

AN INVESTIGATION OF THE COMMUNAL BREEDING SYSTEM
OF THE
SMOOTH-BILLED ANI (*CROTOPHAGA ANI*)

By

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Abstract

In social systems, the distribution of reproduction among group members is termed reproductive skew. This study was intended to address the issues of reproductive skew within the communally breeding smooth-billed ani employing genetic data to complement behavioural data. The aims of the study were: 1) to develop smooth-billed ani specific DNA markers to be used in the assessment of parentage 2) to determine parentage of buried eggs and surviving offspring to assess whether a reproductive skew pattern is seen 3) to comment on adult relatedness within smooth-billed ani groups. Smooth-billed ani microsatellite DNA sequences were isolated and characterized for use in a parentage/kinship analysis. Lambda Zap Express was used to construct a library of recombinant phage which was screened with TG and AAT probes. Five loci were characterized with between 4 and 9 alleles with heterozygosity values ranging from 0.538 to 0.840. The combined total exclusionary power of the five loci was 0.8869. Offspring loss due to egg destruction in the form of burial has been observed in smooth-billed anis. The issue of reproductive skew as a result of egg burial was addressed by estimating parentage of offspring both buried and remaining in the incubated clutch using 5 species specific microsatellite DNA markers. Evidence for an egalitarian system exhibiting very low skew was found. The significant factors affecting the potential for bias were laying order, laying timing and the number of breeding females. The relatedness of members within a social group may affect the amount of reproductive skew observed. Adults within nesting groups were tested for evidence of relatedness using the pedigree analysis program KINSHIP. Ten percent of dyads showed significant relationships. Low

incidence of relatedness provided evidence that smooth-billed ani groups do not contain a large number of young remaining at the natal nest nor was there evidence that regular dispersing within sibling units occurs. There was some evidence that a young male remained on his natal territory while a young female dispersed to a neighbouring territory. Low relatedness between adults and very low reproductive skew were consistent with the prediction of an egalitarian system

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General Introduction

Most bird species breed in behaviourally monogamous pairs that tend to their own young. Social breeding in bird species involves more than a single male-female pair assisting in the production of young at a single nest (Koenig 1981). The terms co-operative or communal breeders have often been used synonymously to describe systems in which breeding adults are helped at the nest by birds other than their mates (Brown 1978).

A distinction can be made between two extreme social systems: helper at the nest and communal-joint nesters. Helper at the nest systems involve one breeding pair with non-breeding helpers (usually offspring) sharing nesting duties but not actually reproducing. Helpers in this system raise young that belong to others as described in such species as Florida scrub jay (Quinn et. al 1999), white winged chough (Rowley 1978) and red-cockaded woodpecker (Haig 1994). The less common communal system (communal-joint nesters) involves groups with more than a single male-female pair breeding and multiple females may lay eggs in a single shared nest. The group may contain a number of monogamous pairs or a combination of males and females engaging in a range of polygynandrous relationships. Duties such as nest building, chick feeding and defense are generally shared by all members and all individuals have some access to reproduction. This system has been described in species such as acorn woodpecker (Koenig and Pitelka 1979), pukeko (Craig 1980), common moorhen (Gibbons 1986), guira cuckoo (Macedo 1992), groove-billed ani (Skutch 1959; Vehrencamp 1977) and smooth-billed ani (Davis 1940; Loflin 1983).

In any social system, a balance between co-operation and conflict will evolve. In avian social systems, co-operation includes the sharing of nesting duties while the conflict centres on reproductive opportunities. There needs to be a partitioning of reproduction between group members. The degree of reproductive bias or the distribution of direct reproduction among same sex members of a social group has been termed reproductive skew (Emlen 1996; Keller and Reeve 1994). A system with high skew is characterized by an individual or pair gaining all of the reproduction in a group, while in a low skew system, reproduction is shared among group members. Species can fall anywhere along a continuum from high to low skew.

Much attention has been given to the conditions under which co-operation or reproductive sharing should occur within a group. The traditional model designed to address reproductive skew in co-operative nesters is outlined by Vehrencamp (1983a,b) and called the optimal skew theory. This theory assumes that selection acts on dominant members of the group to secure more benefits for themselves at the expense of subordinates. Therefore, the optimal skew theory assumes that the dominant individual in the group can and will control the reproductive opportunities of the subordinate. This theory is affected by the benefits of group breeding, the dispersal options available to the subordinate, the genetic relatedness of individuals and the fighting ability of the subordinates (Keller and Reeve 1994). This model works well to describe a helper at the nest system. If offspring have limited dispersal opportunities (decreased adult mortality, increased competition for good territory), they may have little chance of succeeding on their own. Offspring may then be forced to make the best of a bad situation and remain in

their natal territory (Emlen 1996). If parents can benefit from the added helper, they may tolerate the offspring's presence but monopolize all reproduction. Because of the high relatedness between the dominant and the subordinate, this helper stands to gain indirect success due to kin selection. This can be viewed as incentive to stay without conflict. Under these conditions, a dominant has the leverage to enforce complete bias. The theory predicts high skew when relatedness is high. If breeding within a group provides benefits to a dominant individual and a subordinate has dispersal opportunities and is not constrained to remaining in the natal territory, dominant individuals may allow subordinates to breed in order to retain them within the group (Clutton-Brock 1998). If individuals are not related, indirect fitness gains as a result of tending to kin will no longer be available to the subordinate and the dominant will have to concede a certain amount of reproduction in order to gain the benefits of group breeding. Here there is little leverage to enforce strong bias. This should result in a system with lowered skew, termed an egalitarian system.

Therefore, the optimal skew theory describes the amount of reproduction that a dominant should allow to a subordinate in order to gain group benefits while still maximizing its own reproduction. Vehrencamp (1980a,b) acknowledges that the optimal skew theory is limited in its application to groups under more complicated circumstances such as a system in which bias would be preferable for a dominant but is not enforced perhaps because of equal fighting ability among members or impossible physiological controls.

It is unclear within many vertebrate communal systems how much control a dominant individual actually has over a subordinate's ability to reproduce. Clutton-Brock (1998) suggests that there is no unequivocal evidence that dominant female vertebrates make reproductive concessions to subordinates in return for help. The incomplete control theory was put forth to address group systems in which there is no assumption of dominant control over subordinate reproduction (Cant 1998). Asymmetry in dominance may exist within a group, but it is assumed that neither player has the power to permit or prohibit the reproduction of the other. In some cases, breeding with additional females may not be beneficial to a dominant while allowing a subordinate to remain may offset the costs of trying to expel them. If an individual cannot control subordinate breeding, brood size may increase past the optimal level for offspring survival thereby negatively affecting reproductive output (Cant 1998).

Although the dominant individual may not be able to exert complete control over subordinate reproduction, there may be some means of undermining the breeding of other females. This circumstance may lead to competitive behaviour at the nest resulting in such activity as infanticide (Johnstone and Cant 1999; Cant 2000; Macedo and Melo 1999) or egg destruction (Macedo 1992; Mumme et. al. 1983; Vehrencamp 1977). This behaviour could have the function of biasing the surviving clutch in favour of a particular member. Where dominants do not have full control over subordinates, the amount of subordinate breeding likely represents the results of conflict between the two (Clutton-Brock 1998). The theory of incomplete or absent control by dominants may be more suitable than the optimal skew theory for describing many communal-joint nesting

species. Competition between females at the nest is suggestive of a struggle for a limited amount of reproduction.

Study Species

Smooth-billed anis are a communally breeding species of the family Cuculidae. This family contains over 200 species that range from monogamous breeders to host-specific brood parasites. Species of the sub-family Crotophaginae (including the greater ani, groove-billed ani and guira cuckoo) are social breeders (Davis 1942). Smooth-billed anis are glossy black, grackle-sized birds with long tail feathers. Their preferred habitat is open grassland and they feed predominantly on insects and spiders. They forage in groups or pairs by hopping through the open grass stirring up insects.

Smooth-billed anis are found in the Caribbean Islands, Costa Rica, South America and are moving north into Florida. They are considered highly communal having as many as 7 females laying their eggs in a single shared nest (Loflin 1983). They are also known to nest in single pairs. Reproduction can occur at any time of year but most breeding coincides with the rainy season (Aug-Jan). Nests are composed of twigs forming a cup shaped structure 17-26 cm in diameter and 5-9 cm in depth (Quinn and Startek-Foote in press). Nesting sites range from small bushes to large trees and nests are usually protected by thorny branches.

In group nests, eggs are often found beneath a layer of leaves, buried at the bottom of the nest. These eggs are not turned and will not hatch. This egg burial results in

considerable offspring loss and appears to be the result of female-female competition. In groove-billed anis and guira cuckoos, female competition results in eggs being removed from the nest. This breeding conflict may be indicative of a system operating under incomplete or absent dominant control. Egg destruction in groove-billed anis is interpreted as a struggle for reproduction between breeding females and the outcome is a final clutch skewed in favour of the destroying female (Vehrencamp 1977). It is as yet unresolved whether the egg burial seen in smooth-billed anis is comparable to the destruction seen as a result of egg tossing and whether it results in a similar reproductive bias.

To date, a preliminary assessment of the smooth-billed ani breeding system has been made for a Florida population from behavioural data alone (Loflin 1983). This present study was intended to address the issue of reproductive skew within the communally nesting smooth-billed ani employing genetic data to complement behavioural data.

The aims of the study were:

- 1) To develop smooth-billed ani specific DNA markers to be used in the assessment of parentage.
- 2) To determine parentage of buried eggs and surviving offspring to assess whether a reproductive skew pattern is seen.
- 3) To comment on adult relatedness within smooth-billed ani groups, as the degree of relatedness between group members is hypothesized to affect the degree of bias seen.

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Chapter 1

The Characterization of Microsatellite Loci in Smooth Billed Anis

Abstract

Microsatellite DNA sequences from smooth-billed anis (*Crotophaga ani*), were isolated and characterized for use in a parentage/kinship study involving communally nesting groups of smooth-billed anis in Puerto Rico. I used Lambda Zap Express (Stratagene) to construct a library of 350,000 independent recombinant phage and screened it with TG and AAT probes. In total, 85 positive clones (TG-73, AAT-12) were isolated. Primer pairs were designed for 17 sequences and five polymorphic loci were found (3-TG, 2-AAT). The five loci were characterized with between four and nine alleles and heterozygosity values, based on the genotypes of 25 presumably unrelated individuals, ranged from 0.538 to 0.840. Two loci (ANI9546 and ANI500C14) showed deviation from Hardy-Weinberg equilibrium that was likely due to family grouping (genetic subdivision) or nonamplifying (null) alleles. Both loci showed Mendelian inheritance within known parent-offspring relationships. The combined total parental exclusionary power (with neither parent known) of the five loci was 0.8869.

Introduction

The most common mating system observed in bird species involves a behaviourally monogamous pair that breeds, protects a nest and tends to young together. Communal joint-nesting is an unusual breeding system adopted by approximately 3% of avian species. In this system, a group of adults builds a single nest that will contain all of the eggs laid and they will protect and tend to the young as a group. Communal joint-nesting behaviour has been described in such species as pukeko (Jamieson et. al. 1994), acorn woodpecker (Mumme et. al. 1988), guira cuckoo (Macedo 1992), groove-billed ani (Vehrencamp 1978) and smooth-billed ani (Loflin 1983).

Observed nesting groups of smooth-billed anis range from a single pair to 12 adults and nests containing upwards of 30 eggs (present study). Not all eggs laid in a single nest necessarily hatch. Female-female competition at the nest may result in the destruction of other females' eggs through egg burial under nesting material. Therefore, depending upon which adult's eggs remain in the final incubated clutch, there is opportunity for skew in reproductive success between adults within a nesting group.

To estimate the reproductive success of the adults involved, one must assign parentage of the offspring between the nesting adults. In smooth-billed anis, this has been estimated through field observations alone (Loflin 1983). The aim of this study was to isolate genetic markers that could be used to estimate the relationships (parent-offspring, sibling) of the adults and young within a communally nesting group.

Microsatellite DNA sequences have become the genetic marker of choice for studying population genetics, parentage and kinship (Queller et. al. 1993). They are

tandem repeats, less than five base pairs in length (Tautz 1989; Bruford and Wayne 1993) and are found throughout the vertebrate genome (Tautz and Renz 1984). Differences in allele lengths are due to variation in the number of repeat units (Tautz 1989). Microsatellites follow Mendelian inheritance and alleles are codominant. Due to their highly polymorphic nature and amplification using PCR, they are ideal for parentage analyses, particularly when samples yield small amounts of DNA (e.g. tissue from buried or destroyed eggs) (Weber and May 1989; Tautz 1989). One drawback of microsatellites compared to DNA fingerprinting is the effort required to isolate species-specific markers.

The use of cross-species primer pairs to assess microsatellites can save both time and expense. The chance of successful amplification is linked to the evolutionary distance between the two species. The more closely related two species are, the more likely it is that primer regions will be conserved and that those loci that do amplify will be polymorphic (Primmer et. al. 1996).

Cross-species amplification was attempted in smooth-billed anis using microsatellite primers isolated in the yellow warbler (*Dendroica petechia*) (Dawson et. al. 1997), great spotted cuckoo (*Clamator glandarius*) (Martinez et. al. 1998), common cuckoo (*Cuculus canorus*) (Gibbs et. al. 1998) and guira cuckoo (*Guira guira*) (Strange 2000). All primer pairs (except one great-spotted cuckoo and one common cuckoo pair) amplified a product in smooth-billed anis but the patterns were monomorphic. The other two pairs did not amplify a product.

Compared to mammals, microsatellites occur with much lower frequency in the avian genome. This is likely due to the smaller genome size and a decrease in the amount

of noncoding DNA (Primmer et. al. 1997; Wagemann et. al. 1981). For this reason, the isolation of microsatellite DNA sequences in birds tends to require an enrichment protocol to increase the proportion of clones with microsatellites or the screening of a large number of clones. The use of a bacteriophage cloning vector, Lambda Zap Express (Stratagene), for developing microsatellites in bird species has proven quite successful (Hughes et. al. 1997; 1998a; 1998b; Piertney and Dallas 1997). This system greatly increases the number of clones that can be screened at any one time; therefore, we used this approach for the isolation of species-specific microsatellites for smooth-billed anis.

