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MOLECULAR GENETIC (RAPD) ANALYSIS OF BREEDING MAGPIE GEESE

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ABSTRACT.—Magpie Geese (*Anseranus semipalmata*) typically form nesting trios consisting of two females and one male, an uncommon breeding arrangement for waterfowl. To study aspects of their reproductive biology, 20 reproductively active adults representing seven reproductive groupings (broods) were scored for 106 polymorphic primer-specific RAPD bands. Genetic distances between all possible adult pairs were calculated, and these values were analyzed using the Mantel test. In the pairwise comparison of genetic distances, those between males were significantly less than those for other adult combinations ($P < 0.05$). Using the same analytical approach, we found that females that shared a nest were more like one another than were females that did not share a nest ($P < 0.05$). We suggest that a plausible hypothesis to account for the relative genetic homogeneity of the male population is that males seeking to enter the breeding population do so at their natal site. The females they recruit are more closely related to one another than are females in general. *Received 5 April 1995, accepted 29 June 1995.*

LARGE NUMBERS of Magpie Geese (*Anseranus semipalmata*) congregate at seasonal wetlands in tropical northern Australia. The bird's unique morphology (including partially webbed, heavily clawed feet with an opposable fourth toe) led to its taxonomic placement in its own family. This uniqueness also is evident at the molecular level, and recent taxonomies derived from mitochondrial DNA sequences support the view that this species should be assigned to a family of its own (Sraml et al. 1996).

Although abundant in parts of northern Australia, the current distribution of the Magpie Goose is only a remnant of its former range, which has been severely restricted over the past 200 years by the introduction of European-style agricultural practices. Situations that threaten this species could arise again as the result of conflicts between the bird's habitat requirements and changing human activities. Proposed changes associated with tropical agriculture could threaten the conservation status of these birds (Whitehead 1991).

In attempting to sustain adequate numbers of a species, it is important to understand the species' reproductive biology. Unlike most waterfowl, Magpie Geese commonly form breeding trios consisting of two females and one male (Frith and Davies 1961). In situations where females share a nest, all three attendants typically have a direct reproductive (genetic) interest in the clutch, and they are involved in many aspects of care and defense of the eggs and young. Chicks remain with their parents for their first year of life, possibly learning about seasonal resource distribution (Whitehead and Tschirner 1990). The stability of these polygynous groups from year to year distinguishes Magpie Geese from other communal breeders (Brown 1987).

When joined in a reproductive trio, individual females seem to decrease their potential reproductive output as measured by numbers of eggs laid. Egg counts ($\bar{x} = 8.6$) in nests with only one female and one male are not much smaller than those ($\bar{x} = 9.4$) from nests with two females and one male (Frith and Davies 1961). In addition, behavioral observations of trios suggest that males mate with both females and that both females contribute eggs approximately equally to these joint nests. Assignments of young to specific females have been done using restriction-fragment-length-polymorphism (RFLP) analysis of DNA. In making those assignments, conspecific nest parasitism was

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identified in only 1 of 36 young (Horn unpubl. data).

The observation that there are fewer eggs and young per female, when females are associated in reproductive trios, raises an interesting question about the relationship between the females attending the nest. If females sharing a nest are in fact more closely related to one another than are females in general, then the observed decrease in egg output (and lowered numbers of viable young) might be offset by the fact that care and defense of the young are being directed toward offspring of a closely related female (i.e. a female sharing many genes). This could compensate for what might otherwise seem to be an arrangement that would decrease the reproductive output of nest-sharing females.

In this study, we explored the question of adult relatedness in reproductively active Magpie Geese using the molecular-genetic technique called random amplification of polymorphic DNA (RAPD). The RAPD technique (see Hadrys et al. 1992) of examining genotypes (Williams et al. 1990) has been used to identify species and to study variation within and among populations (Huff et al. 1993). This modification of the polymerase chain reaction (PCR) amplifies segments of genomic DNA that are primer-specific and reproducible but of varying lengths. The amplification primers used are random sequences that are 10 nucleotides long. The primer binds to homologous bases, and the PCR-like protocol replicates the intervening base sequence. The variable lengths of DNA amplified in this fashion are inherited as classical Mendelian traits (Williams et al. 1990) and, thus, can be used for genetic analysis. Here, primer-specific amplified DNA bands of reproducible lengths are considered to be attributes just as any other genetically determined trait. We have used the technique to determine the pattern and extent of RAPD variation within a natural, freely ranging bird species. Our data show that males are very similar and that nest-sharing females are more similar to one another than are females that do not share a nest.

