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SHORT COMMUNICATION

Extra-pair paternity in a Neotropical rainforest songbird, the White-necked Thrush *Turdus albicollis* (Aves: Turdidae)

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ABSTRACT. Over the last two decades, several studies have shown that the mating systems of various birds are more complex than previously believed, and paternity tests performed with molecular techniques have proved, for instance, that the commonly observed social monogamy often presents important variations, such as extra-pair paternity. However, data are still largely biased towards temperate species. In our study, at an area of the Brazilian Atlantic Forest, we found broods containing at least one extra-pair young (EPY) in the socially monogamous White-necked Thrush *Turdus albicollis* (Vieillot, 1818). Paternity tests using six heterologous microsatellite loci revealed that four of 11 broods (36.4%) presented at least one extra-pair young (EPY). This rate of EPY is within the range found for other studies in the tropics. This is one of the few studies that present detailed paternity analyses of a Neotropical rainforest passerine. Our findings corroborate the early insights that breeding strategies involving cheating can also be widespread among Neotropical socially monogamous songbirds.

KEY WORDS. Cuckoldry, extra-pair copulation, infidelity, social monogamy.

Over the last two decades, a number of studies have demonstrated that the mating systems of various birds are more complex than previously believed (reviewed in Griffith et al. 2002). For instance, paternity tests using molecular techniques have documented that different levels of extra-pair paternity (EPP) do occur. EPP can be defined as the proportion of offspring sired by only one social parent, usually the female, or the proportion of broods containing at least one extra-pair young (EPY) (Westneat et al. 1990, Ligon 1999, Griffith et al. 2002, Neodorf 2004). The occurrence of EPP and EPY appear to be widespread among passerine birds (Westneat et al. 1990, Griffith et al. 2002, Neodorf 2004), yet this information is largely biased towards populations from temperate zones (Stutchbury & Morton 2008, Macedo et al. 2008), since more research on this subject has been conducted in that region. The Neotropical region holds almost half of the world's avifauna (Myers et al. 2000, Hawkins et al. 2003), and Neotropical bird species differ from temperate species in many important aspects. For instance, they present reduced clutch sizes (Martin et al. 2000, Jetz et al. 2008), extended breeding seasons (Styrsky & Brawn 2011), and occupy different types of habitats, which could potentially affect the frequencies of this breeding strategy (Davies et al. 2003, Brennan 2012). A few studies involving parentage analyses carried out in Neotropical passerines have revealed a wide range of EPY levels, from 0% in Dusky Antbird *Cercomacra tyrannina* (Sclater, 1855) (Fleischer et al. 1997) to 67% in Lesser Elaenia *Elaenia chiriquensis* (Lawrence, 1865) (Stutchbury et al. 2007).

As the lack of parentage studies on Neotropical birds limits broad conclusions about passerine social systems, here we used microsatellite molecular markers to perform paternity tests in a population of the White-necked Thrush, *Turdus albicollis* (Vieillot, 1818), from a well-preserved Brazilian Atlantic Forest area. Our specific objective was to investigate the occurrence of EPY in this Neotropical songbird.

Field work was conducted at Carlos Botelho State Park (24°04'S 47°58'W), São Paulo State, southeastern Brazil. This Park holds 37,644 ha of Atlantic Rain Forest, and together with a set of adjacent conservation units, comprises one of the largest Atlantic Forest remnant in Brazil, with more than 1 million ha.

Altitude varies from 30 to 1,003 m and annual rainfall varies from 777-2,264 mm (average 1,676 mm) (Beisiegel & Mantovani 2006). We searched for nests from September to February during two breeding seasons, 2013/2014 and 2014/2015, by monitoring approximately 7 km of trails, and 3 km of streams, in a sub-montane area varying in altitude from 680 to 850 m (Oliveira-Filho & Fontes 2000). To find the nests we monitored territories defended by males (Martin & Geupel 1993) in areas of primary forest.

The White-necked Thrush is a socially monogamous passerine (Snow & Snow 1963), endemic of the Neotropics, distributed in two disjunct regions: from Mexico to northern Argentina, and from northeastern Brazil to Uruguay (Ridgely & Tudor 1989, Collar 2005). Surveys in the Atlantic Forest have shown that this is one of the most abundant species in this biome (Simpson et al. 2012, Antunes et al. 2013), and the most frequently recorded using mist nets in this forest understory (Alves 2001). It builds bulky cup-shaped nests, mainly in large tree forks, invaginations of tree trunks, or inside bromeliads (Snow & Snow 1963, Collar 2005).