Materials and Methods

Constructing a Library

Smooth-billed ani blood and tissue samples were collected in southwest Puerto Rico by jugular venipuncture. DNA was extracted from blood using a saturated salt extraction protocol while tissue taken from buried eggs was extracted using a standard phenol/chloroform procedure. Two μg of DNA, from each of five unrelated individuals, were combined and digested with *Sau 3a*I. The digested DNA was size fractionated by electrophoresis through a 2% agarose gel to separate the fragments into two size ranges (200-350bp) and (350-500bp) and isolated using a QIAquick Gel Extraction Kit (QIAGEN Inc. Canada). The two size ranges were isolated to allow for easy detection of concatamers, more than one insert per vector, prior to the sequencing stage.

Ligation of the fragments into Lambda Zap Express (Stratagene) was carried out using Stratagene's DNA Ligation Kit at a 2:1 insert to vector molar ratio. The ligation was packaged for infection using Gigapack III Gold Packaging Extract (Stratagene). The ratio of recombinant to nonrecombinant plaques was assessed using blue and white colour selection by alpha-complementation using (IPTG, X-Gal) and was found to be 8:1. The final library was estimated to contain 350,000 independently derived recombinant phage.

Screening the Library

The XLI-Blue MRF strain of *E. coli* (Stratagene) was used for all infections prior to the excision stage. The entire library was plated at 25,000 pfu per 150mm plate, with NZY medium, for the primary screening. All subsequent screenings were plated at 100-1000pfu on 100mm plates.

Plaque lifts were performed using 132mm/82mm diameter nylon membranes (Hybond N- Amersham-pharmacia biotech). Filters were marked to orient the plate to the membrane and ultimately to the autoradiograph. Lifts were performed on chilled plates leaving the membrane in contact with the plate surface for one minute. The filters were then passed through a series of trays in order to denature the DNA (1.5 M NaCl, 0.5 M NaOH / 5 min.), neutralize (1.5 M NaCl, 0.5 M Tris-HCl / 5 min.) and rinse (2X SSC / 30 sec.). Membranes were air dried and baked at 80° C for two hours.

Probing

Membranes were screened using two different probes, a (TG) variable length and (AAT)₁₀. Prehybridization took place for two hours using Westneat's solution (7% SDS, 0.001M EDTA, 0.25M Na₂ HPO₄, 1% BSA). The TG probe was labeled using random primer extension with alpha ³²P dCTP and Klenow fragment (Oligolabeling Kit - Amersham-pharmacia biotech) while the AAT probe was endlabeled with T4 polynucleotide kinase (gamma ³²P dATP). Hybridization was carried out overnight at (65°C - TG) and (35°C - AAT).

Filters were washed with (2X SSC, 0.1% SDS) and were sealed in seal-a-meal bags. Phosphorescent paint was used to orient the filters to the autoradiograph before

aligning to the plate. Film was exposed to the filters overnight (Kodak X-Omat Blue XB-1). Positive signals were lined up with plaques and plugs (2mm diameter) were stored in SM buffer at 4°C.

From the primary probing, 400 variable intensity TG and 12 positive AAT plugs were collected. Second and third probings were carried out to isolate the single plaque that resulted in a positive signal. The aim of the final probing was to verify that the target plaque was pure. In total, 73 TG and 12 AAT plugs were taken through second and third probings.

Inserts were recovered within the plasmid (pBK-CMV) using Stratagene's helper phage (Ex-Assist) and excision protocol (Short et. al. 1988). Forty-nine inserts (41-TG, 8-AAT) were recovered and prepared for sequencing using a standard alkaline lysis protocol (Sambrook et. al. 1989). To check the size and quality of inserts, DNA samples were digested with PST I and HIND III and run on a 1.2 % agarose gel. For samples in which digestion was unsuccessful, a QIAamp Tissue Kit protocol (QIAGEN) was used to remove protein contamination. At this point, recombinants with double inserts could be identified when inserts larger than the target fragment were seen. In total, 17 double inserts were seen, 15 of which were in the 200-350bp size range. These samples were still utilizable. A Sau 3a1 cut site separated the 2 fragments and primers could be designed for both microsatellite sequences (provided there were sufficient flanking regions). Sequences that contained a Pst I or Hind III restriction site, gave two smaller bands when run on an agarose gel as a result of the target band being cut into two pieces.

Thirty-nine clones (31-TG, 8-AAT) were sequenced in one direction by the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University, using a T3 primer (Stratagene). Primer sets were designed for 17 sequences (12 -TG, 5- AAT) using Oligo (Primer Analysis Software 4.06, Rychlik 1992). Primer pairs were chosen with a T_m within the (56 - 60° C) range avoiding complementary sequence between the forward and reverse primers. Oligonucleotide primers were constructed by the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University.

Genotyping

Ten presumably unrelated smooth-billed anis were used for the initial assessment of locus polymorphism. If all 10 individuals were homozygous for the same allele, the amplified locus was presumed to be monomorphic. For loci which proved to be polymorphic, allele frequencies, heterozygosity values and PIC values were calculated based on the genotypes of all sampled adults and of representative young from nests in which no adults were caught (N=25). All PCR reactions were carried out using thin walled tubes. Reactions were performed in a 10 µl volume using either a PTC 100 or PTC 200 DNA Engine Thermal Cycler (MJ Research Inc.). PCR reactions used 25 ng DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM unlabelled reverse primer, 0.16µM unlabelled forward primer, 0.04 µM labeled forward primer, 0.5U Taq polymerase and accompanying buffer (Gibco BRL). Primers were endlabeled using T4 polynucleotide kinase and gamma ^{33}P dATP. PCR cycles used were: 3 cycles of denaturing (94°-2 min),

annealing (52-56°-30 sec), extension (72°-30 sec); 27 cycles of (94°-15 sec), (anneal temp-30 sec), (72°-30sec), followed by a final (72°-2 min).

Amplification products were resolved on 6% denaturing polyacrylamide gel (Biorad Laboratories- 40% acryl:bis solution, 19:1) run at 65 watts for 3 hours. After drying, gels were put on phosphorimager screens and after overnight exposure, were scanned and visualized using the PhosphorImager and ImageQuant software (Molecular Dynamics). Product sizes were determined by running a pUC 18 sequencing ladder (Amplicycle Sequencing Kit- Perkin Elmer) and a locus specific clone as reference. Samples with known allele sizes were also run on every gel as reference for genotyping. All samples, including 26/29 tissue samples from buried eggs, were consistently amplified and genotyped.

Results and Discussion

Of the 17 primer pairs designed, five amplified polymorphic loci (Table 1.1). Allele frequencies, heterozygosity values and PIC values were calculated based on the genotypes of all sampled adults and of representative young from nests in which no adults were caught (N=25).

Smooth-billed ani loci showed from four to nine alleles (Figure 1.1). Alleles from two loci (ANI9546 and ANI450B2) with their characteristic stutter patterns are shown in Figure 1.2. Loci with heterozygosity values < 0.5 are in general not very useful for parentage studies (Marshall et. al. 1998). All observed heterozygosity values for the loci found in this study were greater than 0.5, with the two AAT loci showing the highest values (Table 1.1). Loci with low heterozygosity values can still produce useful information if they provide any ability to exclude potential parents.

Another value indicating the informativeness of a locus is the polymorphic information content value (PIC). While heterozygosity indicates what proportion of the population has 2 alleles at a given locus, the PIC value gives a measure of allelic diversity. A population with all individuals sharing the same 2 alleles at a locus, would have a high heterozygosity level but a low PIC value. A value >0.5 is considered highly informative while a value $< .25$ is only slightly informative (Botstein et. al. 1980). All five smooth-billed ani loci show PIC values > 0.5 . One pair of guira cuckoo primers did amplify smooth-billed ani samples but all individuals showed the same allele pattern indicating perhaps that the primers were amplifying two monomorphic loci. A Hardy-Weinberg equilibrium test was performed for each locus (CERVUS- Marshall et. al.

1998). This test compares the expected frequency of genotypes in a population under random mating to the observed genotypes seen in the sample population. Two loci (ANI450B2 and ANI450C1) were found to be not significantly out of H-W equilibrium even when the genotypes of the entire population were tested (adults and offspring). Locus ANI500C5 was in H-W equilibrium when combined adult genotypes were tested, but was found to be out of equilibrium when the genotypes of the entire population were included. Two loci (ANI9546 and ANI500C14) were found to be out of equilibrium under both circumstances. Deviations from H-W equilibrium are usually indicative either of a population that is genetically subdivided (family groups) or the presence of null alleles.

Null alleles are those which contain mutations in one or both priming sites, leading to the prevention of amplification. A heterozygote containing this allele will appear to be homozygous for the amplified allele and those homozygous for the null will not amplify at all (Callen et. al. 1993). The existence of null alleles is a matter of concern for population studies (Summers and Amos 1997; Primmer et. al. 1995; Pemberton et. al. 1995) and one must be careful to identify such loci for they can greatly affect relatedness estimates. The characteristic signature of the presence of null alleles at a locus is a mismatch between a known parent/offspring and individuals that will not amplify. Both loci ANI9546 and ANI500C14 show an excess of homozygotes giving them positive null frequency estimation values (CERVUS- Marshall et. al. 1998). Both loci show Mendelian inheritance within known parent/offspring relationships and all samples amplified,

suggesting perhaps that the excess homozygotes were due to genetic subdividing or small sample size.

Dinucleotide microsatellites are expected to be the most abundant repeat unit found in the genome with trinucleotides and tetranucleotides less so (Beckman and Weber 1992). Although less common, tri and tetra repeats are thought to be generally more polymorphic. In this study, few AAT clones were isolated (12). Eight samples were sequenced and five primer sets were made. Of these, two were polymorphic and one sequence contained a long repeat but insufficient flanking regions, making primer design impossible. Screening with an AAT probe rather than TG isolated more polymorphic sequences per primary positive (2/12 AAT, 3/73 TG).

Cross-species amplification has been quite successful in some related passerine species (Primmer et. al. 1997). Of the cross-species primers tried, the guira cuckoo was the only species that proved to be polymorphic in smooth-billed anis. This result is not surprising, as the two species are the most closely related of those tried (Hughes 1996). One of the smooth-billed ani microsatellites isolated in this study (ANI450B2) is also polymorphic in the guira cuckoo (Strange 2000).

The heterozygosity and PIC values for the five microsatellites isolated in this study indicate that these loci are informative for parentage analyses. When combined, these loci have a parental exclusion probability of 0.887 (the probability of excluding a single randomly chosen unrelated individual from parentage with neither parent known). They will therefore be useful for making an initial assessment of the breeding behaviour of the smooth-billed ani.

When studying a population which includes potential family members (parents, offspring, siblings) one must keep in mind how these relationships can affect the allele frequencies and heterozygosity values seen at microsatellite loci. Although a genetic analysis program such as CERVUS will give statistical values for all loci, one must still pay attention to known field data and identify potential biases within the sample population. The CERVUS program calculates allele frequencies and heterozygosity based upon the genotypes of the given population. If the genotypes of many related individuals, for example parents and offspring, are used to calculate allele frequencies, then rare family alleles may become over-represented in the population. If parents are homozygous for common alleles, the heterozygosity level for the population may be underestimated.

Additional microsatellite DNA sequences will need to be isolated in order to build a clear picture of the relationships between chicks and adults in this communal system. Although trinucleotides and tetranucleotides may be less common in the genome than dinucleotides, the Lambda Zap Express vector system allows for the screening of a large library thereby making their isolation more efficient.

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Table 1.1: The characterization of microsatellite loci polymorphic in smooth billed anis. Values were calculated from a sample of 25 presumably unrelated individuals using the genetic analysis program CERVUS (Marshall et. al. 1998).

LOCUS	PRIMERS	SIZE bp	NO. OF ALLELES	H _o	H _e	PIC	ANNEAL TEMP °C
ANI9546 (TG)	F-ttaaactatagaaggcggaatg R-acacgcagccgcagcca	202	5	0.538	0.721	0.653	58
ANI500C5 (TG)	F-atcttcagtagtacatgtgc R-tgtgtaatagagcagccag	330	4	0.778	0.723	0.643	55
ANI500C14 (TG)	F-ggggatggtttatttgagg R-ggatacgggtggggcct	200	7	0.538	0.744	0.730	56
ANI450B2 (AAT)	F-gcttcttttaggattaaccgt R-cctggttttagcactgac	226	9	0.821	0.801	0.778	52
ANI450C1 (AAT)	F-gttgttgctgcttttgtttcc R-tgggcaggagggtctctc	169	6	0.840	0.788	0.737	53

H_o - Observed heterozygosity

PIC - Polymorphic information content

H_e - Expected heterozygosity

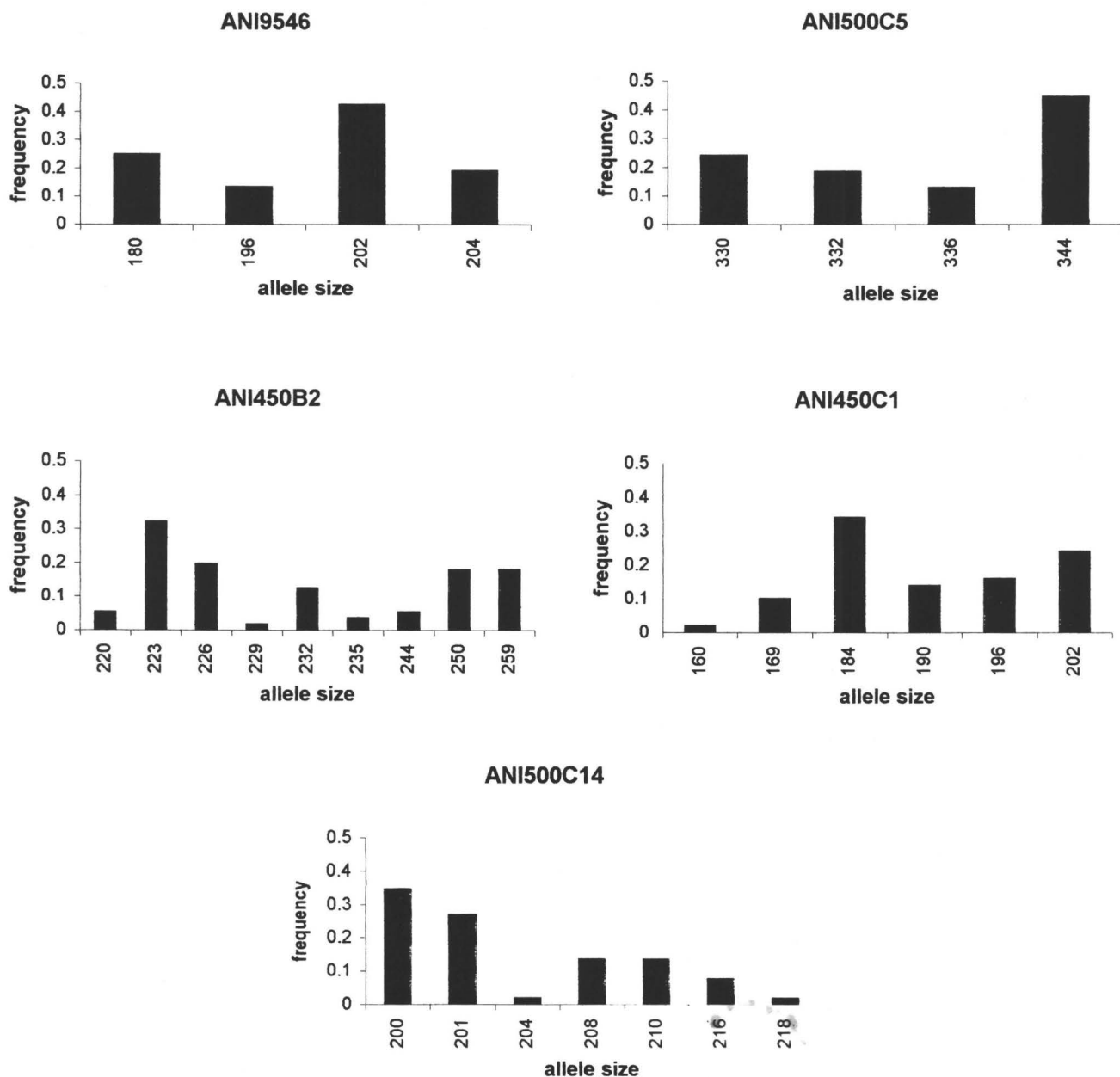


Figure 1.1: Allele sizes (bp) and frequencies from five smooth-billed ani microsatellite loci calculated from a sample of 25 presumably unrelated individuals from a Puerto Rican population.

