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Helpers at a *Sapayoa* nest are kin

by Benjamin M. Van Doren, Jack P. Hruska, Sarah A. Dzielski & Bronwyn G. Butcher

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Summary.—The natural history of the Sapayoa *Sapayoa aenigma*, the sole member of the Sapayoidae and the only New World representative of the 'Old World suboscines', is poorly known. Previously, we reported a pair of adult Sapayoas breeding with assistance from two immature males, but their kinship was unknown. Here, we use double-digest restriction site-associated DNA sequencing (ddRAD-seq) to conduct parentage and relatedness analyses among this group. We found that the members of the adult pair were unrelated, but all other dyads were probably first order (parent-offspring or full sibling). In addition, the helper males were very unlikely to have sired either of the chicks. We conclude that the group consisted of two unrelated adults and two offspring from a previous brood. These results provide important context for social behaviours observed within the group, which included mounting events; such behaviour may be involved in group cohesion.

Sapayoa *Sapayoa aenigma* is the sole member of one of the world's least known bird families, Sapayoidae (Winkler *et al*. 2015). Resident in wet Chocó forests of south-west Panama, western Colombia and north-west Ecuador, *Sapayoa* has perplexed taxonomists for decades; only recently have molecular studies revealed that it is nested within the Old World suboscines (Sibley & Ahlquist 1990, Fjeldså *et al*. 2003, Chesser 2004, Irestedt *et al*. 2006, Moyle *et al*. 2006, Selvatti *et al*. 2015). Nonetheless, its precise relationships within this group remain debatable. Furthermore, a paucity of natural history data limits the potential for comparative studies with other Old World suboscines.

The first descriptions of the nest and nestlings of the Sapayoa suggested that parental care was undertaken by two adults (Christian 2001). However, Dzielski *et al*. (2016) subsequently reported the presence of helpers at a Sapayoa nest, and thus that cooperative breeding occurs. At that time, we were unable to determine if the helpers were related to the breeding pair, which is a common scenario among cooperatively breeding species (Skutch 1999, Cockburn 2006). We also documented unusual social behaviours in the breeding group, which consisted of an adult male, adult female and two immature males. Specifically, all four individuals participated in mounting behaviours, which were always preceded by a solicitation display. Usually, the adult female gave the display before being mounted by a male. Additionally, we recorded occasional mountings between males. The function of these behaviours, which occurred during nest provisioning, is unclear. Were they explicitly reproductive in nature, or did they serve a non-reproductive social function?

We proposed three possible non-reproductive functions for these mounting behaviours: enforcement of a dominance hierarchy among members of the group, maintenance of social cohesion within the group, and experience-gaining through practice. Non-reproductive mounting behaviours have been documented in another species, Acorn Woodpecker *Melanerpes formicivorus*, but their function is unknown (MacRoberts & MacRoberts 1976, Koenig & Walters 2014). Given the prevalence of presumed inbreeding avoidance behaviours

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in birds (Koenig & Dickinson 2004), the possibility that the Sapayoa nest helpers could be both related to the breeding pair and involved in reproduction warrants investigation.

To shed further light on these behaviours and better understand *Sapayoa* reproductive biology, we sequenced genomic DNA from the same family group of Sapayoa studied by Dzielski *et al*. (2016). We posed two primary questions. (1) Were the two nest helpers related to the breeding pair? (2) Did either of the nest helpers sire one or more nestlings?

Methods

Sample collection.—We obtained genetic material from six *Sapayoa aenigma* collected in Darién National Park, Panama. This material is archived at the Cornell University Museum of Vertebrates, Ithaca, NY (CUMV). Four of these—an adult male (CUMV 55871) and female (CUMV 55868) and two immature males (CUMV 55971–972)—were provisioning a single nest containing two nestlings (CUMV 55869–870). Detailed information concerning all individuals is presented in Dzielski *et al*. (2016). Genomic DNA was extracted from blood preserved in ethanol using Qiagen DNeasy Blood and Tissue kits.

ddRAD sequencing.—We used double-digest restriction site-associated (ddRAD) sequencing to identify single-nucleotide polymorphisms (SNPs) across the *Sapayoa* genome for use in parentage and relatedness analysis. We followed the methods of Thrasher *et al*. (2018) to sequence DNA and identify SNPs. The ddRAD libraries from these *Sapayoa* samples were prepared and sequenced along with 232 other samples from species unrelated to this study. We used approximately 200 ng of DNA digested with SbfI and MspI, ligated unique barcode adapters, and pooled samples. We purified DNA, selected fragments of 450–600 bp, and added Illumina sequencing adapters. We pooled samples in equimolar ratios and sequenced the pooled library on an Illumina HiSeq 2500 (100 bp, single end).

