

Extra-pair paternity and the evolution of testis size in a behaviorally monogamous tropical mammal, the large treeshrew (*Tupaia tana*)

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Abstract Monogamy is rare in mammals (<5% spp.) but occurs in greater frequency among primates (15%) and their close relatives, the treeshrews (100%; Order: Scandentia). Two genetic studies of parentage in monogamous primates revealed high rates of extra-pair paternity (EPP), but to date parentage has not been studied in a treeshrew species. I analyzed the genetic parentage of 22 offspring from two populations of large treeshrews in Sabah, Malaysia (NE Borneo), using seven autosomal microsatellite loci and one mitochondrial DNA marker. Half of these offspring were sired by males that were not the presumed partner of the mother (50% EPP), and three litters exhibited evidence of multiple paternity. However, comparative analysis indicated that the high rate of EPP in *Tupaia tana* is not associated with intense sperm competition. Relative testis size of treeshrews was similar to testis size in 22 primate species with uni-male mating systems but smaller than 44 primates with multi-male mating systems. After factoring out the effects of body size and phylogeny, I also found that the evolution of multi-male mating systems was significantly associated with the evolution of larger testis size. Male–

female pairs of *T. tana* occupy joint territories but forage and sleep alone (“dispersed pair-living”), and I argue that this form of behavioral monogamy renders mate guarding ineffective. The adaptive advantages of behavioral monogamy likely differ from the advantages driving EPP in large treeshrews. However, small testis size suggests that behavioral monogamy is not masking a dispersed multi-male mating system in this species.

Keywords Extra-pair paternity · Monogamy · Testis size · Treeshrews · Borneo

Introduction

The claim that 93% of avian species breed monogamously (Lack 1968) has been soundly refuted by evidence supporting the prediction of Trivers (1972) that males and females should exhibit behavioral adaptations for extra-pair mating. An overwhelming 86% of the 130 behaviorally monogamous bird species studied by 2002 exhibited extra-pair paternity (EPP) in greater than 5% of offspring (mean=11% of offspring and 19% of broods, Griffith et al. 2002). Hypotheses for the adaptive function of EPP abound, but predictions of female choice for genetic benefits have received the most empirical support. Females may seek EPP to obtain compatible viability genes (e.g. Johnsen et al. 2000), obtain “good genes” that increase the fitness of their offspring (e.g. Sheldon et al. 1997), or maximize the genetic diversity of their offspring (e.g. Foerster et al. 2003). Observations that EPP is less common in genetically depauperate island populations (Griffith 2000) and more common in genetically diverse populations (Petrie et al. 1998) provide additional support for genetic benefits.

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However, some large-scale studies have failed to detect any genetic benefit of EPP (Schmoll et al. 2003), and comparative analysis indicates that negative direct selection caused by reduced paternal care may be more important than genetic benefits in explaining variation in EPP among avian taxa (e.g. high costs and marginal benefits of EPP in great tits, *Parus major*, Arnqvist and Kirkpatrick 2005; Albrecht et al. 2006).

In contrast to birds, monogamy is generally rare in mammals (3–5% spp., Kleiman 1977), but occurs at greater frequency among canids, rodents, the Euarchonta (tree-shrews, dermopterans, and primates), and bats (McCracken and Wilkinson 2000). The prevalence and adaptive function of EPP in behaviorally monogamous mammals is not well characterized, and several recent studies have highlighted the need to understand behavioral monogamy (also known as social monogamy, or pair-living) and genetic monogamy as separate evolutionary phenomena in mammals. Behavioral monogamy is now generally understood as a close social and/or spatial arrangement of a male–female pair, whereas genetic monogamy refers to situations where molecular genetic analyses confirm exclusive mating between a male and a female (Reichard 2003).

Bi-parental care has been proposed to drive the evolution of obligate behavioral and genetic monogamy in mammals (Kleiman 1977), although other analyses show that most behaviorally monogamous species do not exhibit direct paternal care (Komers and Brotherton 1997). Mating exclusivity and genetic monogamy in some rodent species (Malagasy giant jumping rat, *Hypogeomys antimena*, Sommer and Tichy 1999; California mouse, *Peromyscus californicus* and oldfield mouse, *Peromyscus polionotus*, Ribble 2003) may occur because males provide care that is necessary for female reproduction or enhances offspring survival. However, at least four species with paternal care exhibit EPP (10% in the African wild dog, *Lycaon pictus*, Girman et al. 1997; 44% in the fat-tailed dwarf lemur, *Cheirogaleus medius*, Fietz et al. 2000; 25% in the island fox, *Urocyon littoralis*, Roemer et al. 2001; 52% in the swift fox, *Vulpes velox*, Kitchen et al. 2006). Three genetic studies on behaviorally monogamous mammals without paternal care have recorded EPP rates from 19–57% (alpine marmot, *Marmota marmota*, Goossens et al. 1998; allied rock wallaby, *Petrogale assimilis*, Spencer et al. 1998; fork-marked lemur, *Phaner furcifer*, Schülke et al. 2004), whereas exclusive mating has been confirmed using genetic data for only one such mammal (Kirk's dik-dik, *Madoqua kirkii*, Brotherton and Rhodes 1996).

Experimental studies have also confirmed that mammals with substantial behavioral and physiological adaptations for monogamy (prairie voles, *Microtus ochrogaster*, Carter et al. 1995) exhibit high rates of EPP (multiple paternity in 56% of litters, Solomon et al. 2004). These results suggest

that EPP may be as prevalent in behaviorally monogamous mammals as in birds, especially given the rarity of direct paternal care in mammals (Kleiman and Malcolm 1981). Genetic mating systems may thus often result from a different set of ecological and social pressures than behavioral monogamy in mammals. The adaptive function of EPP has not been firmly established for any mammalian species, but most previous studies have argued that females choose males of superior genetic quality (e.g. territory-holding males in fat-tailed dwarf lemurs, males with longer arms in allied rock wallabies, or heterozygous males in alpine marmots, Cohas et al. 2006). Protection from infanticide through paternity confusion has also been offered as a general explanation for multiple mating in female mammals (Wolff and Macdonald 2004), including some behaviorally monogamous species (e.g. white-handed gibbons, *Hylobates lar*, Reichard 1995).