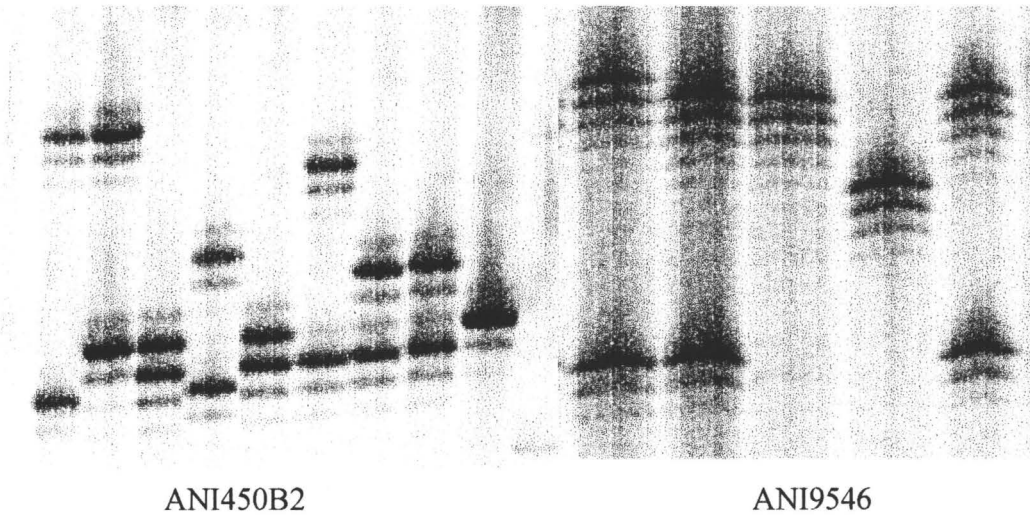


Figure 1.2: Two smooth-billed ani microsatellite loci (ANI450B2- AAT) and (ANI9546-TG) showing a range of allele sizes and characteristic stutter pattern.

Chapter 2

An Assessment of Reproductive Bias in the Communally Breeding smooth-billed ani (*Crotophaga ani*)

Abstract

Offspring loss due to egg destruction in the form of burial has been observed in the communally breeding smooth-billed ani (*Crotophaga ani*). In other species of the sub-family Crotophaginae (groove-billed ani and guira cuckoo), egg destruction occurs as a result of female-female competition and can result in reproductive bias favouring the last laying female. The focus of this study was to address the issue of reproductive skew in smooth-billed anis in relation to egg burial using both genetic and behavioural data. Parentage was assessed in both single pair and group nests to investigate the mating system, ownership of buried eggs and those eggs in the incubated clutch. Evidence for low reproductive skew was found. Although last laying females tended to have just as much or more success as early layers, it appeared that the amount of skew was minimal.

The significant factors affecting the number of eggs buried and therefore the amount of skew were laying order, laying timing and the number of breeding females in the group. In communal species, which exhibit egg tossing, the last laying female suffers minimal to no loss due to egg destruction. In smooth-billed anis, the last laying female in larger group sizes (3-4 females) risked losing eggs to accidental burial due to crowding.

There was evidence for monogamy, polygamy (extra-pair fertilizations) and brood parasitism in both single pair and group nests.

Introduction

True communal nesting has been defined as a system in which more than one female lays eggs in a single shared nest (Koenig and Pitelka 1979). Communal groups may consist of monogamous pairs or combinations of males and females. Various duties are shared such as nest building, defense and the feeding of chicks. This system has been described in such species as guira cuckoo (Macedo 1992), acorn woodpecker (Mumme et. al. 1983), pukeko (Craig 1980), groove-billed ani (Vehrencamp 1977), and smooth-billed ani (Loflin 1983). A variety of mating systems have been adopted by communal breeders. Anis are thought to exhibit monogamy within stable pairs (Vehrencamp 1977; Loflin 1983) while evidence has been found for polygamy in other group nesters (Mumme et. al. 1983; Quinn et. al. 1994).

In communal-joint nesting groups, multiple adults have the opportunity to gain reproductive success from the same nesting attempt. The distribution of direct reproduction among same-sex members of a social group has been termed reproductive skew (Vehrencamp 1983). Systems in which reproduction is shared among members are described as being egalitarian or as having low reproductive skew. The factors contributing to the distribution of reproduction within a group have been the focus of many studies. The optimal skew theory states that the dominant member of the group should maximize their own fitness at the expense of any subordinates (Vehrencamp 1983a,b; Keller and Reeve 1994). It is assumed that the dominant has complete control over subordinate reproduction. If a subordinate has few dispersal options and is related to

the dominant, thereby able to gain indirect fitness from raising kin, the parent can gain benefit from the helper's presence yet monopolize all reproduction. However, if the dominant benefits from a group situation and the subordinate has dispersal opportunities, the dominant may allow some limited subordinate reproduction as incentive to remain in the group (Keller and Reeve 1994).

In many social systems, control of breeding by dominants may actually be incomplete or entirely absent (Cant 1998; Clutton-Brock 1998). Without this control, dominant individuals cannot maximize their own fitness at the expense of subordinates. Subordinates stand to gain reproductive success not as a controlled incentive but because of a lack of control. The fitness of dominants can now be negatively affected by the breeding of other females. Brood size may be negatively correlated with offspring survival (Cant 1998). If all females in a group lay their maximum number of eggs, as brood size increases, one might expect offspring survival to decrease. These circumstances increase the probability for competitive interactions between breeders in the same nest. To maximize their own fitness, individuals will need to undermine the breeding attempts of others.

When the communal breeding behaviour of *Crotophaga* species was first described (Davis 1940; Skutch 1959), field observations detailed the occurrence of wasted reproduction in the form of broken eggs beneath nests or eggs buried under nesting material. It was assumed that the loss was due to laying prior to nest completion or perhaps the accidental movement of eggs during nest maintenance. Recently, it has been discovered through observation that some communally nesting birds will actively

roll or carry eggs out of the nest (Vehrencamp 1977; Mumme et. al 1983). Females appear to deliberately destroy the eggs of other breeders prior to laying their own. Although all group members have an opportunity to breed and therefore exhibit a system with overall low reproductive skew, the result of this egg destruction can be a final clutch biased in favour of the egg-destroying female. In groove-billed anis, Vehrencamp (1977) found that the last laying female in 2-female nests tended to own 63% of the final incubated clutch. Egg ownership was determined through field observations and egg characteristics. There is no indication that anis can identify their own eggs therefore, once a female begins to lay, she can no longer afford to destroy eggs for she may risk losing her own.

Smooth-billed anis are a communally breeding species found in the Caribbean Islands, Costa Rica, South America and Florida (Quinn and Startek-Foote in press). They are in the order Cuculiformes (family Cuculidae; sub-family Crotophaginae) with groove-billed anis and guira cuckoos. They nest in pairs or groups containing up to 12 individuals and have been presumed to breed monogamously (Loflin 1983). During the breeding season, adults in both single pairs and groups bring leaves into the nest to serve as lining before laying eggs. In group nests, eggs are often found trapped in layers at the bottom of the nest separated by leaves. These buried eggs are not turned or incubated and do not hatch. Only a percentage of the total eggs that are laid will remain in the incubated clutch and hatch. Loflin (1983) found that 37.3% of all smooth-billed ani eggs found in 41 nests in a Florida population were lost due to burial. This was higher than all other forms of destruction (predation, weather) combined. Although single pair nests were also

lined with leaves, egg burial was not observed. Egg burial may be a form of female-female competition comparable to the egg tossing seen in groove-billed anis and guira cuckoos.

This study was intended to investigate the communal smooth-billed ani breeding system using both genetic and behavioural data by estimating the parentage of buried eggs and surviving offspring and address the questions:

- 1) Are smooth-billed groups composed of monogamously breeding pairs?
- 2) How does egg burial affect the reproductive success of females breeding within a group and is this reproductive skew predictable by order of egg laying?

Methods

Field Methods

Study site

The smooth-billed ani population studied was located at the Cabo Rojo and Laguna Cartagena National Wildlife Refuges in southwest Puerto Rico. The southwest portion of the island has a dry sub-tropical climate. The Cabo Rojo refuge (587 acres) was once pastureland and is now second growth dry scrub with stands of large mesquite, tamarind and pithecellobium bushes. Various species of cactus are found sporadically and guinea grass covers the open fields. Laguna Cartagena (794 acres), approximately 10 km from the Cabo Rojo site, contains a large freshwater lagoon surrounded by scrubland used for intensive cattle grazing. Sugar cane farms line the refuge. Both refuges contain very open, disturbed habitat, preferred by anis (Loflin 1983; Quinn and Startek-Foote in press).

Sampling

The aim of the field season was to sample breeding adults, follow breeding progress and sample all offspring. The samples used for analysis in this study were collected Sept-Dec 1998. Additional adults trapped in Sept-Dec 1999 were also used at various points in the analysis. Prior to the 1998 field season, the adult population in Puerto Rico was unmarked. Adults were trapped using 2 3/4 inch mesh mist nets (41 ft length) on 7.2m telescoping poles (Meyers and Pardieck 1993). Two trapping strategies

were used. Birds were drawn to nets in response to play-back (either the calls of neighbouring groups or their own) or nets were set up in flight corridors leading to the nest tree. In 1999, adults were also trapped using a hardware cloth cage containing a lure bird and an AI call back. Adults responded with territorial behaviour and entered the cage in an attempt to attack the lure bird. Adult measurements taken were head to tip, culmen, wing length, tarsus, depth of bill, and mass and birds were banded with a unique combination of one numbered metal and 3 colour bands. Blood samples were taken by jugular venipuncture.

Nests

Ten nests were followed (5 single-female and 5 multi-female groups). Six nests were located at Cabo Rojo and 4 at Laguna Cartagena. Nests were found in mesquite, euphorbia and pithecellobium trees with nest heights ranging from 5 ft to 24 ft. Daily nest checks were performed using an extension pole equipped with a mirror and an extension ladder when necessary. Daily nest checks allowed for the tracking of egg laying sequence. All eggs were measured (length, width, mass) and marked with a unique letter. Once eggs began to accumulate, the position of each egg within the nest was recorded. This was intended to follow the incidence of egg burial. Eggs were removed in layers, noting the position of eggs in relation to each other to check for eggs buried beneath the leaf layer or pushed into the side of the nest. Eggs were returned to the nest in the same order as removed. An egg was designated as buried if it followed 3 criteria: 1) it remained unhatched past its projected incubation date (approx. 18 days- Loflin 1983) 2) it

was wedged into twigs or lining and could not be turned 3) it felt cold while eggs in the incubated layer were warm. Buried eggs were removed from the nest by researchers and remaining holes were filled with nesting material. Egg contents were dissected and the brain/heart or entire embryo was taken for DNA samples. No sample was taken from eggs containing only the yolk (no embryonic disc). Seventy-five percent of chicks were linked with the particular egg from which they hatched. To track chick growth, measurements were taken daily (mass, head to tip, tail to tip, culmen, tarsus). Once large enough (2-3 days) chicks were banded with one metal and 2 colour bands. Blood was taken by jugular venipuncture. Samples (blood /tissue) were obtained from 52 chicks and 28 buried eggs from 10 nests. The nest measurements taken were diameter, depth, height from ground, surrounding habitat and percentage of nest coverage. A nesting group was considered to contain all of the individuals on the territory that fed, roosted and attended to the nest together consistently (Loflin 1983). The number of laying females was estimated based on both group size and the total number of eggs laid in the nest (single female 5-7 eggs).

Characterization

Blood/tissue samples were stored in lysis buffer at 4⁰ C. DNA was extracted from blood using a saturated salt procedure and from tissue using a standard phenol chloroform protocol (Sambrook et. al. 1989). DNA was stored in TNE2 at -20⁰ C. Two adult and 2 embryo samples provided poor DNA yields. Fluorometer readings were taken and 25 ng working stocks were made.

Sexing

Smooth-billed anis are sexually monomorphic and therefore all adults and offspring were sexed using molecular techniques. Primers designed by Griffiths et. al. (1997) (P2 and P8) were used to amplify the CHD (chromo helicase binding domain) region on the avian sex chromosome. These primers anneal to conserved exonic regions of the Z and W genes and amplify across less conserved intron regions. The variation in intron length can be detected using gel electrophoresis. A polymerase chain reaction with an annealing temperature of 55⁰ C produced a 375 bp product when run on a 6% denaturing acrylamide gel. The heterogametic female showed two distinct bands (Z-W) 25bp apart, while the homogametic male showed one band representing (Z-Z).