Methods

Known Magpie Goose breeding areas on the Mary River flood plain (12°34'S, 131°20'E) in the Northern Territory of Australia were searched a few days after the peak of hatching in May 1989. Only reproductive groups clearly isolated from other Magpie Geese in open habitats were collected. Unlike many other geese, Magpie Geese do not undergo molt-induced flightlessness, thus retaining the ability to fly throughout the year. Collecting birds was the only feasible method of taking entire groups in this extensive wetland habitat. Collecting isolated groups minimized the potential for mixing goslings between family groups. Family groups comprising at least three adults and accompanying goslings were identified by their behavior when approached by a motorized airboat. Adult group members tended to remain with the goslings. Females led the brood away from the source of disturbance, while males interposed themselves between the brood and observers, and often performed threat displays. Seven groups (designated B, C, D, E, F, G and H) were collected. Adults were shot and 20 g of liver were removed and chilled immediately on salted ice. The sex of adults was confirmed by gonadal examination. Other tissues were used in a variety of studies. Each individual was identified for future reference by the designated family letter, sex, and its number within the trio. In one family group (G) there were four adults (three females and one male), and another adult trio (family C) consisted of two males and one female.

DNA extraction and quantification followed standard protocols of proteinase K and SDS digestion followed by phenol and chloroform extraction of liver pulverized in liquid nitrogen (Maniatis et al. 1982). Following overnight dialysis against TE buffer, extracted nucleic acid was precipitated with ethanol, resuspended in TE, and quantified using absorbance at 260 and 280 nm.

DNA was diluted and optimal concentrations were determined for the RAPD amplification of each individual sample. This involved amplifying four doubling dilutions of each sample in the range of 10 to 40 ng/μL in order to choose the DNA concentration that gave the clearest and most consistent amplification results. Such optimized concentrations were then considered to be "working" dilutions and were used in all further RAPD reactions. Carefully optimizing concentrations of DNA ensures reproducible RAPD amplification runs. Reproducibility was further checked by including a duplicate of one individual (BF-1) in each run and checking these two for identical amplification patterns. RAPD reactions were set up as described by Williams et al. (1990) in final volumes of 25 μL containing 10 mM Tris-Cl; pH 8.3; 50 mM KCl; 2 mM $MgCl_2$; 0.02 μmoles of 10-mer primers (Operon, Alameda, California); 100 μM each of dATP, dCTP, dGTP and dTTP; and 0.5 units of Taq DNA polymerase (Perkin Elmer Cetus, Foster City, California). Primer-specific mixes were made and 24 μL were pipetted into 1.5-ml Eppendorf tubes containing 1 μL of the DNA of a specific individual. A 50-μL paraffin oil overlay prevented evaporation during the thermal-cycling reaction.

Amplification-cycle parameters were: 45 cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C, which were performed overnight on a Perkin Elmer Cetus DNA Thermal Cycler using the fastest available transition between temperatures. Following amplification, samples were analyzed on 1.2% agarose gels. Ethidium bromide was incorporated into the gels to stain the amplified products. Stained gels were photographed while illuminated with an ultraviolet light box.

Only amplifications that produced clear, crisp bands were analyzed. For instance, if primer amplifications resulted in a smeared pattern, neither the gel nor the specific primer was considered for analysis. About one-third of the primers tested did not produce interpretable data. Photographs of interpretable gels were scored as a 1 (band present) or 0 (band absent) for individual birds, and only polymorphic bands produced by primers were tabulated. Gel photographs were scored by two people independently, and only agreed-on scores were used. Fewer than 2% of scores were discarded owing to disagreements.