Variation in pair bonding may also influence mating patterns. Behaviorally monogamous mammals may form associated pairs that maintain proximity and show clear spatial association (e.g., *Peromyscus* spp., Ribble 2003), or dispersed pairs that occupy a joint territory but are not spatially associated during periods of activity (three sengi spp., Rathbun 1979; Rathbun and Rathbun 2006) maned wolves, *Chrysocyon brachyurus*, Dietz 1984; several nocturnal prosimians, Müller and Thalmann 2000. High EPP rates have been predicted in species that exhibit dispersed pair-living because effectiveness of mate guarding may be reduced (van Schaik and Kappeler 2003). Territorial defense alone can function as a form of mate guarding if males chase extra-pair males off their female partner's territory, but such territorial behavior may not be sufficient to prevent cuckoldry. Results from the nocturnal lemurs *C. medius* and *P. furcifer* support this prediction, although nocturnality and female dominance over male partners in these species may reduce the effectiveness of mate guarding more than pair dispersion per se (Schülke and Ostner 2005).

This study examines the genetic mating system of the behaviorally monogamous large treeshrew, *Tupaia tana*, in Sabah, Malaysia (NE Borneo). The large treeshrew is a small (200–250 g), diurnal mammal that inhabits the lowland tropical rainforests of Borneo and Sumatra. This species was previously thought to be primarily insectivorous, but soft, fleshy fruits are an important component of the diet and may play an important role in reproduction (Emmons 2000). Males and females are sexually size-monomorphic, but average male home range size (4.0–6.9 ha) may be significantly greater than female home range size (3.4–4.2 ha) in some populations (Munshi-South et al. 2007). Large treeshrews exhibit sex-specific territorial defense, and male–female pairs maintain approximately concordant territorial boundaries (Emmons 2000; mean of 60–72% of female's territory contained within territory of a

single male, Munshi-South et al. 2007). However, substantial percentages of both male (11%) and female (20%) ranges may overlap the territories of one or more extra-pair individuals. As in other species that exhibit dispersed pair living, male and female partners also typically forage separately on their common territory (Emmons 2000; Munshi-South et al. 2007). Treeshrews also exhibit a unique absentee maternal care system. Females deposit their young in a secluded nest that is subsequently visited only once every 2 days for intense bouts of nursing (Martin 1966; Emmons 2000). Males do not visit the nest, and no evidence exists to suggest that males provide direct parental care. Females are thus unlikely to reject extra-pair copulations due to a fear of reduced paternal care from their spatial partner.

Here, I examine the prediction of high rates of EPP in large treeshrews due to a lack of direct paternal care, spatial access to extra-pair individuals and reduced effectiveness of mate guarding associated with dispersed pair-living. I also investigated the evolution of testis size in large treeshrews. Genetic studies of birds and mammals have revealed that behaviorally monogamous species exhibit a diverse range of genetic mating systems. Females in pairs could copulate exclusively with their partner, mate with one or a few extra-pair males, or mate promiscuously with several males from surrounding territories. Detecting EPP and multiple paternity in large treeshrews would not necessarily indicate the extent of multiple mating and sperm competition, because their litter size is only two. Behavioral monogamy may actually mask a dispersed multi-male mating system in large treeshrews if dispersed pair-living sufficiently reduces the effectiveness of mate guarding. Such a genetic mating system would be predicted to result in substantial sperm competition and the evolution of large testes in this species (Harcourt et al. 1995).

To examine the potential for sperm competition in *T. tana*, I examine relative testis size and behavioral monogamy in 2 treeshrew and 66 primate species using both species data and phylogenetically independent contrasts. Relative testis size is a reliable predictor of sperm competition (Gage and Freckleton 2003), and sperm competition is positively associated with multi-male mating systems in several taxa (reviewed in Harcourt et al. 1995; Parker et al. 1997). However, previous analyses either did not include treeshrews, did not account for phylogenetic dependence, or used a less resolved primate phylogeny (especially for prosimian clades that exhibit dispersed mating systems, Purvis 1995; Purvis and Webster 1999) than the new supertree used in this study (Vos and Moores 2007). Having examined the extent of EPP and the implications for the evolution of testis size, I discuss the evolutionary pressures that may result in a discrepancy between behavioral monogamy, the genetic mating system, and intensity of sperm competition in large treeshrews.

Materials and methods

Study populations and designation of behavioral pairs

I studied a population of large treeshrews in primary lowland rainforest in the Danum Valley Conservation Area, Sabah, Malaysia (Danum; 4°58'N, 117°48'E), from August to December 2002–2004 and a second population in selectively logged forest in the Malua Forest Reserve (Malua; 5°5'N, 117°38'E) from September to December 2003–2004. I trapped large treeshrews at each site with locally made wire mesh traps placed every 25 m along two 500-m transects but in 2004 placed two additional 500-m transects at the logged site to increase captures. I conducted 4-day trapping sessions every 3–4 weeks during the study period; traps were opened at 0600 h and checked twice daily at 1030 and 1500 h. Captured animals were transferred to cloth bags, weighed, and sedated with a 10-mg/kg dose of ketamine hydrochloride injected into the upper thigh (Emmons 2000). I injected animals with a subdermal passive integrated transponder (PIT tag; Biomark, Inc, Boise, ID) for permanent identification, and clipped a tissue sample from the upper ear. Tissue samples were preserved in 95% ethanol and stored at 4°C. If individuals were in good condition and weighed more than 180 g, then I fitted them with radio collars to identify behavioral pairs. Full details on the study site, trapping, and radiotelemetry methods are reported elsewhere (Munshi-South 2006; Munshi-South et al. 2007). All animal handling procedures were approved by the University of Maryland's Institutional Animal Care and Use Committee and the Sabah Wildlife Department.