Genotyping

All samples were genotyped with the 5 smooth-billed ani specific microsatellite loci isolated for this study (Chapter 1). For PCR conditions and locus characterization see (Chapter 1). These genotypes were used for all subsequent genetic analysis.

Parentage Assignment

Genetic and behavioural data were used in combination to estimate the parents/ family groupings of buried eggs and surviving eggs (defined as those eggs that remained in the incubated clutch and hatched). No distinction was made between eggs that hatched and those that went on to fledge chicks for the focus was egg loss due to burial.

Genetic Analysis

Three analytical approaches were used to estimate parents and family groups: 1) allele exclusions 2) parentage (using a 'most-likely' LOD ratio ranking of candidate parents) 3) kinship (log-likelihood scores). True candidate parents were assumed to be only those adults from the same nesting group as the offspring being tested. Single female and multi-female nests were both analyzed in the same manner.

Exclusions

Exclusion analysis was used with nesting groups containing at least one trapped adult. Genotypes of adults were compared to the genotypes of all offspring in their nest. To be a candidate parent, an adult must share at least one allele with the offspring at each of the 5 loci. Allowance was not made for typing error or possible mutation. Any adult not sharing at least one allele at every locus with a given offspring was disqualified as its potential parent.

Parentage-likelihood

Parentage was estimated using the genetic analysis program CERVUS (Marshall et. al. 1998) and included only those nests at which at least one adult had been trapped. CERVUS ranks potential parents based upon the likelihood ratio (probability of sharing alleles by descent/ probability of random sharing) based upon population allele frequencies. LOD scores (log of the ratio) were reported for each pairwise comparison

(adult-offspring). Adults were ranked based upon the highest positive LOD score. The highest score belongs to the most-likely candidate parent. Statistical significance of rank was assessed using CERVUS by calculating the difference between the 2 top ranked scores (this difference is termed delta). CERVUS runs a simulation generating a population of parent/offspring based upon estimated population allele frequencies. The program assigns the delta required to distinguish the most-likely candidate from the second rank with 95% confidence or a relaxed 80% confidence. For cases in which more than one candidate parent remained after exclusion analysis, an individual with a significant top ranked score would be designated the adult more likely to be the parent. A non-significant top rank score was noted as a possible parentage assignment only. A low positive or negative score was not used to disqualify an adult from parentage, as the sharing of common alleles can result in a low or negative score. Offspring were compared not only to the trapped adults from their nesting group but also to the rest of the sampled adults in the population and the adults sampled in 1999. The addition of adult samples from outside the nesting group was used to increase confidence in the ranking results and validate the assumption that true candidate parents are found in the same group as the offspring being tested. Adults from the same nesting group were run according to sex to eliminate the confusion of potentially ranking 2 true parents (male-female) together.

Kinship

The use of kinship analysis focused upon potential sibling relationships. This analysis was performed on adults and young from all nesting pairs and groups. The pedigree analysis program KINSHIP (Queller and Goodnight 1989) was used. A pedigree relationship was defined as full sibling, half sibling or unrelated. KINSHIP will assess the likelihood that the genotype combination of the two individuals being tested could have been produced by the specified relationship (e.g. are 2 individuals full siblings or unrelated). The result is a pairwise matrix reporting ratios or log-likelihood scores. To assess the significance of the ratio, KINSHIP runs a simulation generating pairs of individuals based upon estimated population allele frequencies and meeting the relationship hypothesis, determines the ratio needed to reject the null at $p < 0.05$. A non-significant result is given when insufficient evidence exists to support the primary hypothesis.

The individuals in each nest (offspring and adults) were compared pairwise testing 3 scenarios: (full sib/ unrelated), (full sib/ half sib) and (half sib/ unrelated). A significant value for a full sib/ unrelated test confirmed that the pair was related but did not establish the degree of relatedness. A non-significant (NS) result indicates a lack of evidence for relatedness. A (NS) result for a full sib/ half sib test was not assumed to indicate a lack of evidence for a full sibship because the type II error rate for this test was 0.62 (as calculated by KINSHIP). Therefore, a pair showing a significant ratio for a full/unrelated test but (NS) for a full/half test was concluded to be related but the degree of relatedness was inconclusive. The same lack of resolution was assumed for the half

sib/ unrelated test (type II error rate also 0.62). Therefore, the kinship analysis focused upon the full sib / unrelated test and whether the 2 individuals were related (to any degree) or not related at all. Simulated relatedness values were also generated for full siblings, half siblings and unrelated to assess the general degree of overlap between each pedigree relationship.

Behavioural Analysis

Behavioural data in the form of egg laying chronology were used in addition to the genetic data to assign offspring to parents or family groups. The egg laying information collected during daily nest checks was combined to illustrate the egg laying chronology for each female at nests showing burial. Females were assumed to lay one egg every other day (Loflin 1983; Davis 1940) and therefore it was assumed that a sequence of single eggs appearing every 2 days was indicative of a single-laying female. These data were used in 2 ways:

- 1) to assess whether the genetic assignments were biologically possible e.g. 2 eggs laid on the same day could not have been laid by the same female
- 2) if the genetic assignment of an egg was inconclusive yet the egg fell within a single laying female pattern (egg laid 2 days before and/or after was assigned) the egg may be grouped with others that did have conclusive genetic assignments.

With the combined data, eggs were assigned to a particular female and those laying females were classified as early or late layers. Once egg ownership was established, the occurrence of egg burial (eggs found in layers under leaves or pushed

individually into the side of the nest) was incorporated into the egg chronology pattern for each nest. Based upon the assignments and egg-laying pattern, the number of eggs buried and the number of eggs that remained in the incubated clutch were calculated at each nest. The degree of skew was quantified using the formula: $(Nbv + Nn) / (Nb + Nn)$, where Nb is the number of breeding females in the group, Nn is the number of nonbreeders, v is a measure of variation in reproduction among breeders defined by $[\sum (p_i - 1/Nb)^2] / [Nb-1]$ where p_i is the proportion of all offspring produced by the i th breeder (Reeve and Keller 1995; Pamillo and Crozier 1996) The values in this skew scale range from 0 to 1. When an individual produces all offspring, the skew value is 1, and when reproduction is perfectly equitable among all group members, the skew value is 0.

Mating System

Using the parentage assignments, the mating system of single pairs and groups was assessed to test for genetic monogamy. If matings were monogamous, the alleles observed in all siblings should be accounted for by only 2 parents, each contributing an allele in combination to each offspring, provided brood parasitism had not occurred. An adult involved in a single pair nest should not be excluded from any of the offspring in that nest if the pair is monogamous. For nests in which both parents were sampled, the known adult genotypes were compared in combination to each of the offspring in the nest. For nests in which only one parent was sampled, the alleles at each locus for all offspring grouped as kin were subtracted from those of the assigned parent. If the resulting alleles agreed with a single genotype (a phantom parent), this would support

genetic monogamy. Deviations from these conditions were assumed to provide evidence for systems other than monogamy.

Results

This study focused upon the breeding activity at 10 smooth-billed ani nests (5 single-female and 5 multi-female nests) found on two wildlife refuges in Puerto Rico (Table 2.1). Breeding group sizes ranged from a pair to a group containing 10 adults. The largest number of estimated laying females at a nest was 5. The total number of eggs laid per nest ranged from 5 (single pair) to 37 (10 adults)

The trapping of adults proved to be difficult (Loflin 1983; Startek 1997). Their preferred habitat included large areas of open field, making mist-netting difficult. Adults tended to become wary of nets if another adult had been recently caught and, on occasion, their characteristic slow flight and short wings allowed some birds to free themselves before the net could be lowered. The proportion of adults sampled averaged 50%.

The high incidence of egg/chick predation affected the ability to sample young. Of the 10 nests studied, 7 fell victim to predation. Two nests were attacked at the egg laying stage while 5 had all hatched chicks predated. The heights of these nests ranged from 7- 24 ft above the ground. The two remaining nests (LC Road and Site L) fledged chicks. Two eggs from one single pair nest (Euphorbia) were found beneath the nest site and the adults subsequently abandoned the remaining eggs. The most likely predators of these nests were the brown rat (*Rattus norvegicus*) and feral cat (seen at one nest site). Mongoose are known to attack ground nests in Puerto Rico but typically will not climb

trees. For two nests, (Cartagena, N Lagoon), the only offspring samples obtained were from eggs buried prior to predation events.

A range in the number of buried eggs (0 to 18) was observed between nests. No buried eggs were found in any single pair nests. No burial was observed in one group nest (Joes). Those groups with the largest number of estimated laying females buried the most eggs.

Offspring-parent assignment

Exclusions

To be a candidate parent an adult had to share at least one allele at each of the 5 loci with an offspring. The identity of neither parent was known. The number of presumed candidate parents was only as large as each group size, assuming that true parents are from the same nesting group as the offspring being tested. Offspring genotypes were tested against the genotypes of no more than 3 adults and no more than 2 of any one sex. The exclusion of an adult within a group distinguished between two potential sampled parents or disqualified the only adult trapped in favour of an unsampled parent. Only one single-female nest showed exclusions (Euphorbia). The only adult male sampled was disqualified from parentage for 75% of the young in his nest. In group nests, 63% of all young showed mismatches with at least one adult in the group. Of these, 27% of young were excluded from all trapped adults at their nest, indicating that neither of their parents had been sampled. Therefore, 37% of young (20 individuals) remained with more than one sampled candidate parent. At most, two adults of the same

sex needed to be resolved. The results of the exclusion analysis were the most definitive assessment of parent/offspring relationships. At this stage, an adult showing a mismatch with an offspring was disqualified as a candidate parent.

Parentage-likelihood

Offspring were tested against all adults in the nesting group regardless of the outcome of the exclusion analysis. The remaining adults in the population as well as those adults trapped in 1999 were also included in the analysis (N=43). The delta criterion needed for 95% confidence as calculated by CERVUS was (2.27) and (1.08) for a relaxed confidence of 80%. In total, 38% of young were paired with adults that showed significant top ranked scores. The distribution of the delta scores (difference between the first and second ranked scores) for the top 2 candidate parents for individuals at all nests is shown (Figure 2.1). At single-female nests, 63% of young had adults rank first with >80% confidence while 31% ranked with >95% confidence. At group nests, 21% of young were paired with adults with top ranks >80% confidence and 6% ranked with >95% confidence (Table 2.2). All adults that ranked first with significance belonged to the same nesting group as the offspring being tested. From single-female nests 5-10/16 (80%-95% confidence) of the young were assigned to a parent while at group nests 3-11/52 (80%-95% confidence) of the young were assigned to a parent with significance. In total, 17 young had candidate parents which showed top rank scores (most-likely parent) but without significance. Coltman et al (1998) justified accepting top rank scores with <95% confidence for a number of reasons: other published studies have 1) used simple

most-likely parent criteria without estimating statistical confidence (Meager 1986) 2) have compared LOD scores only using chi squared analysis (Taylor et. al. 1997) and 3) have assigned paternity to non-excluded individuals (Morin et. al. 1994). In this study, top ranked scores without significance were only used if they were supported by genetics and field observations.

In no case did the exclusion data disagree with the parentage-likelihood data i.e. adults with exclusions never ranked first. From the exclusion data, 37% or 20 individuals remained with multiple candidate parents. The ranking of parents provided some additional evidence in 10 cases. In 3 cases (LC Road) significant top rank distinguished between two same sex candidate parents. In 7 cases (LC Road, SiteL) top rank without significance suggested the distinction between two same sex potential parents. Of the remaining 10 individuals with multiple parents, 8 belonged to one nest (Joes) in which only 1 adult from a group of 4 was sampled. The remaining 2 unresolved individuals were from Site L .

Caution must be taken when assessing rank outcomes. The success of likelihood ranking is affected by the sharing of common alleles. This may have been the reason for some low positive scores in single-female nests.

Kinship

Kinship analysis was used to separate offspring found in group nests into sibling groups. All adults and offspring within a nest were compared pairwise to test for related versus unrelated individuals. Relatedness values were calculated to assess the degree of

overlap between each pedigree relationship (Figure 2.2). The full sib distribution had a mean of 0.4863 (0.2327) with a range of (-0.4 to 1), half distribution mean 0.2361(0.2366) range (-0.5 to 0.9) and unrelated distribution mean -0.0032 (0.2408) range (-0.5 to 0.8). There was considerable overlap between these simulation distributions. The half sib distribution almost completely overlapped both the full and unrelated distributions. Blouin et. al. (1996) tested how the number of loci used for analysis can affect the resolution and misclassification between categories. They found that when using 5 loci with heterozygosity of 0.75, the fraction of unrelated pairs misclassified as full sibs was 0.214. The misclassification of unrelated as half was 0.33. The average heterozygosity for the 5 loci used in this study was 0.70. A conservative approach was taken testing only for full sib/ unrelated to conclude whether two individuals were related to any degree or unrelated.

The overall distribution of log-likelihood scores showed a range from -2 to 10.5(Figure 2.3). The log ratio required for significance at the $p < 0.05$ level was 0.4661. Single-female nests showed 85% of pairs as related. Within 3 single-female nests (Creek, Driveway, Site E) all young were related to each other. In the 2 remaining single-female nests (Euphorbia, Corner), young had low log-likelihood values. There were asymmetrical relationships between all young suggesting the involvement of more than one breeding pair. In the group nests, 44% of dyads proved to be related. These significant log-likelihood scores did not separate young clearly into sibling groups. There was considerable overlap between the relationships. In 2 groups (Site L, LC Road), the same sex adults were related to each other indicating that all of their offspring should

show relatedness. All of the young at Joes nest were related to each other again suggesting that the parents might be related. The more informative result was the (NS) or unrelated relationships. At the LC Road nest, one individual who was excluded as the genetic offspring of either sampled adult was clearly not related to any other young in the nest. At N Lagoon, the two young assigned to one female were related to each other but not to the remaining offspring, who was assigned to the other female adult. At Site L, some young showed low log-likelihood scores with many other offspring suggesting that they may have belonged to the unsampled female. Overall, the kinship analysis gave better information regarding individuals that did not belong together than it did with respect to grouping young by their relatedness.

