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Evidence of genetic monogamy in the lemur Indri (*Indri indri).*

A study based on paternity analysis and history of indri family groups in the Maromizaha New Protected Area, Madagascar

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Abstract

Monogamy is a rare strategy among mammals but relatively common among primates. The study of evolution of monogamy in mammals and primates is lacking empirical studies that assess the relationship between a pair-living social organization and genetic monogamy. Sexual or genetic monogamy can only be assessed by performing molecular analyses and investigating rates of Extra-Pair Paternity (EPP). Studying the occurrence of EPP can provide valuable insights into reproductive strategies and their adaptive value. The indri is a pair-living primate that lives in stable groups. Their social units are composed of the reproductive pair and up to four more individuals, but extra-pair copulation (EPC)

can occur. This raises the question of whether this event may or may not lead to EPP. Here, we investigated whether a pair-living social organization corresponds to genetic monogamy in indris (*Indri indri*). We analyzed the paternity of 12 offspring from 7 pairs using a set of six microsatellite loci on fecal samples (mean number of alleles 11.7 ± 1.8 (mean ± SD). We found that in 92% of cases the genetic profile of the offspring matched the paired male of the group for all the loci considered. In the only case of paternity mismatch, the paternity assignment remained inconclusive. Our results show that in indri genetic monogamy is the norm and support the hypothesis that pair-living social organization is associated with low EPP rate. Also, our results are in contrast with the hypothesis of infertility as a reason to engage in EPC for this species.

Keywords: Extra-Pair Copulation, Extra-Pair Paternity, primates, genetic monogamy, indri

Research Highlights

- In 92% of cases, the paired male of the group did not have any locus mismatch with the offspring. In the only case of paternity mismatch, we were not able to assign the sire identity.
- Our finding suggest that genetic monogamy is the norm in the indri, although EPC can occasionally occur.

Introduction

Monogamy is a mating system with a social and a genetic component, both linked to the social organization of a species. A social organization commonly associated with a monogamous mating system is pair-living, defined as a permanent and continuous association of one adult female and one adult male in space and time (Fuentes, 2000; Kappeler & van Schaik, 2002).

Behavioral and genetic studies on pair-living species of birds and mammals demonstrate that monogamy is a much more flexible and variable mating system in respect to what was previously thought (Kleiman, 1977; Black, 1996; Tecot, Singletary & Eadie, 2015), and different levels of monogamy can be recognized depending on the level of analysis: a pair-living social organization does not necessarily imply sexual exclusivity between partners (*sexual monogamy*), nor that all offspring are sired by the social father (*genetic monogamy*) (Reichard, 2003). While behavioral studies can help in describing a pair-living system (Kappeler & van Schaik, 2002), DNA analyses of the offspring and their parents are necessary to provide evidence of genetic monogamy, which usually refers more strictly to the mating system (Reichard, 2003). Genetic fingerprints of individuals from wild populations can provide important insights into the relationship between pair-living social organization and genetic monogamy, as well as a better understanding of the evolution of this mating system in primates and mammals in general.

In the existing literature, two terms have been used to define evidence of offspring sired by a different male than the social father: Extra Pair Paternity (EPP) and Extra Group Paternity (EGP), referring to offspring fathered by a male outside the reproductive pair or

the group, respectively. EPP and EGP are interchangeable when considering pair-living monogamous species (Isvaran & Clutton-Brock, 2007), as in our study; we decided to apply the term EPP because we focus on the reproductive pair of the group.

Birds are the typical example of a taxon in which more than 90% of species were considered sexually monogamous because of their pair-living organization (Lack, 1968). However, molecular analysis have confirmed that EPP is widespread across birds (Griffith, Owens & Thuman, 2002). Contrary to birds, pair living is very rare among mammals (3%) (Kleiman, 1977), and, when present, it is more frequently related to genetic monogamy (Lukas & Clutton-Brock, 2012, 2013). According to a study including 22 mammal species generally considered to have a monogamous mating system, the variation of EPP among species seems to be related to the social structure, with pair-living species having the lowest rates of EPP (Cohas & Allainé, 2009). The strength of pair bonding has also been suggested to possibly play a role (Cohas & Allainé, 2009; Clutton-Brock & Isvaran, 2006).