Whenever we found a nest we marked its location with a GPS (Garmin 62ST), and when nestlings were about five days old we sampled a drop of blood (10 μL) from each bird through a small incision at the tip of a claw. We also placed one mist net close to each nest to capture the parents when they flew into the nests to feed the nestlings, and blood samples were also obtained from them following the same procedure. Blood was stored in 1.5 ml tubes in 100% ethanol, and each animal received a unique combination of polyetilene colored bands for subsequent identification of adults by visual observations. After blood sampling and marking, nests were monitored by performing daily one hour focal observation sessions to confirm that we had sampled adults that were the social parents of the nestlings, and not individuals that happened to be close to the nests.

The DNA was extracted using a phenol-chloroform-isoamyl alcohol protocol (Sambrook et al. 1989), and animals were sexed by amplification of the homologous copies of the CHD gene (chromo-helicase-DNA-binding) using the primers P2/P8 (Griffiths et al. 1998). We also used the primer (P0) (Han et al. 2009) in the same PCR reaction. The latter anneal to a unique sequence of W chromosome, resulting in a third band for females, usually 100 bp longer than CHDW, improving the resolution of sex identification. PCR reactions followed Anciães & Del Lama (2002), and were resolved in 3% agarose gels.

For paternity tests we used six microsatellite loci previously evaluated in Common Blackbird, *Turdus merula* (Linnaeus, 1758) (Simeoni et al. 2009), and Forest Thrush, *Turdus lherminieri* (Lafresnaye, 1844) (Arias et al. 2012): Asμ15, Cuμ 28, Cuμ 32, Dpμ 01, PatMP2-43, and TG04-012 (see Table 1). PCR reactions had a final volume of 10 μl, containing 0.2 mM of dNTPs, 1X amplification buffer, 3 mM MgCl2, 0.2 mM of each primer, 1 U Taq polymerase, and 100 ng of DNA. The reactions were performed in an Eppendorf thermocycler programmed for an initial denaturation at 94°C for 3 minutes, and 29 cycles of 94°C for 30 seconds, 30 seconds in the annealing temperature (see Table 1), and 72°C for 30 seconds, followed by a final extension at 72°C for 5 minutes. As allelic patterns could be clearly determined, alleles were scored for each locus in 7.5% polyacrylamide gels (Schwarzová et al. 2008), run at 47 W during 1:40 hours, and results were visualized by silver staining (Comincini et al. 1995). We estimated the observed and expected heterozygosity, probability of heterozygosity deficit, and the probability of linkage disequilibrium using the software GENEPOP 4.0 (Raymond & Rousset 1995). We also evaluated the possibility of null alleles, allelic dropout, and scoring errors due to stuttering using Micro-Checker (Van Oosterhout et al. 2004). The accuracy of the loci to perform the paternity tests was estimated by the probability that two random individuals in the population could present identical allelic composition (the probability of identity), and by calculating the probability that the set of loci could not exclude a pair of candidate unrelated parents from an random offspring, using Cervus 3.0 (Kalinowski et al. 2007). All the above estimates were performed using only the adult individuals. The identifications of EPY were conducted through direct observation of allele inheritance (Fleischer 1996, Mitrus et al. 2014), and resulted when at least one young in a nest was sired by at least one extra-pair parent based on at least two loci. This is because mismatching at only one locus can be potentially caused by mutations or null alleles (Westneat & Mays 2005, Liu et al. 2015).

Table 1. Microsatellite loci used for paternity tests in *Turdus albicollis*, primer sequences, annealing temperature (T), and source.

Locus	Primer sequences (5′-3′)	T(°C)	Source
Asμ15	F: AATAGATTCAGGTGCTTTTTCC R: GGTTTTTGAGAAAATTATACTTTCAG	55.8	Bulgin et al. 2003
Cuμ 28	F: GAGGCACAGAAATGTGAATT R: TAAGTAGAAGGACTTGATGGCT	45.3	Gibbs et al. 1999
Cuμ 32	F: AGGAGAGTGAAAGAAAAGGG R: GAATTCTCAGCATGACAAATC	45.3	Gibbs et al. 1999
Dpμ 01	F: TGGATTCACACCCCAAAATT R: GTTTCTTAGAAGTATATAGTGCCGCTTGC	45.0	Dawson et al. 1997
PatMP2-43	F: ACAGGTAGTCAGAAATGGAAAG R: GTATCCAGAGTCTTTGCTGATG	55.8	Otter et al. 1998
TG04-012	F: TGAATTTAGATCCTCTGTTCTAGTGTC R: TTACATGTTTACGGTATTTCTCTGG	58.5	Replogle et al. 2008