Previous radio tracking of 46 individuals revealed that *T. tana* form dispersed pairs across a range of ecological conditions (Munshi-South et al. 2007). Having previously described the location and shape of adult territories for each site in each year using radiotelemetry data and spatial analyses, I designated behavioral pairs of *T. tana* when at least 50% of a female's territory was contained within the territory of a single male. No individuals had more than one behavioral partner, and percent overlap with extra-pair individuals (mean overlap \pm SE = 11% \pm 2%) was generally low (Munshi-South et al. 2007). Partners stayed on the same territories throughout each field season, a period long enough for one to three reproductive events. Only two individuals (F14 and M35) persisted for more than one study period, but the spatial arrangement of home ranges was similar across years. I did not observe any divorce or territory-switching among large treeshrews. Two males disappeared within the first month of the 2003 study period in primary forest and were subsequently replaced by new males that occupied similar home ranges (Munshi-South et al. 2007). Incomplete sampling or radio collar failure prevented designation of pairs for all adults, particularly in

primary forest in 2002 and selectively logged forest in 2003. When radio-tracking data were not available, I used trapping locations to identify presumed mates ($N=3$ behavioral fathers: M03, M07, and M08, Table 1). The presumed mates identified using this latter method were trapped on a known female's home range multiple times, and were surrounded by same-sex home ranges identified through radiotelemetry (i.e., process of elimination aided designation of these males).

The length of the female receptive period has not been described for wild *T. tana*, but has been reported as only 1–3 h for captive *T. belangeri* (Martin 1968). *T. tana* females can produce up to nine litters annually in captivity, but in the wild have one to three litters per year during and shortly after peak annual fruit abundance (Emmons 2000). Due to the absentee maternal care system of *T. tana*, I could identify juveniles only after weaning when they were trapped outside the nest. Juveniles were identified by their small size (mass < 180 g based on growth curve in Emmons 2000) and the presence of milk teeth or newly erupted, unworn adult teeth. I trapped 15 juveniles in primary forest

and seven juveniles in selectively logged forest during the study period (Table 1).

Genetic parentage analysis

DNA was extracted from ear tissue samples using Qiagen DNeasy tissue kits (Qiagen, Valencia, CA, USA). Seven previously described microsatellite DNA loci named JS22, JS132, JS183, JS188, JS196, SKTg19, and SKTg22 were amplified using the PCR conditions in Munshi-South and Wilkinson (2006). Fluorescently labeled alleles were separated on an Applied Biosystems 3100 DNA Analyzer and sized and scored using Genotyper 2.5 (Applied Biosystems, Foster City, CA, USA). Locus JS183 exhibited a homozygote deficiency consistent with the presence of null alleles (Munshi-South and Wilkinson 2006), therefore I ran all analyses with and without this locus because null alleles can substantially influence molecular parentage analyses (Dakin and Avise 2004).

I also used a 324 bp segment of the mitochondrial DNA (mtDNA) control region to limit the number of candidate

Table 1 Behavioral and genetic parentage of 15 offspring in primary forest and 7 offspring in selectively logged forest from 2002–2004

Offspring	Year	Haplotype	Genetic mother	Behavioral father	No. Loci Excluding IPP	Genetic father	Paternity
Danum							
f02	2002	na	F06*	undetermined	na	M06**	EPP ^b
f07	2002	Ttdlp2	F08**	M08	2	M01**	EPP
f09	2002	Ttdlp2	F06*	undetermined	na	M10**	EPP ^b
f22	2004	Ttdlp2	F26*	M40	2	M50**	EPP
f23	2004	Ttdlp4	F21*	M39	2	unassigned	EPP ^b
f28	2004	Ttdlp5	F26*	M40	0	unassigned	IPP ^b
f36	2004	Ttdlp5	F10**	M29	1	M19*	EPP
M04	2002	na	F01*	M03 ^a	1	M03**	IPP
M11	2002	Ttdlp2	F04*	M07 ^a	0	M07**	IPP
M14	2002	Ttdlp2	F04**	M07 ^a	2	M06**	EPP
M17	2003	Ttdlp2	F14*	M19	2	unassigned	EPP ^b
M24	2003	Ttdlp3	F10**	M08 ^a	2	unassigned	EPP ^b
M25	2003	Ttdlp2	F14*	M19	0	unassigned	IPP ^b
M37	2004	Ttdlp9	F26*	M40	0	M40**	IPP
M49	2004	na	F26**	M40	0	M40**	IPP
Malua							
f33	2004	Ttdlp4	F29**	M35	0	M35**	IPP
f39	2004	Ttdlp3	F35**	M31	0	M31**	IPP
f40	2004	na	F38**	undetermined	na	unassigned	IPP ^b
M43	2004	Ttdlp11	F29*	M35	2	M31**	EPP
M45	2004	Ttdlp12	F29**	M35	1	M31**	EPP
M46	2004	Ttdlp3	F38**	undetermined	na	unassigned	IPP ^b
M48	2004	Ttdlp13	unassigned	M35	0	M35*	IPP ^b

Paternity was designated as either intra-pair (IPP), extra-pair (EPP), or unassigned, based on the number of loci excluding paternity of the behavioral father and a maximum likelihood analysis of paternity. The likelihood analysis was based on a simulation with a genotyping error rate of 1%, and thus identified IPP for offspring m04 despite one locus excluding the behavioral father.

^a Behavioral father designation based on trapping data

^b Suspected parentage, i.e., one parent unassigned.