Behavioural Data-egg laying chronology

Egg laying chronology was used to assess the biological possibility of the genetic assignments (assessed only for group nests). For each nest, the assignments resulting from the genetic analysis were supported by the egg laying sequences observed i.e. eggs assigned were at least 2 days apart. The assignment of 4 unresolved young was supported by egg chronology. In one case (Site L) an egg was laid on the same day as two other eggs that were assigned to the two sampled females. This suggested that the unsampled female was the parent of the third egg. Two unresolved young (Cartagena) were assigned to the early layer and one offspring with no DNA sample fit into the laying sequence of one female (egg before and after assigned).

Nest Summary-egg burial

LC Road

The LC Road group contained 4 adults (2 sampled) and 7 young. All young were assigned to one of two adults sampled (Figure 2.4). The first 4 eggs laid were infertile. There was clear evidence for an early and late layer. The late layer was assigned 5 eggs. The infertile eggs may have belonged to the other layer (only 2 eggs assigned) for a total of 6 eggs (without DNA this could not be confirmed). Two eggs were laid on the same day during one female-laying sequence. One of these eggs did not match either adult or show relatedness to any other offspring. This may be the result of brood parasitism. Three early laid eggs were buried under a leaf layer by day 9. The fourth buried egg (buried after day 20) was pushed into the side of the nest. If the infertile eggs belonged to the female assigned 2 eggs (as deduced), then the early layer lost 4/6 eggs to burial with 2/6 in the incubated clutch. The late layer lost no eggs to burial and had 5/5 eggs in the incubated clutch.

N Lagoon

Due to predation, only 3 samples from 15 eggs were obtained at this nest. The egg laying sequence is shown in (Figure 2.5). All three young were assigned to one of 2 females. No leaf layer was seen covering eggs. No burial of early laid eggs occurred. The 2 buried eggs became pushed into the side of the nesting material (one from each female) with complete burial occurring by day 19. Although the total number of eggs in

the incubated clutch for each female could not be assessed (predation), each female did lose one egg to burial.

Site L

At Site L, 3 adults were trapped from a 5-adult group. Twenty-four eggs were laid (Figure 2.6). Twenty-two young were each assigned to one of the 3 females with 2 offspring left unassigned. Both unresolved eggs were in the accumulated clutch. There were 2 early laying and one late laying females. Five early laid eggs were buried under a leaf layer by day 2. Six eggs were accidentally buried in the nest lining. Complete burial of these eggs occurred by day 24. Egg loss due to burial was 3/8 and 4/8 for early layers (including unresolved eggs) and 4/8 eggs belonging to the late layer were lost. The incubated clutch contained 11 eggs.

Cartagena

The sampling of offspring was limited by predation at this nest and therefore genetic assignment was limited (Figure 2.7). Layered burial began at day 12 and resulted in 13 buried eggs. Six eggs laid in a sequence were infertile. Six eggs were in the process of being buried in the side of the nest when the predation event occurred. Egg losses could not be assigned to specific females but all eggs buried under the leaf layer were laid early in the sequence. The eggs in the process of being accidentally buried were randomly distributed in the laying sequence.

Joes

No egg burial was seen. All eggs remained in the incubated clutch.

Overall, burial and skew results for group nests in which egg ownership by breeding female could be estimated are presented in (Table 2.3). The reproductive skew values calculated for each of these nests are (LC Road = 0.16), (Site L = 0.0048) and (N. Lagoon = 0.0128).

Egg Burial

Two types of egg burial loss were observed in nests: layered burial and individual random burial. Leaves were regularly brought in to the nest during egg laying. Layered burial was characterized as an accumulation of eggs that were covered by a layer of leaves in the bottom of a nest and would not resurface. This type of burial targeted early laid eggs. Incubation in smooth-billed anis involves much movement of eggs within the nest. Eggs are completely mixed top to bottom. On occasions when individual random burial occurred, there were 15 to 17 eggs already accumulated in the nest. Mixing during incubation caused random eggs to become lodged in the side of the nest. This type of burial was not seen in single female nests (7-egg maximum). Random burial did not target any particular eggs. This appears to have been due to a crowding effect. In this study, 58% of buried eggs were lost due to layered burial, while 42% were lost to random burial. In two nests (Site L, Joes) there was evidence of chicks being crushed in the bottom of the nest. In both of these nests, 11 chicks hatched but only 9 and 10 survived. No chick loss due to crowding occurred in single-female nests. Nest dimensions (diameter, depth) for all groups and pairs were compared and found not to be significantly different from one another ($X^2 = 1.5704$ and $X^2 = 1.43$, $df = 9$). Since the

nests are the same size, an increased number of eggs or chicks in the nest must lead to this crowding effect.

Mating System

The assignment of parent and offspring relationships allowed for the assessment of the mating system in both single-female and multi-female groups. The assumption was that smooth-billed anis were monogamous (Davis 1940; Loflin 1983). The alleles seen in the offspring were subtracted from those found in the assigned adult to assess whether one phantom mate could have been responsible for the remaining alleles, indicating evidence of monogamy. In three single-female nests (Creek Driveway, Site E), two parents from each nest accounted for all of the alleles seen in the offspring. In the remaining two nests, offspring could not be accounted for by two adult genotypes. In one case (Euphorbia), the sampled male adult mismatched three out of four young and the offspring were not all related. This provided evidence for a lack of genetic monogamy in a single pair nest. This may have been a case of brood parasitism or extra-pair fertilization. There was evidence for monogamy in group nests. At Site L, L.C. Road and N. Lagoon, young assigned to adults were accounted for by one phantom genotype. At site L, the trapped male could account for all of the offspring assigned to one particular female suggesting a monogamous pair. The male trapped at N. Lagoon could account for the alleles of young assigned to both sampled females. This suggests a polygynous relationship, although the remaining male was unsampled and hence could not be excluded. The young at Joes nest could not be accounted for by single pairs of

genotypes suggesting polygamy or extra-pair fertilizations. There is evidence that monogamy does exist in both single-female and multi-female groups but polygamy may also be present. There is evidence that brood parasitism may occur.

Discussion

The ability to estimate parent-offspring relationships in wild populations can provide information on population structure, social structure and breeding behaviour. Genetic markers allow for the assessment of relationships within wild populations for these types of data may be very difficult or virtually impossible to collect in the field.

In this study, parent-offspring relationships were estimated for 10 smooth-billed ani nesting groups using both microsatellite analysis and field data. The ability to exclude adults as genetic parents of particular young proved to be reasonably successful. Allele mismatches seen in multi-female nests resulted in 63% of young being excluded from 1 of 2 potential parents. The parentage-likelihood analysis resulted in 38% of young being paired with a significant top ranked adult (most likely parent). This analysis provided further evidence for parent-offspring relationships for 10 young, leaving 20% unresolved. The kinship analysis proved to be the least informative analysis. The high incidence of relatedness between offspring in a nest meant distinguishing between sibling groups was difficult.

There are a number of issues to consider when assessing the ability to accurately assign relationships. In this study, 5 microsatellite loci (heterozygosity = 0.70) were used. After comparing a series of computer simulations and mouse breeding experiments, Blouin et al (1996) discovered that in order to distinguish between full siblings and unrelated individuals with 90% confidence, 10 loci $H_e = 0.75$ would be needed. Forty loci would be needed to distinguish full siblings from half siblings with

the same confidence. A compromise of 20 loci would allow for distinction between full / unrelated with 98% confidence and full/half with 82% confidence. To exclude with 99% confidence using loci with a low number of alleles (2 to 3) Chakraborty et al (1998) reported needing at least 50 loci. With an increase in the number of loci used, the similarity between individual genotypes decreases. Therefore, the more loci used the greater the confidence in the assignment.

There are other factors besides the number of loci that greatly affect the ability to accurately assign parentage: number of candidate parents; incomplete sampling; relatedness of individuals. Taylor et al (1997) and Coltman et al (1998) conducted parentage studies similar in nature to this study involving wild populations (wombats, pinnepeds) with incomplete adult sampling using 9 and 6 microsatellite loci. Both studies concluded that parentage could not be assigned with 95% confidence using the loci available. Both studies were attempting to distinguish between large numbers of candidate parents (wombat $N = 58$), (pinneped $N = 180$). The statistical confidence in assignment decreases with the number of candidate parents included. With an increase in the number of potential parents, the number of unresolved relationships is expected to increase (Neff et al 2000; Marshall et al 1998). In the case of communally breeding smooth-billed anis, the number of candidate parents is limited to the number of adults in the group. In smooth-billed ani groups often neither parent is known, but the existence of breeding groups eliminates the need to screen every adult in the population as a possible parent.

The issue of incomplete sampling is an important factor in this study.

Approximately 50% of adults within breeding groups were sampled. The number of unsampled adults affects the number of unresolved relationships seen (Marshall et al 1998). In this study, as in a study of armadillos conducted by Prodoehl et al (1998), the use of field data complimented genetic data to increase the confidence in assignments when adults in the population were unsampled. Perhaps the greatest improvement to this study would have been a larger proportion of sampled candidate parents.

The relatedness of individuals to either the candidate parent or the offspring can affect relationship assignment. The relatedness between candidate parents can decrease the ability for exclusions (Double et al 1997). Adult relatedness was assumed to be a factor in the inability to distinguish sibling groups using kinship analysis in this study.

Overall, using the total evidence collected, 80% of young were assigned to probable potential parents. It is difficult to estimate accurately the number of loci that would be required to improve the analysis. Based upon the literature, if the adults in this population prove to be related, but the number of candidate parents remain small, the use of upwards of 20 loci would allow for a more complete assessment of half to full sibling relationships.

The number of smooth-billed ani nests used for this study was 10. It would be helpful to increase this sample size. These 10 nests were observed in one field season. In 1994, 11 smooth-billed ani nests were found (Startek 1997) and in 1999, 12 groups were found within the same study site boundaries (J. S. Quinn and J. Haselmeyer p. commun.). It may be that more nests will not be found in the given area in one season and the area of

study may need to increase. Similar data collected over a number of years will need to be combined to increase the overall sample nest size.

Nest Use

All 10 smooth-billed ani nests were analysed using a combination of genetic and behavioral data and the results from single-female and multi-female nests provided different types of information. All 5 single-female nests provided information regarding the smooth-billed ani mating system. The 5 group nests provided insight into reproductive skew issues related to egg burial. In 4 out of 5 group nests, genetic data were strongly supported by the behavioral data, while for 1 nest, the genetic information available was very limited due to egg predation. This nest was very still very useful for assessing the timing of burial in relation to a general laying pattern.

Reproductive Skew

Under no circumstances in group nests, did every egg laid remain in the incubated clutch and hatch successfully. In all group nests, egg loss or chick loss was observed. This loss of offspring was the result of interactions between breeders within the group. Competitive behaviour between breeding females has been described in great detail for the communally nesting acorn woodpecker and the groove-billed ani. In both of these species, egg loss in the form of tossing rather than burial occurs under very similar circumstances as those seen in the smooth-billed ani. In both the acorn woodpecker and groove-billed ani, the last female to lay has been observed removing eggs accumulated in

the nest by an earlier laying female. The ownership of eggs in the incubated clutch of woodpeckers was found to favour the egg-removing female (Mumme et al 1983). The same pattern was observed in the groove-billed ani for which Vehrencamp (1977) reports that in two-female nests, the second female owned 63% of the incubated clutch and in three-female nests, the division was (1st 24% - 2nd 30% - 3rd 46%). Egg ownership in both studies was assessed in the field using egg classification and the identification of gravid females. Loflin (1983) also assigned egg ownership in smooth-billed anis using field techniques and reported skew results very similar to the groove-billed anis (2nd female 60%) (1st 22% - 2nd 31% - 3rd 41%). In this study, similar results were found (Table 2.3). The last layer in a two-female nest owned 71% and 54% of eggs in the incubated clutch, while in one three-female nest, the ownership results were (1st 38% - 2nd 31% - 3rd 31%). One two-female nest showed no burial at all. In all three species, the reproductive skew observed is very low. The overall skew values calculated for groove-billed anis and acorn woodpeckers fall below 0.05, on a scale of 0 to 1 (Vehrencamp 2000). The skew values calculated for this study ranged from (0.004-0.16). All females in the group appear to have breeding opportunities although they may not always be equally successful in any given nesting attempt. The last female to lay either does as well or better than the earlier layer. The early layer rarely has greater ownership than the late layer, but can do equally well. Therefore, although some offspring loss is always observed in group nests, the amount of skew observed as compared to that seen in despotic systems (skew values approaching 1.0) is minimal.

Egg Burial

In smooth-billed anis almost all offspring loss related to female interactions is the result of egg burial. Layered burial targets early laid eggs, while accidental burial can involve any egg in the incubated clutch. In this study of 36 buried eggs, 58% were lost due to layered burial, while 42% were accidentally buried. All eggs lost to the late laying female were lost due to accidental burial. Egg burial is not reported in acorn woodpeckers, but the incidence of accidental burial was mentioned in reference to groove-billed anis (Vehrencamp 1978; Skutch 1959). In large groove-billed ani nests, it was noted that some eggs might become lost in the bottom of the nest. This is referred to as a rare event. In smooth-billed anis, accidental burial is not seen in single pair nests (5 - 7 eggs). The occurrence of accidental burial in nests with large numbers of eggs suggests a crowding effect. The lack of importance given to accidental burial in groove-billed anis may be related to the finality of egg tossing behaviour. Groove-billed ani females lay 4 to 5 eggs and approximately 3 are tossed out for every early laying female (Vehrencamp 1977). So even in a three-female nest, the total clutch size should rarely rise above 9 eggs, and the most common group size includes two females (Vehrencamp et al 1988). With eggs tossed out of the nest, more room is made for the incubated clutch. In smooth-billed anis, egg burial (both layered and accidental) may reduce the inner depth of the nest making the circumstances for crowding even more probable. Early layers lose more eggs to layered burial than accidental, but later layers lose only to accidental burial under certain circumstances.

How does crowding within nests affect chick survival? In two smooth-billed ani nests 11 chicks hatched, yet the smallest were crushed in the bottom of the nest under

older chicks. This did not happen in single pair nests with 7 chicks. This was also observed in groove-billed ani nests in which 5% of the last hatched chicks on average were usually found dead in the nest of larger groups (Vehrencamp 1978). In acorn woodpeckers, last laid chicks were often lost due to brood reduction (Mumme et al 1983). Smaller nestlings had decreased survival in relation to food competition. Therefore, last laying females risk losing their last laid offspring to chick competition.