Trim, filter and demultiplex.—Again following Thrasher *et al*. (2018), we assessed read quality using FASTQC (www.bioinformatics.babraham.ac.uk/projects/fastq) and trimmed sequences to 97 bp using fastX_trimmer (FASTX-Toolkit). We removed reads containing a single base with a Phred quality score of <10 (using fastq_quality_filter). We additionally removed sequences if more than 95% of the bases had a Phred quality score of <20. Using process_radtags, we demultiplexed the sequences to obtain sequences specific to each individual.

De novo assembly.—We assembled the sequences de novo using the Stacks pipeline, following Thrasher *et al*. (2018). First we used denovo_map.pl to assemble the reads into a catalogue with a minimum stack depth of 5 (m) and allowing up to five mismatches between loci within an individual (M) and five mismatches between loci when building the catalogue (n). Corrections were made by running rxstacks. Loci with a log likelihood less than -20 (lnl_lim -20) or that were confounded in 25% of the population were removed (conf_lim 0.25). We then reran cstacks and sstacks with this new catalogue.

SNPs identified.—We identified SNPs using the populations module within Stacks, following Thrasher *et al*. (2018). All of our samples were considered in one population and a locus was processed if it was present in 95% of the individuals (r) at a stack depth of ≥10 (m). The data were restricted to the first SNP per locus (write_single_snp) and a minor allele frequency of 0.25 was required to process a nucleotide site at a locus (min_maf).

Parentage and relatedness.—We performed a paternity analysis using Cervus (Marshall *et al*. 1998, Kalinowski *et al*. 2007) to determine whether the father of each nestling was the adult male or one of the helpers. We assumed that the adult female was the mother of both nestlings because she was the only female present at the nest. We also performed a parentage analysis for the nest helpers to determine the likelihood that they were offspring of the adult pair. Finally, we calculated pairwise relatedness coefficients for all individuals

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using the R package SNPRelate (Zheng *et al*. 2012). We multiplied coefficients by two so that they scaled from 0–1, with 0.5 being the theoretical expectation for full siblings or a parent-offspring relationship. We calculated bootstrapped 95% confidence intervals for each comparison with 10,000 iterations, sampling with replacement from included loci.

Results

Post-filtering, we retained 672 SNP loci for analysis. Paternity analysis using Cervus showed that the adult male was far more likely to be the father of the nestlings than either immature male (Table 1). When we considered the adults as possible parents of the helpers, we found that the number of mismatched loci between adults and helpers was comparable to that of adults and nestlings (Table 2). This strongly suggests that the adults were also the parents of the immature male helpers, and is supported by low non-exclusion probabilities (Table 2). Finally, relatedness scores between individuals of the group were relatively high (mean 0.35 ± SD 0.12) and similar for all comparisons, with one exception: the adult male and female showed far lower relatedness scores than all other comparisons (Table 3).

Discussion

These data show that the adult male and female were less closely related to each other than to all other members of the breeding group. In addition, they confirm that both chicks were offspring of the pair and support the hypothesis that the immature male helpers were young from an earlier brood. A scenario in which young relatives assist an experienced adult pair is common among avian cooperative breeders (Skutch 1999, Cockburn 2006). Among the Old World suboscines, multiple species of broadbills have been observed to breed cooperatively, at least occasionally (Lambert & Woodcock 1996, Bruce 2003). The lack

TABLE 1
Paternity analysis of nestling Sapayoas *Sapayoa aenigma*, comparing adult and immature males.