*Parentage assigned at 80% likelihood

**Parentage assigned at 95% likelihood

mothers based on shared mtDNA sequences. Primers were designed from conserved segments of the control region in the northern treeshrew, *T. belangeri*, and the sister taxon to treeshrews, the Malayan colugo, *Cynocephalus variegatus* (GenBank Accession Nos. AF217811 and AJ428849, respectively, Schmitz et al. 2000; Murphy et al. 2001; Arnason et al. 2002), using the Primer 3 computer program (Rozen and Skaletsky 2000). PCR amplification was performed in 9 μ l volumes containing 1 μ l template DNA, 0.125 U *Taq* polymerase (Invitrogen), 1X PCR buffer (Invitrogen), 0.3 mM of each dNTP, 2.5 mM $MgCl_2$, and 0.55 μ M of each primer (forward primer JMSTbel386 5'-ACCTCCGT GAAATCAGCAAC-3' and reverse primer JMSTbel1110 5'-TTCTTGT TTTTGGGGTTTGG-3'). PCR was performed on a Peltier thermocycler programmed for 30 amplification cycles with denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. I sequenced PCR products using the BigDye Terminator 3.1 and a 3100 DNA Analyzer (Applied Biosystems). Sequences were edited and aligned using Sequencher 4.1.2 (Gene Codes, Ann Arbor, MI, USA) and Bioedit 7.0.4.1 (Hall 1999).

Parentage likelihood analyses were conducted separately for treeshrews from the two study sites using Cervus 2.0 (Marshall et al. 1998; Slate et al. 2000). All Cervus analyses were based on a simulation with 10,000 cycles assuming five candidate parents, complete parental sampling and genotyping, and a 1% genotyping error rate. Neither parent was known a priori for any offspring, so I conducted a stepwise parentage analysis. The Cervus simulation predicted an assignment success rate for mothers of 74% at the strict criterion and 99% at the relaxed criterion, and 100% for both criteria for sire assignment when the mother was known. First, I assigned genetic mothers to offspring when the certainty calculated by Cervus for one female exceeded 80% (relaxed criterion) or 95% (strict criterion). I limited the number of candidate mothers for each offspring in the maternity analysis based on shared mtDNA control region haplotypes, because Cervus is more successful at assigning parentage when there are fewer candidate parents. Thirteen mtDNA haplotypes defined by ten segregating sites were identified from the 324 bp segment of the control region sequence (Table 2). Genetic mothers were not assigned to all offspring after an initial analysis where candidate mothers were limited by shared haplotypes. Therefore, I re-ran the analysis with all adult females as candidate mothers for offspring not successfully assigned genetic mothers during the first analysis. The results from this mitochondrial screening procedure did not identify different mothers than a separate Cervus analysis that included all maternal candidates but did provide higher likelihood values for some assigned mothers.

Mothers assigned to offspring were then carried over to the paternity analysis as known parents, and genetic fathers

were assigned at either the strict or relaxed criterion. Offspring assigned both parents were designated as the result of either intra-pair paternity (IPP) or EPP based on whether their genetic father was also their behavioral father as defined above. I also recorded the number of loci excluding the behavioral father as the genetic father for each offspring. In cases where multiple loci excluded the behavioral father but a genetic sire was not assigned in the likelihood parentage analysis, I designated parentage as EPP. When no loci excluded the behavioral father but a genetic sire was not assigned, I designated parentage as IPP. When I omitted locus JS183 from the analysis due to possible null alleles, I found reduced support for some parentage assignments but no support for alternative parental relationships. The only exception was the assignment of two potential sires at 80% certainty for offspring f28, but neither could be definitively assigned. Once offspring were assigned to genetic parents, I tested for a difference in EPP rates between primary and logged forests using a Pearson's χ^2 test. I used a *t* test and *F* test of unequal variances, respectively, to examine whether mean and variance in the number of offspring sired by males was significantly different from the mean and variance in offspring assigned to females.

Female *T. tana* give birth to litters of two offspring, so when littermates were trapped I examined the possibility of multiple paternity using the parentage analyses above and genetic estimates of pair-wise relatedness. I used the program ML-RELATE (Kalinowski et al. 2006) to calculate maximum likelihood estimates of pair-wise relatedness between genetic mothers and offspring, genetic fathers and offspring, putative full siblings, and putative half-siblings identified by the parentage analyses. Maximum likelihood estimates of relatedness are generally more accurate than other estimators at determining specific relationships (Milligan 2003), and this particular implementation accounts for the influence of null alleles on relatedness calculations (7% null alleles estimated for locus JS183, Kalinowski and Taper 2006). Lower pair-wise relatedness values for littermates than for parent-offspring or full-sibling dyads were considered evidence in favor of multiple paternity.

Testis size analysis

To examine the potential for sperm selection in treeshrews and primates, I collated primate species data on testis size and body size from earlier reviews of all mammals ($N=14$ spp., Gage and Freckleton 2003), all primates ($N=28$, Harcourt et al. 1995), and strepsirrhine primates ($N=24$, Schülke et al. 2004). Testis size for male *T. tana* ($N=15$ individuals) and the plain treeshrew (*Tupaia longipes*, $N=3$) trapped during this study were calculated using the

Table 2 Frequency and characteristics of 13 mitochondrial control region haplotypes among 41 large treeshrews from primary and selectively logged forest

Haplotype	<i>N</i>	Prop.	nt1	2	9	16	41	158	233	287	299	302
Ttdlp1	1	0.02	G	G	C	C	C	G	T	T	A	G
Ttdlp2	12	0.29	C	T
Ttdlp3	10	0.24	C	T	.	T
Ttdlp4	5	0.12	C	T	.	T	G	.
Ttdlp5	3	0.07	C	T	.	T	.	.	.	C	.	.
Ttdlp6	1	0.02	C	T	.	T	.	A	G	.	.	.
Ttdlp7	2	0.05	.	T	.	T
Ttdlp8	1	0.02	C	.	.	T
Ttdlp9	2	0.05	.	T
Ttdlp10	1	0.02	C	T	G	T
Ttdlp11	1	0.02	C	T	.	T	A
Ttdlp12	1	0.02	C	T	.	T	A
Ttdlp13	1	0.02	.	.	.	T	.	.	G	.	G	.

