellent field assistance. Thanks are extended to the staff and volunteers at the Southwestern Research Station in Portal, Arizona. J.P.B. received financial support from Sigma Xi (Grants-in-Aid of Research) and the American Museum of Natural History.

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Received 10 July 1995, accepted 2 October 1995.