The factors that appear to affect the number of buried eggs or the skew in the incubated clutch are laying order and timing. The effect of laying order is straightforward. Earlier layers generally have a percentage of their eggs buried under a layer of leaves, while the late layer does not.

Timing of Egg Laying

(Synchrony versus Asynchrony)

The onset of the last laying female signals the end of tossing or layered burial therefore, it is the best interest of the earlier female to lay her eggs as synchronously as possible with the last-laying female. Evidence of this can be seen in two smooth-billed ani nests (Joes, N. Lagoon) in which laying was synchronous and no burial under a layer of leaves occurred. Laying synchrony in acorn woodpeckers also results in a lack of egg destruction (Koenig et al 1995). In this way, the early layer increases her potential proportion of the incubated clutch. The compromise is that with synchronous laying, the age of all chicks should be very similar and therefore, the earlier layer would lose the advantage of having the oldest chicks in the nest. Synchronous laying for the second female offers a different outcome. The greatest advantage to the second layer is the

decrease in age difference between chicks. The late layer decreases the risk of losing her last laid chicks. Mumme et al (1988) suggested that the result of egg destruction in woodpeckers actually simulates synchronous behaviour and reduces the losses that would occur through laying/hatching asynchrony. For smooth-billed anis, there are real disadvantages to synchronous laying for a second female. If early females' eggs are not buried, the clutch/brood size can become large, increasing the risk of egg loss due to accidental burial or chick loss due to crowding as seen in N. Lagoon and Joes.

With an asynchronous pattern, the early layer stands to lose a proportion of eggs to layered burial. However, any offspring that do survive will be larger and likely better able to compete for food. The second laying female in an asynchronous system, can reduce the overall clutch size by burying eggs prior to her own laying and dominate the number of eggs in the incubated clutch. Although under these circumstances, her last laid chicks may be outcompeted (L. C. Road and Site L.) by any offspring that were not buried.

The other factor to consider is the number of laying females. If a late female breeds with a larger number of females, even if many early eggs are buried, the laying after burial may still lead to a large clutch size and increase the risk of accidental burial (Site L and Cartagena). The modal number of breeding female acorn woodpeckers and groove-billed anis is 2 (Koenig and Stacey 1990; Vehrencamp et al 1988). As group size increases, the number of fledglings per female decreases.

The best strategy for an early laying smooth-billed ani to adopt in order to increase the number of her eggs in the incubated clutch, is to nest in small groups with

synchronous laying (as seen in the N. Lagoon nest with skew value 0.004). The most profitable strategy for a late laying smooth-billed ani is to nest in a small group and lay asynchronously, burying all early laying efforts (as seen in the LC Road nest with skew value 0.16).

Infertile Eggs

In 3 group smooth-billed ani nests infertile eggs were found early in the nesting sequence. In 2 nests these eggs were laid in a sequence characteristic of a single female (every other day). Koenig (1980 a, b) observed a high incidence of ‘runt eggs’ associated with early layers in acorn woodpeckers. They were not found in single pair nests. Two reasons have been suggested for the laying of infertile eggs: 1) to act as a synchronizing signal for laying 2) caused by temporary disturbance to reproductive organs (Koenig 1980a). There is no direct evidence for either of these hypotheses. In the smooth-billed ani study nests, up to 6 infertile eggs were laid by potentially the same female. This would be a very costly synchronizing signal. Davis (1940) suggested that communal breeders may tend to be spontaneous ovulators and that ovulation may be triggered by something other than courtship. Perhaps females are ready to lay eggs prior to obtaining a copulation for fertilization. Koenig (1980) suggested that disturbance during inter-individual contact i.e. subordinate and dominant interactions, may result in the laying of infertile eggs. Although there is no direct evidence, perhaps the infertile eggs seen in the smooth-billed ani nests were the result of competitive behaviour between females or perhaps they are indicative of an inexperienced female laying prior to obtaining a

copulation. The occurrence of these eggs early in the laying sequence may indicate an experience differential between early and later laying females.

Single Pairs versus Groups

There has been many studies conducted regarding the factors that affect whether communal adults breed in groups or pairs. When successful, single pairs often fledge the greatest number of chicks per female (Koenig 1981; Vehrencamp 1978). Four of five single pair smooth-billed ani nests were successfully raising up to 7 chicks when they were predated. All chicks were being adequately fed (daily chick measurements) but all chicks were lost to a predator. The 2 nests that fledged chicks were group nests. The number of chicks fledged per female was 4 and 5. The number of fledglings per female was greater for single pairs but all nests were destroyed. The fact that some individuals do nest in pairs rather than groups likely indicates that under some circumstances, single pair nests are successful otherwise one might expect that selection would act against their existence and smooth-billed anis would always be observed breeding in groups. In future studies, with data collected from more single pair and group nests, one could investigate whether increased protection from predation appears to provide an advantage to nesting within a group rather than as a pair.

Mating System

Various mating systems have been observed in communally breeding birds. Acorn woodpeckers are characterized as being opportunistically polygandrous or promiscuous (Koenig and Stacey 1990; Koenig and Pitelka 1979). Groove-billed anis

show conspicuous pair bonds and mate guarding and are assumed to maintain monogamous pairs (Vehrencamp 1986). Loflin (1983) described pair bonding and the dominance of a monogamous system in smooth-billed anis. Evidence for polygamy and brood parasitism as well as monogamy was found by Startek (1997) in a preliminary genetic study of smooth-billed anis. In this study, adults were observed roosting in pairs and courtship feeding, often interpreted as an act to maintain a pair bond (Lack 1940). Three single-female and 3 multi-female nests provided evidence for monogamy. There was evidence for brood parasitism at one single-female and one group nest. An adult from one single pair nest was excluded as the genetic parent for 75% of the young in his nest. This smooth-billed ani nest was subsequently abandoned and the eggs were found beneath the nest. Vehrencamp (1978) claimed that the most common cause of abandonment seen in groove-billed anis was parasitism by other anis. Eggs were found below the nest and the adults disappeared. This may be indicative of a case of brood parasitism in smooth-billed anis. The probability that a single egg laid in one group nest, that could not be assigned to the breeding adults, was the result of brood parasitism was supported by both genetic and egg chronology data.

Conclusion

The overall results of this study indicate that breeding in smooth-billed anis is indicative of an egalitarian system. Unlike in a despotic system, all females appear to have breeding opportunities but the success of each female in any given nesting attempt may vary. Although the last laying female tends to own more or an equal proportion of

the incubated clutch, the amount of bias is minimal and is likely influenced by a number of factors that change from one nesting attempt to another. The main factors affecting this bias appear to be laying order, synchrony of laying and the number of laying females. A second type of burial (accidental) provides risks to late laying smooth-billed anis not emphasized in other communal nesters. Smooth-billed anis show a range of mating systems such as monogamy, polygamy and brood parasitism which is consistent with their family history, for within the Cuculidae family there are species which exhibit breeding systems from monogamy to host specific parasitism (Davis 1940).

Although an increase in the nest and adult sample sizes would increase the information gained, the nests used in this study allowed for an initial assessment of reproductive skew and the effects of egg burial in smooth-billed anis using both genetics and behaviour. Once increased adult sampling and the isolation of more loci are established, this approach for assessing family relationships will be much more accurate and less labour intensive than the extensive observations needed to assign parentage in the field.

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Table 2.1: Group and nest composition of a population of Puerto Rican smooth-billed anis.

TERRITORY NAME (REFUGE)	EST. GROUP SIZE	EST. NO. LAYING FEMALES	TOTAL NO. EGGS LAID	NO. OF SAMPLES COLLECTED			NO. EGGS BURIED
				<u>adult</u>	<u>chick</u>	<u>embryo</u>	
LC Road (LC) ^c	4	2	12	2	7	0	4
Joes (LC) ^c	4	2	13	1	11 ^b	0	0
Cartagena (LC) ^c	10	5	37	1	0 ^a	8	18
N.Lagoon (LC)	4	2	15	3	0 ^a	3	2
Site L (CR)	5	3	24	3	11	11	11
Site E (CR)	3	1	7	1	7 ^b	0	0
Euphorbia (CR)	2	1	5	1	0 ^a	4	0
Driveway (CR)	2	1	7	2	7 ^b	0	0
Creek (CR)	2	1	7	2	7 ^b	0	0
Corner (CR)	2	1	5	0	5 ^b	0	0

^a nest predated before egg hatched

^b nest predated before chicks fledge

^c nest contained infertile eggs

embryo samples taken from buried eggs

Table 2.2: The percentage of offspring for single and group nests that were paired with an adult showing a significant top ranked LOD score (at two levels of confidence).

CONFIDENCE LEVEL	SINGLE NESTS	GROUP NESTS
> 80%	63%	21%
> 95%	31%	6%

Table 2.3: The percentage of eggs buried and those remaining in the incubated clutch for breeding females in three smooth-billed ani groups.

NEST	NO. LAYING FEMALES	TOTAL NO. EGGS LAID	EGGS LAID			TOTAL NO. EGGS BURIED	% OF EGGS BURIED			% OF INCUBATED CLUTCH		
			<u>^a1st</u>	<u>2nd</u>	<u>3rd</u>		<u>^a1st</u>	<u>2nd</u>	<u>3rd</u>	<u>^a1st</u>	<u>2nd</u>	<u>3rd</u>
LC Road	2	12	6	5	-	4	67	0	-	29	71	-
Site L	3	24	8	8	8	11	50	38	50	38	31	31
N.Lagoon	2	15	7	8	-	2	14	13	-	46	54	

^a laying order of females

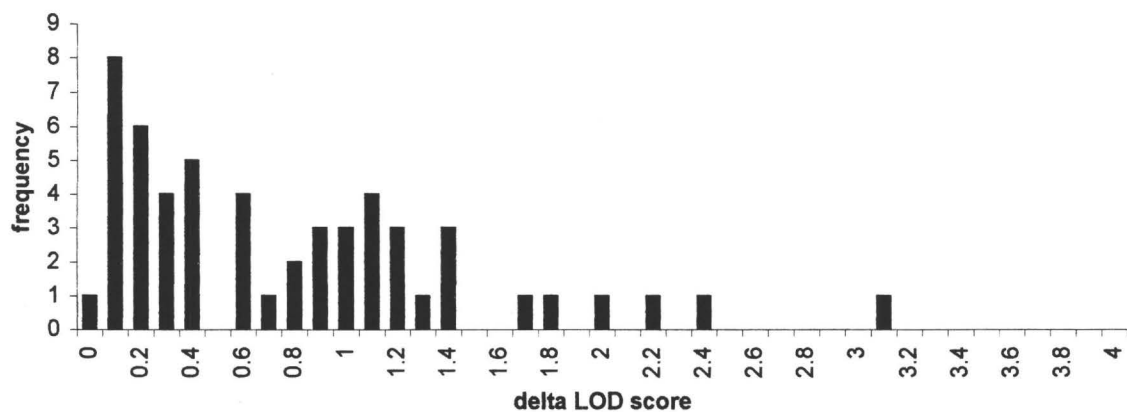


Figure 2.1: The distribution of delta scores of 'most-likely' parent candidates for all smooth-billed ani nests. Thirty-eight percent of young were paired with adults that showed significant top ranked scores. (95% confidence (delta =2.27), 80% confidence (delta =1.08))

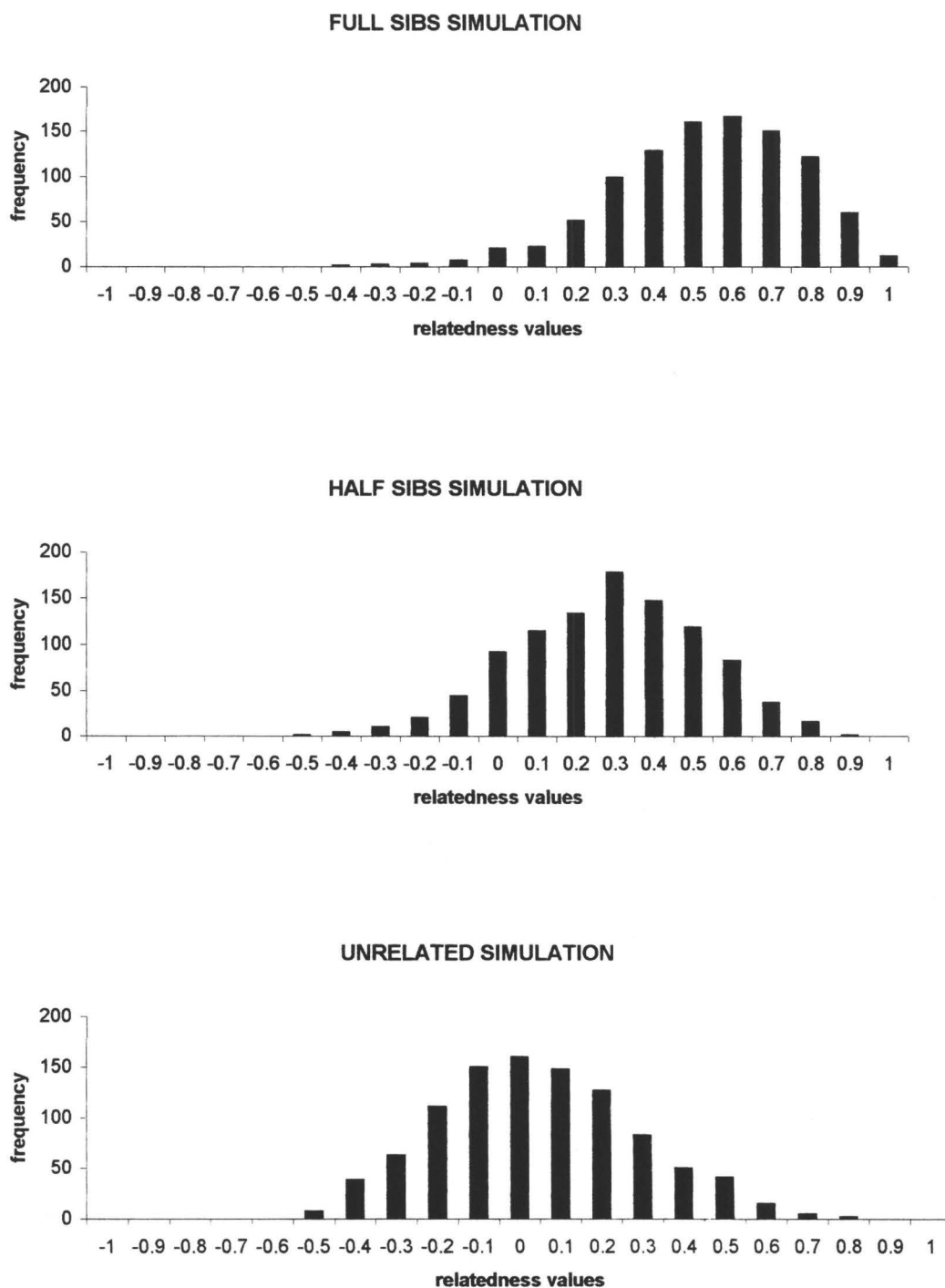


Figure 2.2 : The distributions of relatedness values for full sibling, half sibling and unrelated individuals generated by KINSHIP (Queller and Goodnight 1989). Mean and SD values: Full 0.4863 (0.2327), Half 0.2361 (0.2366) Unrelated -0.0032 (0.2408).