The haplotypes are characterized by nucleotide substitutions at 10 variable sites in a 324 bp sequence (alignment gaps and missing data excluded; pg. 75). The consensus sequence follows: SKTCAGGGCCATTGAYTGAAGATCGCCACACNYBKTGWCCCHYKTAATAAAGACATCTC GATGGATTCRTGACTAATCAGCCCATGCCTAACATAACTGTGSTGTCATGCCYTTGGTATTTTAAATTTAGGGGTGGTATCACT CAACAGGGCCGGGAGGCCTCGTCCCAGGCAAACCTGATTGTAGCTGGACTTAACTTGAATATTCTTTAATCGCATATAAACCATAAGGCTGTAATCTTTCCATGCTCGATGGACATAACAAATCATCAATACAGACCCAAACAYAAACCCAACCCRACGCACGTACACG TACACGTACACG

formula $1/6 \times \pi \times \text{Length} \times \text{Width}^2$ (Hosken 1998). Only species values for which the behavioral mating system could be identified were used. The mating system for each species was designated as either behaviorally monogamous, polygynous, or multi-male. Mating system designation was based on the information in the testis size references above or Komers and Brotherton (1997). Following Schülke et al. (2004), species where males are solitary and dispersed were classified as exhibiting multi-male mating systems. I used analysis of covariance (ANCOVA) with log body size as the covariate to examine whether log testis size differs between mating systems.

Species data cannot be treated as statistically independent because species are related through descent from common ancestors (Felsenstein 1985). Hence, I also used Comparative Analysis by Independent Contrasts (CAIC) v. 2.6.9 (Purvis and Rambaut 1995), to convert species data into phylogenetically independent contrasts. I used a recent, highly resolved supertree phylogeny of all primates with branch lengths (Vos and Moores 2007). The CAIC analysis did not include any treeshrew data, because branch lengths between primates and treeshrews have not been calculated using the same methodology employed in the phylogeny above. Including a single treeshrew clade as an outgroup also would not substantially increase the size of the comparative dataset. This phylogenetically controlled analysis was run primarily to corroborate general findings from the species-level analysis that did include two treeshrew species. To remove the effects of body mass on testis size, I first calculated the least squares regression equation between log body mass and log testis size using the species

data (log testis size = $1.26 + 0.71 \times \log \text{mass}$, $F_{1,66} = 152.2$, $P < 0.0001$, $R^2 = 0.70$). I then used this regression formula calculated from the species data to calculate residual testis size. These residual values were then tested against mating system categories using the BRUNCH algorithm in CAIC. The BRUNCH algorithm requires a dichotomous categorical variable, so monogamy and polygyny were lumped together as uni-male mating systems and compared to multi-male mating systems. The fundamental distinction between these two categories is whether females mate with multiple males during the same reproductive period. I used a *t* test to examine whether these categorical contrasts were significantly above zero, as predicted if the evolution of multi-male mating systems is associated with the evolution of larger testis size in primates (see CAIC manual, Purvis and Rambaut 1995). JMP version 5.0 (SAS Institute 2003) was used for all statistical analyses.

Results

Genetic parentage analysis and rates of EPP

Both behavioral and genetic parents could be assigned for 8 out of 15 (67%) offspring in Danum's primary forest (Table 1). In selectively logged forest at Malua, both behavioral and genetic parents were assigned for four out of seven (57%) offspring (Table 1). The parentage analyses identified EPP among treeshrews in both primary and selectively logged forest. Of the eight offspring in primary forest for which both genetic and behavioral parents were

assigned, four resulted from EPP and four from IPP (50% EPP, Table 1). Of the four offspring in selectively logged forest with complete parentage information, two resulted from EPP and two from IPP (50% EPP, Table 1). Overall, 6 out of 12 offspring with complete parentage information resulted from extra-pair matings (50% EPP, Table 1).

EPP was suspected in five additional primary forest cases where a behavioral father was not identified but an extra-pair male was positively designated as the genetic father by the paternity analysis, or paternity by the behavioral father was excluded by multiple loci (64% EPP overall in primary forest if these five cases are included). Three additional cases of IPP were suspected in logged forest: two offspring mothered by F38 but for which behavioral and genetic fathers were not identified, and one offspring sired by M35 for which a genetic mother could not be identified (f40, m46, and m48; 29% EPP overall at Malua, if these three are included). In these cases, one parent on the offspring's territory was not trapped, but most of the surrounding adults were sampled but not assigned genetic parentage. Overall, 11 out of 22 offspring resulted from extra-pair matings if incomplete parentage assignments were included (50% EPP, Table 1).

The behavioral father was not excluded by any loci in eight cases of IPP, whereas the behavioral father was excluded by two loci in seven cases of EPP (Table 1). EPP rates were identical between sites when only complete parentage assignments were included but did not significantly differ between sites even if the incomplete parentage assignments were included (Pearson's $\chi^2_{1,20}=1.93$, $P=0.16$). Female adults were assigned significantly more genetic offspring ($N=12$, mean \pm SE=1.75 \pm 0.28 offspring; $t_{28}=2.45$, $P=0.02$) than male adults ($N=18$, mean \pm SE=0.83 \pm 0.23), but variance in reproductive success was not significantly different between the sexes ($F_{1,28}=0.24$, $P=0.63$).

Average pair-wise relatedness (mean \pm SE) between genetic mothers and offspring ($N=18$, $r=0.36\pm 0.04$), genetic fathers and offspring ($N=13$, $r=0.36\pm 0.05$), and full-siblings ($N=6$, $r=0.37\pm 0.05$) was more than twice the average recorded for half-siblings ($N=12$, $r=0.12\pm 0.04$). Three putative littermate pairs were identified when two offspring shared the same genetic mother, were trapped within a few days of each other and were similar in mass at time of capture. In two cases (m11–m14 in primary forest, $r=0.09$; f33–m43 in selectively logged forest, $r=0.0$), littermates had different genetic sires and low pair-wise relatedness values, suggesting that multiple paternity occurs in *T. tana*. The offspring pair f22–f28 ($r=0.0$) in primary forest may represent another case of multiple paternity, although the genetic sire of f28 was not assigned. These results indicate a minimum multiple paternity rate of 32%, assuming no other cases of multiple paternity in incompletely sampled litters.