A matrix was produced showing the presence or absence of 106 bands for 20 individuals. Two measures were calculated between individuals: a simple band-sharing similarity and a genetic distance (Nei and Li 1979). Band-sharing similarity was calculated by summing the number of bands shared between each pair of individuals (Table 1, upper right). Pairs with high scores are most similar genetically. After determining that each string of 1 and 0 scores was a unique array (meaning that each individual had a unique string of traits), we calculated all possible pairwise genetic distance values (Nei and Li 1979):

$$d_{xy} = 1 - [2n_{xy}/(n_x + n_y)], \quad (1)$$

where n_x and n_y are the numbers of bands amplified in individuals x and y, respectively, and $2n_{xy}$ is the number of bands shared by those individuals. In this analysis, smaller numbers are associated with more genetically similar individuals, whereas larger numbers suggest genetic dissimilarity (Table 1, lower left).

RESULTS

We used 87 primers to amplify the DNA from a population of 20 reproductively active adult birds. Of these, 53 produced scorable gels and 37 produced polymorphic patterns, yielding 106 scorable polymorphic bands. An average of 6.2 bands was produced per primer, but only polymorphic bands for a given primer were scored. A typical scorable gel is shown in Figure 1, where polymorphic as well as monomorphic bands can be seen. Primers with 3′ endings of either C or G were, in general, far more likely to produce interpretable bands than were primers whose 3′ endings were A or T.

In the summed band analysis, the absence of a band is not considered as a similarity because the two individuals being compared may lack the band for different reasons (e.g. a single base mutation preventing primer binding vs. a gene deletion). In Table 1 (upper right), the summed band-sharing values range from 27 to 69 with a mean of 50 (106 possible). For females who were sharing a nest ($n = 8$), the shared-band average value was 52.5 (49.5%). Among all other female comparisons ($n = 83$), the number of shared bands averaged 47.6 (44.9%). The male-male comparisons ($n = 15$) had an average band-sharing score of 56 (52.8%), and in the nest (family C) consisting of a trio of two males and one female, the two males shared 55 of 106 (51.8%) of the bands. The most dissimilar pair involved two females from different nests; they shared only 27 of 106 (25%) polymorphic bands. In general, the other low scores also involved comparisons of females that did not share a nest. The highest score (69 of 106, or 65%) also was between two non-nest sharing females.

Calculations of genetic distance yielded values ranging from 0.183 to 0.573 (Table 1). The extreme values were derived from two females from different nests. Values for females sharing a nest ($n = 8$) averaged 0.313, whereas females not sharing a nest averaged 0.358 ($n = 83$). Values from male-male comparisons ($n = 15$) averaged 0.279. The genetic distance matrix data were subjected to a Mantel test (Schnell et al. 1985) to assess genetic similarity among adults of different breeding status relative to the population at large. Reproductively active males were more similar genetically than were adults in general (Mantel $t = 2.301$, $P < 0.05$). In addition, females sharing a nest were more alike genetically than were females in general ($t = 2.161$, $P < 0.05$). Females sharing a nest, however, were no more genetically similar to one another than were random pairs of adults, without regard to nest association or sex ($t = 1.210$, $P > 0.05$).

An interesting situation was found in family G, which consisted of three adult females, one adult male, and nine goslings. From morphometric data, the three females were all of reproductive age, yet RFLP findings indicated that seven of nine goslings could be assigned to GF-2 or GF-3. Although some inconsistencies could not be resolved, it was not possible to unambiguously assign any of the goslings to GF-1, suggesting that G-1 may not have contributed any eggs to this nest (Horn unpubl. data).

TABLE 1. Number of primer-specific polymorphic RAPD bands (out of 106) shared between pairs of reproductively active adults (upper right) and genetic distance among adults (lower left). Individuals identified by family letter (B to H), sex (F or M), and number within family.