We collected blood samples from 22 nestlings and 22 parents from 11 nests. The number of sampled nestlings per nest were 1 (n = 2 nests), 2 (n = 7), or 3 (n = 2) – in one nest with two eggs, only one egg hatched. The number of alleles per loci varied from 3 to 6 and none of the loci pairs were significantly linked. We also did not detect heterozygosity deficit (Table 2), null alleles, allelic dropout or scoring errors due to stuttering. The probability of identity of two random individuals in the population was 0.04%, and the probability that the set of loci could not exclude an unrelated pair of parents was 2.5%.

Table 2. Number of alleles per locus (Na), observed (Ho), and expected (He) heterozigosities, and probability for heterozygote deficit (p).

Locus	Na	Ho	H_E	p
Asµ15	6	0.545	0.583	0.421
Cuµ 28	5	0.636	0.766	0.108
Cuµ 32	3	0.227	0.210	>0.999
Dpµ 01	3	0.545	0.552	0.577
PatMP2-43	4	1.000	0.612	>0.999
TG04-012	4	0.454	0.504	0.339

We detected cases of EPY in four of the 11 sampled nests (36.4%), which included one nest with one nestling, two cases in nests with two nestlings, and one case in a nest containing three nestlings. In three of these four nests we confirmed the social parents feeding the offspring. Raw genotypic data and allelic mismatches among nestlings and their social parents are presented in Appendix 1.

Cases of extra-pair mating have been reported for all species of *Turdus* studied so far. In a population of Clay-coloured Thrush, *Turdus grayi* (Bonaparte, 1838), from Panama (09°N), 52.6% of the broods contained EPY (Stutchbury et al. 1998), and in a population of the American Robin, *Turdus migratorius* (Linnaeus, 1766), from Illinois, USA (40°N) this figure was 72% (Rowe & Weatherhead 2007). Although we have analyzed a smaller number of broods, the frequency of EPY (36.4%) found for White-necked Thrush was smaller than these studies. However, this still can be considered high, as in Griffith et al. (2002) review 35% of 34 socially monogamous passerine species have exhibited EPY rates above 36.4%, indicating a potential phylogenetic effect within the *Turdus*.

A possible explanation for the exceptionally high levels of EPY in American Robin can involve breeding synchrony, a parameter commonly evoked to explain variations of EPY in passerine birds (Westneat et al. 1990, Griffith et al. 2002, Neodorf 2004). When breeding is synchronic many females in a population are fertile at the same time and males may have limited opportunities to obtain extra-pair females, resulting in lower rates of extra-pair copulations (Griffith et al. 2002, Neodorf 2004). Although synchrony has not been estimated for this set of species, inferences can be made based on the duration of breeding seasons, i.e., populations or species with shorter breeding seasons should present more synchronic breeding (Stutchbury & Morton 1995). We have observed active nests of White-necked Thrush during a period of four months and at least one marked pair nested twice in the season. Clay-coloured Thrush breeds during approximately three months in Panama (Stutchbury et al. 1998), and they also can nest twice (Dyrcz 1983). The breeding season of American Robin also lasts about four months (Rowe & Weatherhead 2007), and they typically nest twice in the season (Howell 1942, Young 1955). These data do not provide strong support for the idea that longer breeding seasons results in higher EPY rates in tropical species of *Turdus*.

In conclusion, the percentage of nests of White-necked Thrush in which at least one individual providing parental care have been cheated is within the range found for other Neotropical passerines, e.g. 10% for the Banded Wren *Thryothorus pleurostictus* (Sclater, 1860) (Cramer et al. 2011), 36.7% for Yellow-shouldered Blackbird *Agelaius xanthomus* (Sclater, 1862) (Liu 2015), 52.6% for Red-throated Ant-tanager *Habia fuscicauda* (Cabanis, 1861) (Chiver et al. 2015), 54.8% for Cherrie's Tanager *Ramphocelus costaricensis* (Cherrie, 1891) (Krueger et al. 2008), and 67% in Lesser Elaenia (Stutchbury et al. 2007). This is one of a few studies that have performed detailed paternity analyses in a Neotropical bird, and our findings add knowledge to the early insights that breeding strategies involving EPY can be disseminated also among Neotropical socially monogamous songbirds.