In four out of six cases of EPP with full parentage assignment, extra-pair sires occupied territories that were directly adjacent to their extra-pair mate in that year (Fig. 1). The two exceptions were extra-pair offspring sired by M01 and M19 in primary forest in 2002 and 2003, respectively. Another pair's territory separated these males from their extra-pair mates. M01 occupied a territory where no adult female was captured in 2002, but M19 also reproduced with his behavioral partner in 2003 (F14, Fig. 1).

Comparative analysis of testis size in treeshrews and primates

Relative testis size of behaviorally monogamous species, including *T. tana* and *T. longipes*, was consistently smaller than relative testis size in species with multi-male mating systems (Fig. 2). ANCOVA confirmed that log testis size increased with log body size in all species, ($F_{1,67}=39.4$, $P<0.0001$), and there was a significant difference in testis size ($F_{1,67}=15.9$, $P<0.0001$) between species with behaviorally monogamous (adjusted mean from ANCOVA \pm SE=3.29 \pm 0.09), polygynous (3.50 \pm 0.33), and multi-male mating systems (3.86 \pm 0.05). The interaction between body mass and mating system was not significant ($F_{1,67}=0.04$, $P=0.96$), indicating that the regression lines for monogamous, polygynous, and multi-male mating systems did not have significantly different slopes (Fig. 2).

Analysis of phylogenetically independent contrasts of residual testis size (controlled for body mass) and behavioral mating system indicated that the evolution of multi-male mating systems in primates is significantly associated with the evolution of larger relative testis size ($N=15$ contrasts, mean \pm SE=0.13 \pm 0.03; $t_{1,14}=4.81$, $P<0.0001$).

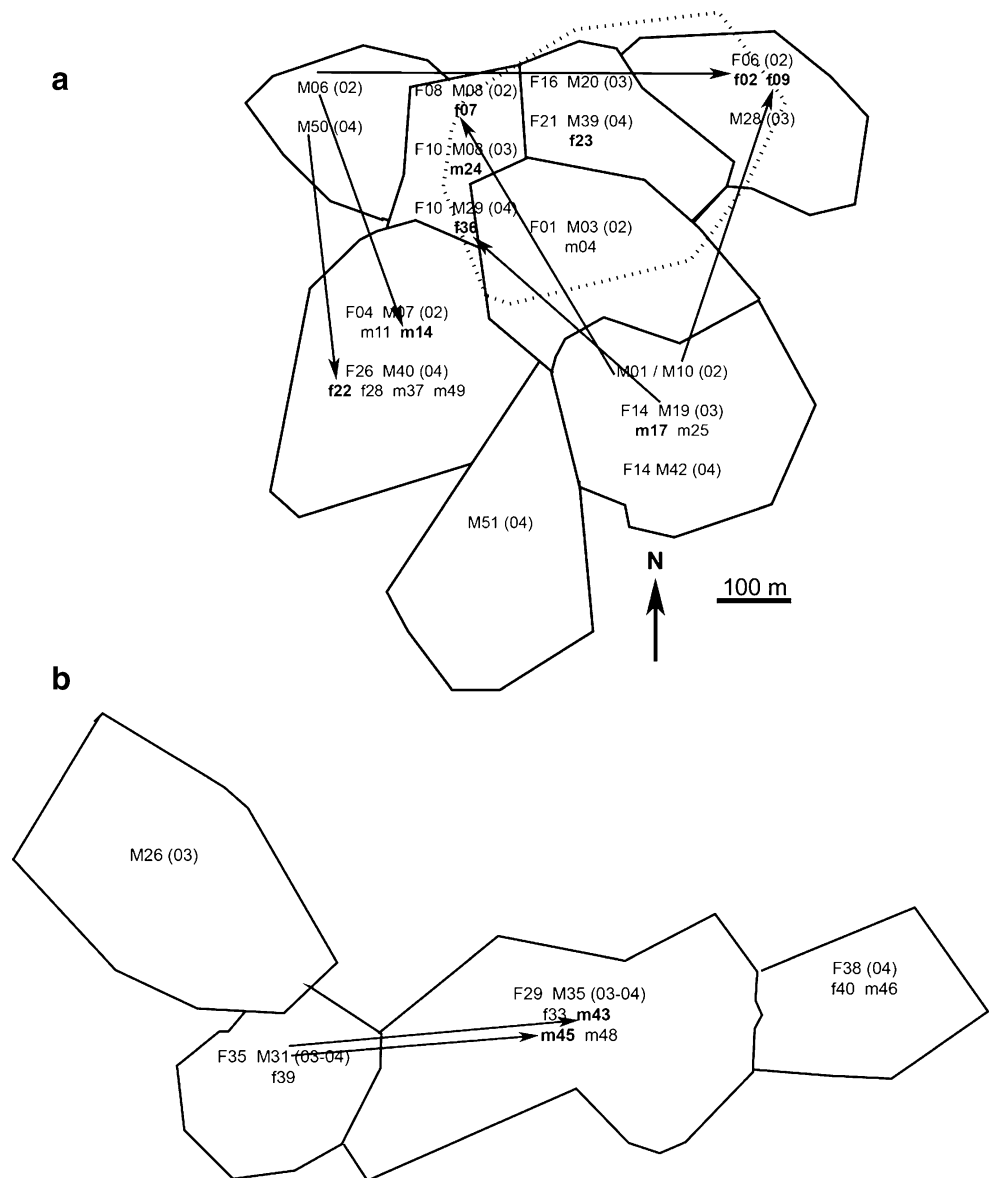
Discussion

Genetic mating system of the large treeshrew

Genetic analysis of parentage in the large treeshrew revealed one of the highest rates of EPP (50%) recorded for a behaviorally monogamous mammal. Only EPP rates reported from wild populations of the swift fox (52%, Kitchen et al. 2006), and the lemurs *C. medius* (44%, Fietz et al. 2000) and *P. furcifer* (four out of seven offspring, Schülke et al. 2004), are of comparable magnitude. We also found evidence for multiple paternity in large treeshrews, indicating that female *T. tana* may mate with more than one male in a single breeding period.