	BF-1	BF-2	C3	DF-1	DF-2	EF-1	EF-2	FF-1	FF-2	GF-1	GF-2	GF-3	HF-1	HF-2	BM	C1	C2	DM	FM	HM
BF-1		55	37	46	43	40	42	42	44	35	43	39	44	45	56	52	51	45	53	35
BF-2	0.287		43	55	51	40	63	59	50	50	61	46	50	56	53	53	62	53	54	55
C3	0.456	0.400		42	48	44	41	49	41	38	46	49	35	49	49	46	40	42	41	45
DF-1	0.368	0.294	0.404		52	27	50	52	57	42	47	44	41	50	50	49	46	48	54	42
DF-2	0.396	0.320	0.352	0.320		41	54	49	51	49	50	54	43	53	53	46	56	51	44	58
EF-1	0.431	0.431	0.388	0.573	0.423		45	36	37	33	36	44	36	36	44	53	54	42	38	44
EF-2	0.404	0.225	0.416	0.333	0.302	0.375		59	48	48	59	52	50	50	57	55	55	53	50	64
FF-1	0.406	0.259	0.346	0.314	0.340	0.468	0.261		48	46	53	56	48	54	55	53	45	53	46	53
FF-2	0.385	0.333	0.416	0.276	0.321	0.464	0.351	0.351		58	51	52	48	69	54	47	52	50	47	50
GF-1	0.481	0.333	0.451	0.410	0.340	0.500	0.351	0.369	0.263		61	48	52	54	50	43	46	44	45	56
GF-2	0.396	0.239	0.374	0.365	0.333	0.474	0.259	0.310	0.328	0.241		57	55	58	57	52	51	51	51	62
GF-3	0.439	0.368	0.345	0.389	0.303	0.391	0.316	0.350	0.277	0.350	0.277		61	58	58	53	53	53	48	57
HF-1	0.388	0.333	0.477	0.414	0.400	0.472	0.333	0.352	0.352	0.315	0.291	0.243		54	51	47	53	45	46	46
HF-2	0.382	0.282	0.345	0.333	0.304	0.475	0.333	0.299	0.183	0.300	0.262	0.268	0.292		58	51	51	55	48	59
BM	0.285	0.310	0.339	0.333	0.304	0.385	0.273	0.293	0.300	0.333	0.273	0.262	0.324	0.264		58	56	60	57	54
C1	0.314	0.304	0.368	0.346	0.370	0.310	0.287	0.304	0.362	0.397	0.316	0.305	0.358	0.332	0.263		55	51	58	51
C2	0.320	0.236	0.429	0.371	0.283	0.297	0.292	0.382	0.316	0.368	0.322	0.310	0.308	0.328	0.283	0.293		63	56	61
DM	0.379	0.309	0.411	0.352	0.321	0.405	0.310	0.309	0.333	0.386	0.322	0.310	0.383	0.294	0.250	0.328	0.228		58	59
FM	0.307	0.296	0.418	0.301	0.385	0.450	0.333	0.370	0.357	0.375	0.327	0.351	0.371	0.350	0.271	0.263	0.286	0.268		51
HM	0.480	0.290	0.376	0.412	0.262	0.390	0.218	0.308	0.333	0.279	0.232	0.274	0.365	0.259	0.299	0.327	0.243	0.263	0.321	