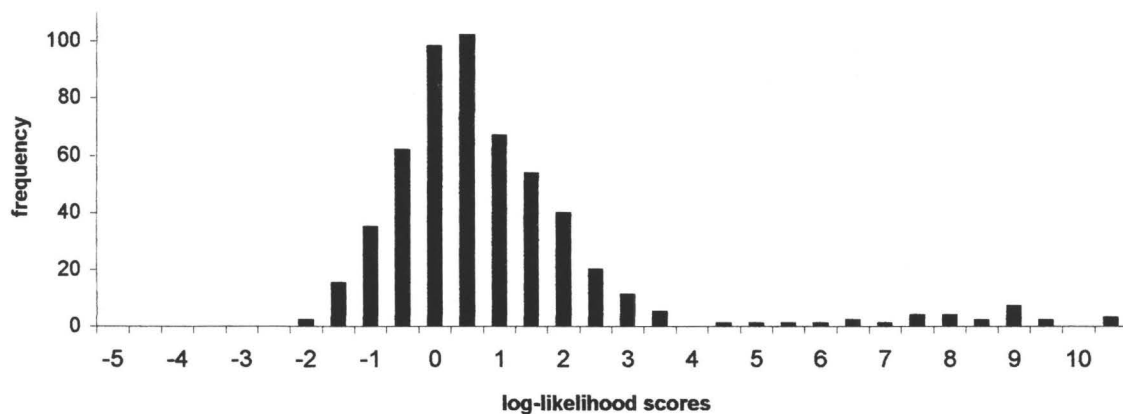


Figure 2.3: The distribution of log-likelihood ratios for pairs tested for a full sibling against unrelated relationship for all nests. The ratio required for evidence of related individuals $p < 0.05 = 0.4661$ as calculated by KINSHIP. Sixty-four percent of all pairs showed significant log-likelihood scores indicating evidence for relatedness.

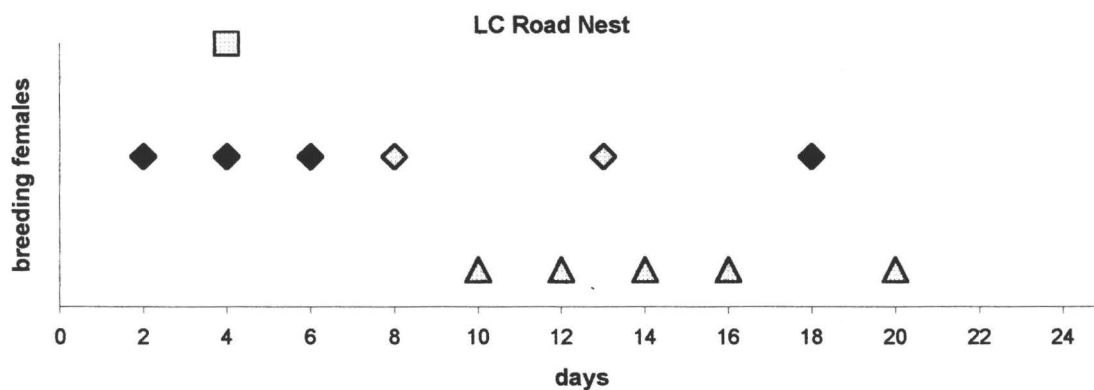


Figure 2.4: The egg laying chronology of the LC Road nest showing the timing of laying and incidence of burial separated by breeding female. The dark markers indicate buried eggs. Different symbols indicate eggs laid by different females.

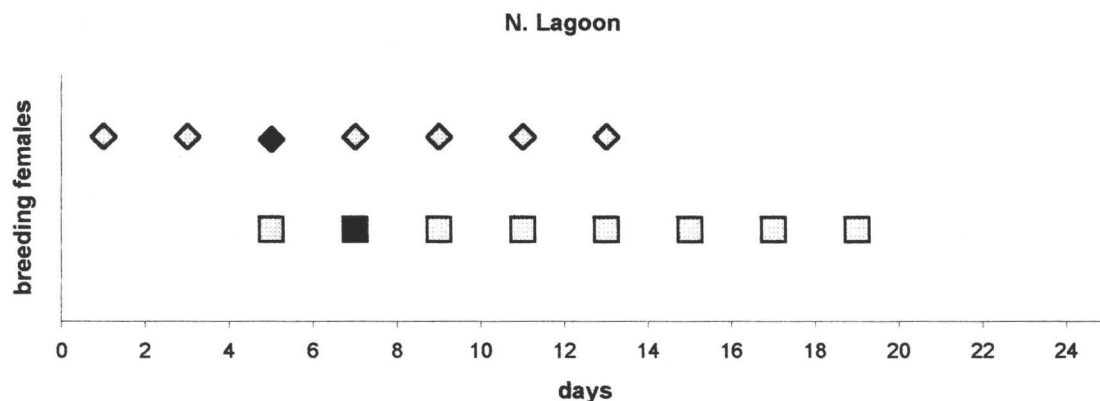


Figure 2.5: The egg laying chronology of the N. Lagoon nest showing the timing of laying and incidence of burial as separated by breeding female. Dark markers indicate buried eggs. The ownership of the eggs laid following day 9 are hypothesized only as the eggs were predated before DNA samples could be collected. Different symbols indicate eggs laid by different females.

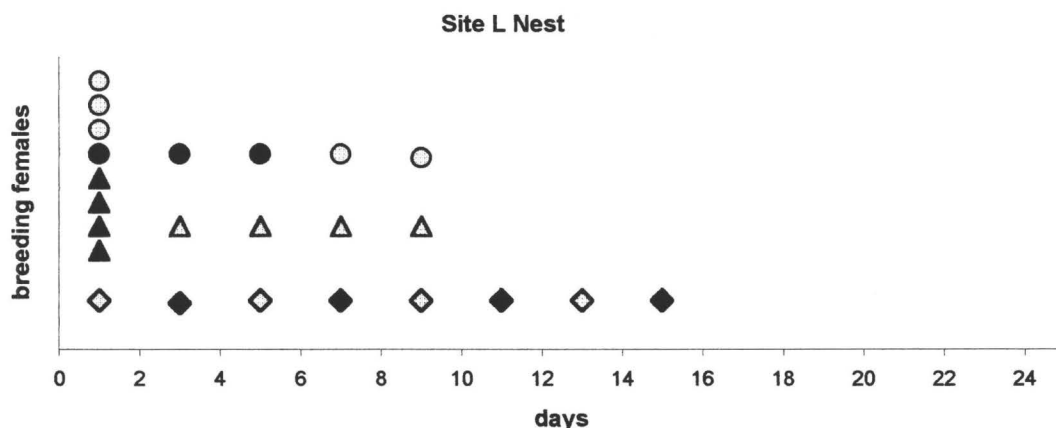


Figure 2.6: The egg laying chronology of the SiteL nest showing the timing of laying and the incidence of burial as separated by breeding females. Dark markers indicate buried eggs. The eggs laid on day 1 represent eggs already accumulated when the nest was found. Different symbols indicate eggs laid by different females.

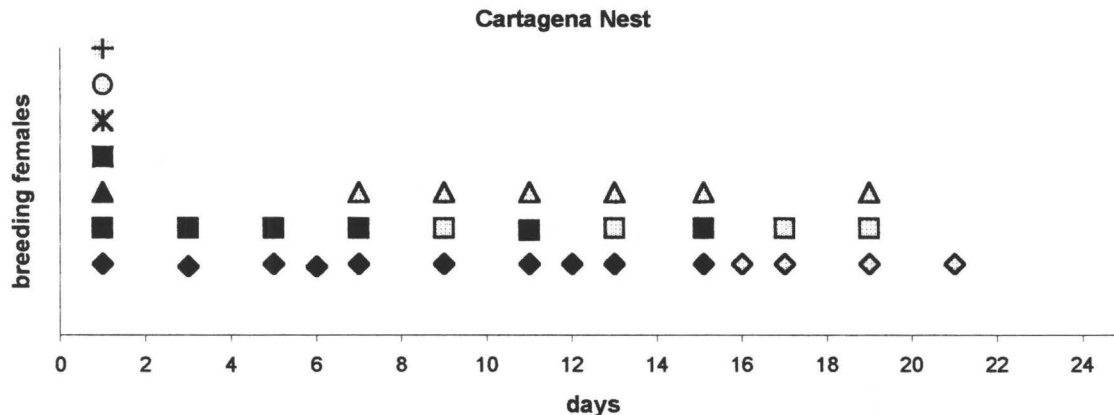


Figure 2.7: The egg laying chronology of the Cartagena nest showing the timing of laying and incidence of burial. Due to a lack of DNA samples (egg predation) the chronology does not separate egg laying by breeding female but rather only indicates which eggs in the sequence were buried (dark markers). The differing symbols were used for clarity only. The eggs on day 1 represent the accumulation of eggs present when the nest was found.

Chapter 3

The Relatedness of Adult Smooth-billed Anis in Communally Nesting Groups

Abstract

The relatedness of members within a social group may affect the amount of reproductive skew observed. The relatedness of smooth-billed ani adults within communally breeding groups was estimated using genotypes at microsatellite loci. Five smooth-billed ani specific loci were used to genotype 27 adults from 9 breeding groups. Adults within groups were tested for evidence of relatedness using the pedigree analysis program KINSHIP. Ten % of pairs showed significant relationships. Four groups contained both related and unrelated individuals while the remaining five groups contained no related individuals (58% of adults were sampled). Low incidence of relatedness provided evidence that smooth-billed ani groups do not contain large numbers of young remaining on the natal territory. There was also no evidence of regular dispersing within sibling units. One group provided evidence that young males will remain within the natal territory although breeding status is unknown. A young female dispersed from her natal nest to a neighbouring territory and was found in a group containing a male who was likely a second order relative. Adults tended to breed in the same territory in subsequent years and familiarity rather than kinship may be grounds for repeated nesting with the same female. Smooth-billed anis exhibit low reproductive skew and therefore the low incidence of relatedness seen between adults within groups agrees with the prediction of an egalitarian breeding system.

Introduction

Living in social groups involves a balance between co-operation and conflict. Conflict can evolve due to the partitioning of reproduction among group members. The distribution or amount of bias in reproduction within a group has been termed reproductive skew (Vehrencamp 1983a,b; Keller and Reeve 1994; Emlen 1996). The amount of skew observed in a system can vary from complete monopolization of reproduction by an individual (despotic system) to roughly equal reproductive sharing divided among group members (egalitarian system)(Vehrencamp 1983a). Dominant individuals in a group that can control subordinate breeding opportunities, should monopolize all reproduction at the expense of subordinates when they can, however if a dominant benefits from subordinate presence and the subordinate has dispersal options, the dominant may share reproduction as a staying incentive. This is the basis of the optimal skew theory (Vehrencamp 1983a,b; Reeve and Keller 1994).

The main factors that influence reproductive skew in groups are 1) the benefits of group breeding 2) dispersal options available to subordinates and the degree of success expected 3) asymmetry in dominance among group members 4) genetic relatedness between group members (Emlen 1996).

The dispersal patterns of young greatly influences the composition of groups (the relatedness of individuals). Offspring have four dispersal options 1) stay in the natal territory as a helper (forego breeding) 2) stay in territory and breed 3) disperse with relatives (siblings) 4) disperse alone. In non-social bird species, offspring usually

disperse from the natal territory and claim their own breeding territories the following breeding season. The young in some communal species stay in the natal territory during the breeding season. Often the delay of dispersal occurs when the costs of securing a suitable vacancy and breeding independently are high (Emlen 1994). The factors affecting dispersal are 1) shortage of good territory (related to habitat type or decreased adult mortality) 2) shortage of potential mates 3) low probability of success (experience) (Emlen 1994). In the communally breeding acorn woodpecker, up to 10 helpers are observed in groups along with breeders. These helpers are the young from the previous year (Koenig and Pitelka 1979). Thirty-one percent of the adults are non-breeding helpers. This high incidence of delayed dispersal is thought to be due to limited dispersal options because of conditions of severe environmental constraint (Koenig et. al. 1998). Jamieson et al (1994) pointed out that the number of helpers seen in pukeko species between the north and south islands of New Zealand differed. In the north, where helpers were seen, there was decreased adult mortality and low dispersal options for young while in the south, where the occurrence of helpers is rare, there was increased adult mortality and therefore increased dispersal opportunities. In groove-billed anis, the few helpers that remain on the natal territory were consistently the youngest of the year that had no real chance of dispersal (Koford et. al. 1990).

In some communal species, the dispersal of young is sex biased. In the groove-billed ani 12% of young stayed in the natal territory the year after hatching and almost all were male (Bowen et. al. 1989). Twenty-seven percent of males stayed in the natal territory while 8% of females stayed (Koford et. al. 1990). In acorn woodpeckers any

young found in a natal territory were usually the sons of the breeding individuals (Koenig and Stacey 1990).