Several males assigned extra-pair offspring in the genetic analysis did not have known behavioral mates (four out of seven, Fig. 1), suggesting that pursuing EPP is a successful reproductive strategy for males that do not have the option

Fig. 1 Schematic location of pair territories and capture sites at **a**) Danum in 2002–2004, and **b**) Malua in 2003–2004. Note that diagram represents relative territorial arrangement for ease of interpretation; see Figs. 1, 2, 3 and 4 in Munshi-South (2006) for actual spatial overlap between territories. Identity of adult pair members is denoted by *F* (female) and *M* (male), followed by their year of residence in that territory in parentheses. Each pair's offspring are listed directly underneath their parents and denoted by *f* (female) or *m* (male). **Bold offspring names** denote extra-pair paternity, and **arrows** point from extra-pair fathers to their genetic offspring. The **dashed polygon** represents the anomalous schematic territory of M39 in 2004, which was much larger than other territories recorded in this study



of mating within a behaviorally monogamous pair. In two cases, males without known partners mated with already-paired females located one territory-length away. I could not observe copulations directly, but the multiple observations of Emmons (2000) at the same site of short-term male forays into extra-pair female territories suggests male initiation of extra-pair mating. The observation that males exhibit significantly less spatial overlap with their female partners than females exhibit with their male partners also indicates that males have enhanced opportunities to pursue extra-pair copulations (Munshi-South et al. 2007). Selective pressures to seek EPP should be greater on males than females, given that the average number of offspring sired by male treeshrews in this study was significantly lower than offspring genetically assigned to females. Males that do not gain EPP may have low or no reproductive success during their lifetime.

Testis size, behavioral monogamy, and EPP

Comparative analysis of testis size revealed that primates with multi-male mating systems have relatively larger testes than behaviorally monogamous or polygynous treeshrews and primates (Fig. 2). These results were independent of body mass and phylogeny, and generally agree with previous analyses (all primates, Harcourt et al. 1995; Kappeler 1997; strepsirrhine primates, Schülke et al. 2004). However, the analysis reported in this paper used a much more highly resolved, and more accurately dated, phylogeny (Vos and Moores 2007) than these previous studies, resulting in a substantially larger number of independent contrasts calculated for testis size and mating system. The increase in sample size was most apparent for the strepsirrhine primate clades that behaviorally resemble treeshrews.

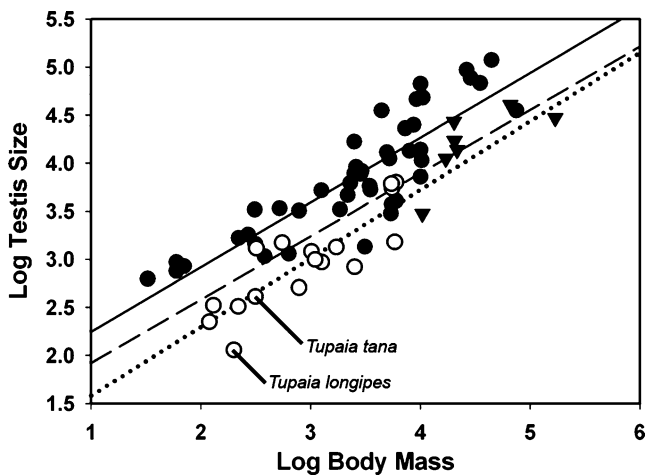


Fig. 2 Relationship between log testis size and log body size in 2 treeshrew and 66 primate species. *White circles with the dotted regression line* correspond to behaviorally monogamous species, *black triangles with the dashed regression line* denote polygynous species, and the *black circles with the solid regression line* represent species with multi-male mating systems

Large relative testis size is a reliable predictor of sperm competition in species with multi-male mating (Parker et al. 1997; Gage and Freckleton 2003). For example, all solitary and group-living lemur species have very large testes for their body size (Kappeler 1997), presumably due to scramble competition mating systems that result in substantial sperm competition (Eberle and Kappeler 2004b; Schülke and Ostner 2005). Many of these lemur species exhibit dispersed mating systems that limit the effectiveness of mate-guarding (Müller and Thalmann 2000), and thus one could predict that species exhibiting dispersed pair-living would also exhibit large testis size due to multi-male mating. However, high EPP rates in large treeshrews and two nocturnal lemurs with small testes indicate that greater sperm competition does not necessarily result from extra-pair copulations within dispersed pair-living systems.

Male–female pairs of large treeshrews typically travel separately during the day and exhibit high rates of EPP. Small relative testis size in this species, however, suggests that females do not copulate promiscuously during one receptive period. Additionally, half of all young were sired by their behavioral father despite the dispersion of male–female pairs. *T. tana* males may maintain greater spatial proximity with their female partners during her short receptive period, as has been reported for captive pairs kept in separate cages in another treeshrew species (*Tupaia belangeri*, Martin 1968). Alternatively, males may limit the incidence of multi-male mating simply by patrolling their territory and expelling male intruders. Behavioral monogamy most likely evolved in large treeshrews due to reduced predation and/or conspecific foraging competition (Schülke 2005; Munshi-South et al. 2007), but protection against

cuckoldry should not be discarded as a secondary evolutionary benefit of maintaining a shared territory with one female.

Discordance between high EPP rates and small testis size could also result from evolutionary constraints on testis size or abnormally high population densities in degraded habitats that lead to more promiscuous mating (Schülke and Ostner 2005). Sperm morphometry, and particularly sperm size, could also be more important than sperm number for fertilization in treeshrews and other mammals. Sperm length is positively correlated with testis size in mammals, although the relationship is phylogenetically dependent (Gage and Freckleton 2003). I argue below that high population density is not responsible for EPP in *T. tana*, but additional data on testis size and sperm morphometry in treeshrews are needed before other explanations can be ruled out.