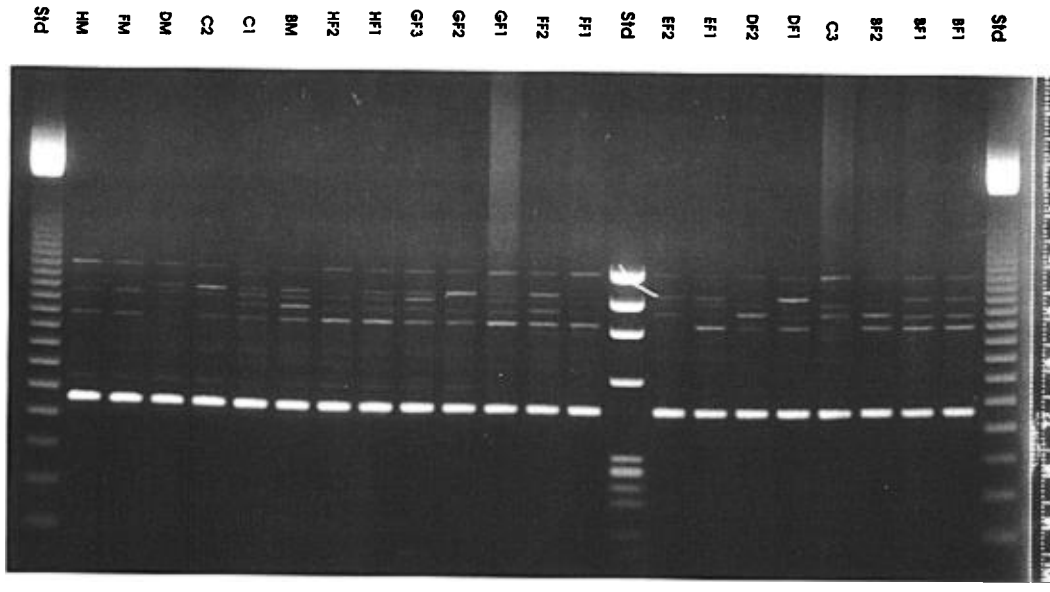

FIG. 1. Agarose gel (1.2%) stained with ethidium bromide. Twenty reproductive adult Magpie Geese DNA samples amplified with primer I05. Lanes labeled Std contained DNA of known molecular weights and are used as a markers.

DISCUSSION

The RAPD technique proved to be a reliable method for evaluating polymorphisms within this population. Once the appropriate DNA dilutions were selected, the random primers that worked (i.e. amplified bands that were readable on gels) could be used to screen numerous individuals rapidly. The scored arrays of presence or absence of primer-specific amplified bands of particular length could be used to identify individuals as unique, thus allowing comparisons of genetic similarity among individuals. We are aware of the limitations to interpreting RAPD-derived bands (e.g. reliance on the distance of band migration [hence size] to claim band identity and the fact that bands of DNA of similar length may not necessarily have the same base sequence [Hadrys et al. 1992]). Yet, the technique can readily distinguish among individuals within a population. This information can be used to evaluate genetic variation within a population and, hence, population structure. In our case, we used RAPD to explain how the genetic variation within a breeding population related to the reproductive behavior of a species.

The numbers of primer-specific bands amplified in our study were not as high as reported by others (Huff et al. 1993); however, this is likely to be a reflection of the resolution of agarose gels, rather than any inherent property of the DNA of Magpie Geese. The average genetic distance between all possible pairs of adult Magpie Geese (0.338) was greater than those reported by Huff et al. (1993) within two natural populations of a grass species. This suggests that there is more genetic diversity in our population of freely ranging adult birds than there was in the obligate outcrossing grass populations studied by Huff et al (1993).

Unfortunately, little is known about dispersal patterns in Magpie Geese. Therefore, it is difficult to relate our observation of genetic similarity among males (which is suggestive of natal philopatry) to independently derived data on choice of sites for reproduction. In many waterfowl species, banding and other movement studies have shown that females are more likely to return to the natal area than are males (e.g. Hepp et al. 1989, Gauthier 1990). However, it is known from one radio-telemetry study that adult male Magpie Geese tend to nest in the same breeding colony in consecutive years (Whitehead unpubl. data). That study also included two males that failed to breed but still returned to the vicinity of the same colony where they had been captured in association with a nest the previous year. Although it can be concluded that adult males tend to display

strong fidelity to a colony in which they have nested previously, there are no data indicating that this fidelity is confined to the natal area. Comparable dispersal data for female Magpie Geese are not available.

In the absence of behavioral information, explanations for high relatedness among female Magpie Geese nesting jointly are inevitably speculative. For example, if sisters tend to remain together after ejection from the family group, they may also enter a breeding group together. Alternatively, daughters of "widowed" females may remain with their mothers when recruited to a breeding group by a new male. The manner in which females enter breeding groups, together with many other aspects of the breeding system of the Magpie Goose, will remain enigmatic until a long-term study of a substantial marked population is conducted, including more comprehensive genetic research. The primary significance of our study is the guidance it provides for framing relevant hypotheses and designing robust tests of those hypotheses.

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