Optimal skew theory states that dominants should monopolize breeding, limiting subordinate reproductive opportunities when dispersal options are low. The related young should forego breeding and help raise kin. Young do get breeding opportunities in their natal nest under some circumstances. Of 12% of groove-billed anis that remained in the natal nest, two thirds bred (Bowen et. al. 1989). The factors thought to affect the ability of offspring to breed in their natal nest are 1) dominance asymmetry (reproductive suppression due to dominance) 2) kin asymmetry (mothers and daughters are asymmetrically related to each other's offspring provided monogamy occurs and parents have more to gain by monopolizing reproduction than young have to lose by yielding) 3) incest avoidance. In both acorn woodpeckers and groove-billed anis, offspring in the natal nest remain as helpers unless the breeder of the opposite sex disappears or new unrelated immigrants join the group (Bowen et. al. 1989; Koenig and Stacey 1990; Koenig and Pitelka 1979). This behaviour is suggestive of incest avoidance. Incest between offspring and parents or siblings within a group does occur as seen in the moorhen (McRae 1996) and pukeko (Craig and Jamieson 1990). Deleterious effects of inbreeding were observed in the moorhen reflected by a decreased survival rate of offspring produced by father-daughter incest while no examples of decreased survival due to inbreeding were seen in pukeko.

For many young dispersal options are available. There may be opportunities to disperse with siblings or alone. There may be great advantages to dispersing with

siblings. Leaving the natal territory in unisex units helps decrease the chances of inbreeding. When territories or other resources are limited and must be fought for, coalitions may be advantageous. Sixty-five % of male acorn woodpeckers immigrate in the company of other related males with 50% of females dispersing in female units (Koenig and Pitelka 1979). Females that dispersed together were more likely to acquire high quality breeding territory and once acquired, were less likely to be evicted (Mumme et. al. 1988). These sibling units were egalitarian with both sisters gaining reproductive success from breeding and gaining indirect success from each other. Large coalitions of male lions were found to gain better access to groups of females (Packer 1991). In larger groups, only a few individuals have breeding opportunities therefore if individuals are related they can gain success through indirect fitness. Groups of non-relatives were egalitarian. Their group size remained small reducing their ability to acquire better territory.

Smooth-billed anis are a communally breeding species that are found in the Caribbean Islands, South America, Cost Rica and Florida (Quinn and Startek-Foote in press). They nest in pairs or groups. Little is known about the relatedness of individuals within these breeding groups. As in the groove-billed ani, reproductive skew is low, suggestive of an egalitarian system (Loflin 1983; Chapter 2). Relatedness is thought to be low in groove-billed anis with only a few of the youngest males remaining in the natal nest and no evidence of joint dispersal (Koford et. al. 1990). In a system exhibiting low reproductive skew or shared breeding, one might predict that individuals within a group would be unrelated.

The objective of this study was to estimate the relatedness of smooth-billed ani individuals within breeding groups using microsatellite analysis.

Methods

The smooth-billed ani samples used in this study were collected at the Cabo Rojo and Laguna Cartagena wildlife refuges in southwest Puerto Rico. All fieldwork was conducted during the peak in ani breeding activity Oct-Dec 1998 and Nov-Dec 1999. Adults were trapped using 2 3/4-inch mesh mist nets (41ft length) on 7.2m telescoping poles. Prerecorded ani call back was used to attract adults to the nets. Adults sampled in 1999 were also trapped using a hardware cloth cage containing a hand raised lure bird. Blood was taken by jugular venipuncture and adults were banded with a unique metal and colour band combination. Nine groups, each containing 2 or more adults were analyzed (Table 3.1). Nests with a total group size of 2 adults (a pair) were not included in analysis.

Three groups analyzed were sampled in 1998 and 6 groups were sampled in 1999. An average of 58% of adults were sampled from each group. Four individuals observed in the 1999 field season had been trapped in 1998 (CAN111, CAN112, CAN144, CAN079). Three individuals were adults known to have bred in 1998 (CAN111, CAN112, CAN144) while one hatched in the 1998 season (CAN079). Two females (CAN111, CAN112) were members of a group that bred in 1998 on the N. Lagoon territory. They were seen as part of the S. Panel group in 1999, which was observed on the same territory. Both females were tested for relatedness with individuals from each group. A female (CAN079) was born at Site L in 1998 and was observed as part of the Hillside group in 1999 in a neighbouring territory. The 1999 Home group contained a

female adult (CAN144) that bred as part of a pair in 1998 (Chapter 2). Data regarding breeding activity were collected for only those individuals sampled in 1998.

DNA was extracted from blood using a saturated salt protocol. As smooth-billed anis are sexually monomorphic, all adults were sexed using molecular techniques. CHD (chromo helicase binding domain) region primers P2 and P8 (Griffiths et al 1997) were used to amplify regions of the Z and W genes incorporating a variable intron region resulting in length variation. Females showed two distinct PCR products (Z-W) while males amplified one product (Z-Z).

All 27 adult samples were genotyped using smooth-billed ani microsatellite loci (Chapter 1).

Genetic Analysis

Potential relationships between nesting adults were assessed using the pedigree analysis program KINSHIP (Queller and Goodnight 1989). This program calculates pairwise likelihood values based upon population allele frequencies and tests 2 opposing relatedness hypotheses (i.e. are 2 individuals more likely to be full siblings or unrelated). Critical values to test the significance of the ratios were generated for $p < 0.05$ by a program simulation. A pairwise matrix reports log-likelihood ratios by group.

The type II error rate for attempting to distinguish full siblings from half siblings was 0.6 while the rate for distinguishing full siblings from unrelated individuals was 0.2 (as generated by KINSHIP). For this reason, a conservative approach was taken and

individuals were only tested for fullsib/unrelated. The adults within each group were compared to each other testing for full sibling against unrelated relationships. This test concluded whether 2 individuals were related to any degree (significant ratio) or unrelated (non-significant ratio).

Results

The 27 adult samples used for analysis were from 9 different groups containing 2 or more sampled adults (Table 3.1). The log-likelihood ratio required for significance ($p < 0.05$) was 0.534 as calculated by KINSHIP. In total, the likelihood ratios from 5 pairs of individuals proved to be significant indicating that the individuals within these pairs were related (Table 3.2). Two significantly related pairs were from the same nesting group (Hillside) while the 3 remaining dyads were from 3 separate groups.

Groups

L.C Road (1998)

Two males (CAN121, CAN124) from a 4 adult group were related. Both of these adults bred in 1998 (Chapter 2) and produced young. Both individuals shared at least one allele at all loci indicating the potential for a father-son relationship. One male (CAN124) was observed in the 1999 field season in the same territory used in 1998 in the company of a male (CAN118) who was born in the 1998 nest.

Site L (1998)

Two females (CAN089, CAN093) and one male (CAN073) were sampled from a 5-member group. The 2 females were related to each other but neither was related to the male. Both females bred in 1998 and produced young (Chapter 2). The females shared at least one allele at each locus indicating the potential for a mother-daughter relationship.

From this group, only CAN089 was observed in 1999 on the same territory with a group of unbanded birds.

S. Panel (1999)

Five females and one male were sampled from a group estimated to contain 10 individuals. This group contained 2 females (CAN111, CAN112) that bred and nested together in 1998 on the same territory as the 1999 group, but they were not related to each other. One female (CAN111) was related to a female (CAN171) in the 1999 group. They both shared an allele at each locus. The breeding activities of these females in 1999 were unknown. All other dyads in the group were unrelated.

Hillside

Six adults were sampled from a group with an estimated size of 9 (3 males and 3 females sampled). Two dyads involving a male and female each were related. A male (CAN169) and a female (CAN170) shared at least one allele at each locus. The breeding activity of either is not known. The second dyad included a female (CAN079) and a male (CAN168). Individual (CAN079) hatched in 1998 in a neighbouring territory (Site L) and individual CAN093 was assigned as her mother (Chapter 2). Her father was unknown. No other adults banded in 1998 were observed with her in the 1999 group. CAN168 could be a potential father to CAN079 as his alleles combined with those of the mother (CAN093) account for those seen in CAN079. Furthermore, all offspring at Site L

in 1998 were banded, therefore although CAN168 is related to CAN079, he is unlikely to be a sibling from the same natal year. All other dyads in the group were unrelated.

In all remaining groups (N=5), all sampled individuals were unrelated.

Discussion

Five microsatellite loci were used to estimate the relationships of adults within breeding smooth-billed ani groups (average locus heterozygosity=0.70). Blouin et al (1996) estimated that when using 5 loci with heterozygosity 0.75, the fraction of unrelated pairs misclassified as full sibs was 0.214. In order to distinguish between full and half siblings, 20 loci would be required for classification with 82% confidence. Therefore, the number of loci used in this study would likely not adequately distinguish between full and half siblings but should provide a reasonable estimation of related to unrelated individuals.

Twenty-nine adults from 9 breeding groups were compared and 10% of the dyads were significantly related. They tended to be related dyads grouped with other unrelated dyads. In systems with many helpers at the nest i.e. the acorn woodpecker system in which 31% of adults are helpers (Koenig et. al. 1998) or the pukeko system in which 71% of young remain permanently in the natal territory (Craig and Jamieson 1990), one would expect to find high incidence of relatedness within groups. Koford et al (1990) found that in groove-billed ani groups, where helpers are not as common only 12% of young stayed in the natal nest, the overall relatedness within groups was not high. The low incidence of relatedness found within smooth-billed ani groups indicates that they, like the groove-billed anis, do not appear to retain many young at the natal territory.

In the acorn woodpecker, joint-unisexual sibling dispersal is thought to allow siblings to gain high quality territories and share reproduction (Mumme et al 1988). Only

7% of females and 1% of males within groups were unrelated. Although male-male and female-female dyads were related in smooth-billed anis, the number was certainly not at the level that might be expected if sibling dispersal was a common strategy.

Kinship results did show all possible related pair combinations by sex (male-male, female-female, male-female). Although there is a low incidence of young remaining in the natal territory in groove-billed anis, those young that do remain, tend to be male (Bowen et al 1989). In the smooth-billed ani study nests, group (LC Road) contained 2 related males. The dyad could potentially be father-son. The next year (1999) a male that fledged from their 1998 nest was seen on his natal territory with one of breeding males. The breeding status of this male was unknown. Young male groove-billed anis tend to wait for unrelated females to join the group before breeding. Perhaps one of the 2 related males (CAN121, CAN124) originally belonged to a pair that allowed a son to take on an outside mate and increase the group size. Parents should tolerate this increase in group size until a threshold is reached at which time the increased group size begins to negatively affect success (Bowen et al 1989). Therefore, groups that remain on a territory for several years will likely contain some relatives, likely the young males that have stayed and bred.

A bias towards males remaining in the natal territory means that females tend to disperse. The only female young from 1998 seen in 1999 dispersed to a neighbouring territory. This dispersing female was related to a male in the group. This male could be her father (see results). Female acorn woodpeckers will not breed if they are in a group containing a male that bred during their natal year (Koenig and Pitelka 1979). Natural

selection is expected to favour the evolution of incest avoidance mechanisms, as the outcome of an incestuous mating is usually deleterious (Emlen 1995). Inbreeding avoidance should lead to a decreased tendency to pair with a close relative of the opposite sex. It would seem maladaptive for a female to disperse to a new territory with her father. This male is also likely not a sibling from the same natal nest (as they were all banded). Adults in 3 1998 groups remained in the same territory in the 1999 season although they were not all seen with group members from the previous season. If dispersal tends towards adjacent territories as is seen in the pukeko (Craig and Jamieson 1990), then relatedness between neighbouring groups may be seen. Perhaps this female dispersed to a group containing a half-sib from another nesting event or a cousin. The distance of juvenile dispersal can be difficult to assess for it is almost impossible to distinguish between young that did not survive and those that left the study site. Loflin (1983) found that of 209 young banded, only 6 were found on the study site. If neighbouring groups contain relatives from different natal nests, then these related individuals might come into contact almost by chance.

The female-female related dyad seen in Site L could be the result of a mother-daughter or perhaps a sister-sister union. Both females were known to breed in 1998 and therefore there is no evidence of reproductive suppression or delayed breeding due to incest avoidance (as might be expected if the father was still present in the group). Loflin (1983) observed young dispersing singly, in twos or threes. This dyad could also represent a sister-sister relationship. The equitable sharing of reproduction could be the result of common gain through indirect fitness (siblings) or the result of an incomplete

control system in which both females are competing for a limited amount of reproduction.

The only adults seen breeding as part of the same group 2 years in a row were 2 unrelated females (CAN111, CAN112). Mumme et al (1988) suggests that perhaps familiarity rather than kinship explains the tendency for females to group together over consecutive years. It is possible that joint-nesting coalitions are formed between females that have established a social bond. An egalitarian system in which reproduction is shared is often found within groups of unrelated individuals (Vehrencamp 1983). Smooth-billed anis exhibit a breeding system in which reproductive opportunities are available to all group members and skew is low. Therefore the low incidence of relatedness found within smooth-billed ani nesting groups agrees with the prediction of an egalitarian system.

Conclusions

Only 10% of smooth-billed ani dyads were related (to any degree) with 58% of adults sampled. There was no evidence of many related members within groups as might be expected from a system in which many young remain at the natal territory or regularly disperse in sibling units. Related dyads included males and females and there was no tendency for sex-bias relatedness. There were indications that male young may remain on the natal territory although their breeding status was not determined. Some adults tend to breed in the same territory for consecutive seasons and evidence that female young disperse to neighbouring territories may result in relatedness between neighbouring

groups. Familiarity rather kinship may be grounds for repeated nesting with the same female.

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Table 3.1: The groups of adult smooth-billed anis tested for within group adult relatedness.

GROUP (Refuge)	YEAR SAMPLED	EST. GROUP SIZE	NO. ADULTS SAMPLED	SEX OF ADULTS SAMPLED	
LC Road(LC)	1998	4	2	2 M	
Site L (CR)	1998	5	3	1M	2F
N. Lagoon (LC)	1998	4	3	1M	2F
S. Panel (LC)	1999	10	6	1M	5F
Home (CR)	1999	5	4	1M	3F
S. Fence (CR)	1999	7	3	1M	2F
Hillside (CR)	1999	9	6	3M	3F
Greenbean (CR)	1999	4	2	1M	1F
UPlands (CR)	1999	6	2	1M	1F

Table 3.2: The individual smooth-billed anis involved in a significantly related dyad ($p < 0.05$) within a breeding group.

GROUP	RELATED INDIVIDUALS (SEX)		NO. ADULTS SAMPLED	EST. GROUP SIZE
LC Road	CAN121 (M)	CAN124 (M)	2	4
SiteL	CAN089 (F)	CAN093 (F)	3	5
S. Panel	CAN111 (F)	CAN171 (F)	6	10
Hillside	CAN079 (F)	CAN168 (M)	6	9
	CAN170 (F)	CAN169 (M)		