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Appendix 1. Raw microsatellite genotypic data for 11 *Turdus albicollis* families. Discordant alleles among nestlings and both of the social parents are in bold.

Nest #	Individual	Sex	Loci											
			A5a	A5b	C28a	C28b	C32a	C32b	D1a	D1b	P243a	P243b	T412a	T412b
Nest 01	Parent	M	115	115	168	172	125	125	149	149	147	153	144	144
	Parent	F	115	115	170	170	125	127	149	149	147	153	144	144
	Nestling		115	115	170	**170**	125	125	149	149	147	153	**140**	**142**
	Nestling		115	115	**164**	170	125	125	149	149	147	153	**142**	144
Nest 02	Parent	M	115	115	170	172	125	125	149	149	147	153	144	144
	Parent	F	115	119	164	170	125	125	149	151	147	153	142	142
	Nestling		115	115	164	172	125	125	149	151	147	153	142	144
	Nestling		115	115	164	172	125	125	149	149	147	153	142	144

Continues

Appendix 1. Continued.

Nest #	Individual	Sex	Loci											
			A5a	A5b	C28a	C28b	C32a	C32b	D1a	D1b	P243a	P243b	T412a	T412b
Nest 03	Parent	M	109	119	164	164	125	125	149	151	147	153	142	144
	Parent	F	115	117	168	168	125	127	151	151	143	153	140	144
	Nestling		119	**119**	164	**170**	125	127	149	151	147	153	142	144
Nest 04	Parent	M	115	115	168	172	125	125	149	151	147	153	144	144
	Parent	F	119	119	170	170	125	125	149	149	147	153	142	144
	Nestling		115	119	170	172	125	125	149	151	147	153	142	144
	Nestling		115	119	170	172	125	125	149	151	147	153	142	144
Nest 05	Parent	M	109	115	164	170	125	125	149	151	151	153	142	142
	Parent	F	115	115	164	176	125	125	149	151	143	153	142	144
	Nestling		115	**119**	164	176	125	125	149	151	**147**	153	144	**144**
	Nestling		115	**119**	164	164	125	125	151	151	**147**	153	142	142
Nest 06	Parent	M	115	117	170	170	125	131	149	151	147	153	144	144
	Parent	F	115	119	168	172	125	127	149	149	147	153	144	144
	Nestling		115	117	168	170	125	131	149	151	147	153	144	144
	Nestling		115	119	168	170	127	131	149	149	147	153	144	144
Nest 07	Parent	M	115	115	168	170	125	125	149	151	147	153	144	144
	Parent	F	115	115	168	172	125	125	149	151	147	153	142	144
	Nestling		115	115	168	170	125	125	151	151	147	153	144	144
Nest 08	Parent	M	115	115	172	172	125	125	149	149	147	153	142	144
	Parent	F	115	121	168	170	125	125	151	151	147	153	142	142
	Nestling		115	121	168	172	125	125	149	151	147	153	142	144
	Nestling		115	**119**	**164**	170	125	125	149	151	147	153	142	**146**
	Nestling		115	121	168	**168**	125	125	149	151	147	153	142	**146**
Nest 09	Parent	M	115	115	168	168	125	125	151	153	147	153	142	144
	Parent	F	117	119	168	172	125	125	149	149	143	153	144	144
	Nestling		115	119	168	168	125	125	149	153	147	153	144	144
	Nestling		115	119	168	172	125	125	149	151	147	153	144	144
Nest 10	Parent	M	115	119	168	176	125	127	149	151	147	151	142	144
	Parent	F	115	119	170	170	125	125	151	153	147	153	144	146
	Nestling		115	115	170	176	125	127	151	153	147	151	142	**142**
	Nestling		115	119	**166**	170	125	125	149	153	147	153	144	144
Nest 11	Parent	M	111	115	168	172	125	125	149	149	147	153	142	144
	Parent	F	117	119	164	170	125	125	151	153	147	153	144	144
	Nestling		111	117	164	172	125	125	149	153	147	153	142	144
	Nestling		115	117	164	168	125	125	149	151	147	153	142	144
	Nestling		111	117	164	168	125	125	149	153	147	153	142	144