Offspring ID	Candidate father	Mismatched loci: adult female and candidate male (no. compared)	Trio LOD score	Most likely father
Chick (male)	Adult male	23 (669)	45.62	*
	Immature male 2	95 (669)	-286.55	
	Immature male 1	105 (668)	-349.47	
Chick (female)	Adult male	36 (671)	67.15	*
	Immature male 2	104 (671)	-281.34	
	Immature male 1	117 (670)	-315.58	

TABLE 2
Parentage analysis of nestling and immature Sapayoas *Sapayoa aenigma*, showing number of loci inconsistent with parentage by adult male and female.

Offspring	Mismatched loci: adult female (no. compared)	Mismatched loci: adult male (no. compared)	Mismatched loci: both adults (no. compared)	Parent pair non-exclusion probability
Chick (male)	9 (668)	13 (669)	23 (670)	1.86×10^{-71}
Chick (female)	24 (670)	12 (671)	36 (672)	1.30×10^{-74}
Immature male 1	10 (668)	15 (669)	26 (670)	2.62×10^{-78}
Immature male 2	9 (669)	14 (670)	24 (671)	5.63×10^{-76}

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TABLE 3
Relatedness matrix scaled from 0–1. Parentheses show bootstrapped 95% confidence intervals.

	Adult female	**Adult male**	**Chick (female)**	**Chick (male)**	**Immature male 1**	**Immature male 2**
Adult female	1	0 (0–0.135)	0.341 (0.27–0.401)	0.42 (0.371–0.5)	0.427 (0.38–0.5)	0.5 (0.397–0.5)
Adult male	0 (0–0.135)	1	0.417 (0.372–0.472)	0.417 (0.366–0.5)	0.406 (0.354–0.5)	0.409 (0.358–0.5)
Chick (female)	0.341 (0.27–0.401)	0.417 (0.372–0.472)	1	0.356 (0.304–0.423)	0.329 (0.264–0.396)	0.356 (0.291–0.5)
Chick (male)	0.42 (0.371–0.5)	0.417 (0.366–0.5)	0.356 (0.304–0.423)	1	0.234 (0.165–0.322)	0.344 (0.276–0.413)
Immature male 1	0.427 (0.38–0.5)	0.406 (0.354–0.5)	0.329 (0.264–0.396)	0.234 (0.165–0.322)	1	0.287 (0.209–0.359)
Immature male 2	0.5 (0.397–0.5)	0.409 (0.358–0.5)	0.356 (0.291–0.5)	0.344 (0.276–0.413)	0.287 (0.209–0.359)	1

of relatedness between the adult male and female—the breeding pair—is also consistent with observations in other species and the 'incest avoidance' hypothesis.

Our results lend support to the hypothesis that mounting events between individuals in this Sapayoa family were not explicitly related to reproduction. We believe that the most likely explanation for this behaviour is to maintain cohesiveness of the breeding group. The adult female expended considerable effort soliciting mountings from both adult and immature males, and in turn these individuals consistently provisioned the nest (Dzielski *et al*. 2016). If soliciting mountings reinforces group bonds or otherwise encourages males to persist in the provisioning effort, the female would benefit from a reduced burden to deliver food. Mountings between males could likewise be explained by the group cohesiveness hypothesis. In contrast, a dominance hierarchy (see, e.g., Briskie 1992, Eason & Sherman 1995) is less likely to explain these observations because males did not vie for access to the female, and a mounting was always preceded by a solicitation display given by the receiving individual.

The most likely scenario is that all related individuals were first-order relatives (full siblings or parents and offspring), yet relatedness values for most pairs fell below the expected 0.5 value. In practice, relatedness values estimated using SNP data can vary far from their theoretical expectation, especially when the sample consists of only a small number of closely related individuals (Wang 2017).

Conclusion

Overall, this information provides valuable context for the observations reported by Dzielski *et al*. (2016) and indicates that *Sapayoa aenigma* will breed cooperatively with assistance from their kin. This scenario may occur in other Old World suboscines (e.g. multiple broadbill species), but cooperative breeding in these taxa remains to be studied in detail. *Sapayoa* also requires further study, especially the apparently rich array of social interactions that occur in this species.

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