Fewer mates and lifetime breeding opportunities compared with polygynous or promiscuous primates are more likely explanations for small testis size in *T. tana* than the explanations discussed above. In general, a single male's mating opportunities are maximized when females have short breeding seasons, enter estrus simultaneously, and are clustered in space (Isvaran and Clutton-Brock 2007). Behaviorally monogamous fork-marked lemurs (*P. fuscifer*) also exhibit small testis size despite a high EPP rate, presumably because males only mate with their female partner and up to five female neighbors (only half of which are receptive in any given year, Schülke et al. 2004). In contrast, male gray mouse lemurs (*Microcebus murinus*) with relatively large testes roam over the territories of four to 21 females (Eberle and Kappeler 2004b), and females mate with one to seven males up to 11 times during their single night of receptivity (Eberle and Kappeler 2004a). Sperm competition is likely to be a much more potent evolutionary force in the latter species, where large ejaculate size will favor the sperm of a single male competing with up to six other males in the reproductive tracts of several females. Male treeshrews may mate with only their female partner, up to five neighboring females (Fig. 1), and potentially a small number of more distant females, each of which reproduce asynchronously one to three times a year (Emmons 2000). The intensity of sperm competition in treeshrews is thus more likely to resemble that in behaviorally monogamous lemurs like *P. fuscifer* than in wide-ranging promiscuous species with large testes like *M. murinus*.

Explanations for EPP in large treeshrews

High rates of EPP in behaviorally monogamous species may result from specific ecological conditions, such as high breeding density or synchrony, or adaptive evolutionary benefits to females (Griffith et al. 2002). Adaptive explanations for EPP can be further divided into direct benefits

provided by extra-pair males, and indirect benefits from genetic quality or genetic variation. Direct benefits from paternal care, improved foraging, infanticide prevention, or predation prevention are largely precluded by the reproductive biology and ranging patterns of *T. tana* (Munshi-South et al. 2007). Extensive radio tracking showed that female *T. tana* do not spend significant time foraging or engaged in other activities on extra-pair home ranges, so they cannot receive direct benefits from extra-pair males. However, given the number of unpaired males that sired extra-pair young (Fig. 1), the possibility that behaviorally monogamous females mate with extra-pair males to avoid continuous male harassment (Wolff and Macdonald 2004) cannot be ruled out for large treeshrews.

The prevalence of EPP in *T. tana* raises the question of the prevalence and effectiveness of mate guarding. Brotherton and Komers (2003) argued that behavioral monogamy in mammals can primarily be explained by the benefits of male mate guarding strategies, and predicted that most female mammals do not seek extra-pair copulations because of the costs of aggressive conflicts (e.g. dik-diks, Brotherton and Manser 1997). However, *T. tana* and many nocturnal prosimians form dispersed pairs (presumably to avoid foraging competition, Schülke and Ostner 2005; Munshi-South et al. 2007), and this avoidance behavior may render mate guarding ineffective. Male *T. tana* will likely maximize their reproductive success if they mate with their behavioral partner but also pursue extra-pair copulations, rather than make large temporal and energetic investments in mate guarding. However, males may attempt to monopolize reproductive opportunities with their partner by intensively guarding females during their short receptive period. I was unable to observe such consort behavior in the wild, but Martin (1968) noted that captive male *T. belangeri* showed intense, short term (i.e., less than 1 day) interest in estrous females kept in adjacent cages.

This study provides the first genetic parentage analysis for a monogamous mammal that inhabits tropical rainforests. EPP in tropical birds is generally uncommon, possibly due to asynchronous breeding limiting the opportunity for mate assessment, or relatively larger territories and lower breeding densities than in temperate environments (Fleischer et al. 1997; Stutchbury and Morton 2001). The high rates of EPP recorded for *T. tana* populations are at odds with results from tropical birds. Relatively asynchronous breeding in tropical birds may limit the abilities of males to pursue EPP and females to assess extra-pair males. Low EPP rates may further explain the relatively smaller testis size in tropical vs temperate songbirds (Stutchbury and Morton 1995). However, the correlation between EPP and breeding synchrony in birds is difficult to separate from other causal factors (Griffith et al. 2002), and smaller relative testis size does not necessarily imply that

EPP is rare (this study and Schülke et al. 2004). Only three previous studies have been conducted on monogamous mammals in the tropics, and all three were conducted on sympatric species in a dry deciduous forest in Madagascar: two lemurs that exhibited high rates of EPP and very short breeding seasons (2 weeks, Fietz et al. 2000; Schülke et al. 2004) and a genetically monogamous rodent with substantial male parental care (Sommer and Tichy 1999). I studied treeshrews from August to December to maximize offspring captures, because Emmons (2000) recorded the highest reproductive output for *T. tana* during these months. However, young were recorded in nearly all months of the year in Emmons' study, suggesting that *T. tana* reproduce relatively asynchronously. Breeding synchrony thus does not adequately explain EPP in the large treeshrew or other mammals studied to date.

High density of breeding adults is another ecological explanation for high rates of EPP, but has not received robust support in comparative avian studies (Griffith et al. 2002). EPP was detected in an insular fox species with one of the highest population densities ever recorded for a canid, presumably because territorial proximity and limited opportunities for dispersal in an insular habitat facilitated promiscuous mating (Roemer et al. 2001). High population density may also explain why swift foxes occasionally form polyandrous or polygynous "trios" (Kitchen et al. 2006). The *T. tana* population in selectively logged forest exhibited just as much EPP as the population in primary forest, despite longer distances between neighboring pairs in logged forest than in primary forest (Munshi-South 2006). Two sympatric tupaiids occur at lower population densities than large treeshrews (*T. longipes* and *T. gracilis*, Emmons 2000), and would provide an interesting test of the hypothesized association between breeding density and EPP.

Conclusions

This study provides the first genetic analysis of a treeshrew mating system, and the first results from a behaviorally monogamous mammal in a tropical rainforest. Treeshrews exhibited one of the highest rates of EPP recorded for a pair-living mammal. EPP was more common among males that did not have the option of mating with a female partner. The dispersed pair system of *T. tana* may render male mate guarding ineffective and lead to the high rates of EPP observed in this species. High EPP rates in treeshrews and pair-living primates were not associated with large relative testis size, indicating that sperm competition is not an important evolutionary force in behaviorally monogamous treeshrews or primates. Small testis size further suggests that behavioral monogamy is not masking more promiscuous multi-male mating in these species.

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