Among mammals, primates include the highest frequency of monogamous species (29%; Lukas & Clutton-Brock, 2013), usually organized in pair-living groups with immature or even mature offspring (Reichard & Boesch, 2003; Kappeler & van Schaik, 2002). Few studies have investigated the genetic paternity in putatively monogamous primates (not necessarily pair-living as we defined it) and some of them show that EPP occurs, including in tarsiers (*Tarsius lariang*, Driller, Perwitasari-Farajallah, Zischler & Merker, 2009), lemurs (*Hapalemur griseus alaotrensis,* Nievergelt, Mutschler, Feistner & Woodruff, 2002; *Cheirogaleus medius*, Fietz et al. 2000; *Phaner furcifer*, Schülke, Kappeler & Zischler, 2004), gibbons (*Hylobates lar*, Barelli et al. 2013; *Nomascus gabriellae*, Kenyon, Roos, Binh &

Chivers, 2011). To date, Azara's night monkey (*Aotus azarae*) and Muller's Bornean gibbon (*Hylobates muelleri*) are the only two primate species in which no evidence of EPP was found; however, in the latter case, only four offspring were tested (reviewed in Lambert, Sabol & Solomon, 2018).

The occurrence of EPP has consequences on individuals' reproductive success. Several non-exclusive hypotheses have been proposed to explain the presence of EPP, such as to guard against infertility of the social mate, to increase the genetic diversity of the offspring with consequences at population level, to provide "good genes" for the offspring, and to gain direct benefit given by access to resources (Birkhead & Møller, 1992; Griffith et al., 2002; Møller & Thornhill, 1998). However, there is still a lack of empirical data to support these hypotheses (Griffith et al., 2002; Akçay & Roughgarden, 2007), especially among primates.

The occurrence of EPP is directly related to the possibility of engagement in Extra-Pair Copulation (EPC). A social context with easy access to potential mates within and between groups increases the probability of EPCs and, as a consequence, can lead to higher rates of EPP (Cohas & Allainé, 2009). In pair-living species, the opportunities of engaging in EPC are lower because of more efficient control of the partner over potential mates. In territorial species, the overlap between territories and the rate of intergroup encounters can influence the chances of being in contact and interacting with potential mates, as suggested in gibbons (*Hylobates lar,* Reichard & Sommer, 1997).

It seems that there is a relationship between genetic monogamy and the strength of the pair bond (Huck, Fernandez-Duque, Babb, Schurr, 2014), and recent reviews proposed

the hypothesis that social organization and mate guarding are the main factors influencing a monogamous mating system in primates (Lambert, Sabol & Solomon, 2018, Reichard 2018). In this study, we investigate the genetic relationship among reproductive pairs, potential sires, and offspring to explore whether pair living is associated with genetic monogamy in the indris (*Indri indri*).

Bonadonna and colleagues (2014) reported the first observation of EPC in indris between a neighboring paired female and a paired male within the same population of this study. This event raised the question of whether EPC may lead to EPP; our study aims to address this question with subsequent eventual reconsideration of the monogamous mating system of this species at the genetic level.

The indri is a diurnal lemur living in family groups composed of up to six individuals, usually consisting of the reproductive pair and their offspring (Pollock, 1975, Bonadonna et al., 2014). The pair bonding in indris is remarkable. The reproductive pair is the basal unit of a group (Pollock, 1975) and we have records of individuals being together for at least a decade (unpublished data).

This species can be considered a slow breeder. The gestation period reported for indris is 157 days (Godfrey, Samonds, Jungers, Sutherlan, Irwin, 2004) and another study reported 176 days (Weir, 2014) with interbirth intervals of two to three years (Weir, 2014), females give births to one offspring at a time between May and July, and individuals presumably become potentially reproductive at the age of three, when they start singing (unpublished data). Group members tend to be cohesive, and usually the individuals of the reproductive pair stay within a distance between 0 and 13 m from each

other (Pollock, 1975). Each group occupies a stable and exclusive territory and intergroup encounters are very rare (Bonadonna et al. 2017). The indri is one of the few singing primates and, besides having a function in signaling territory occupancy and defense (Torti et al., 2013), the song also plays an important role in the mating and social system of this species. The temporal and spectral characteristics of the song can signal the pair-bond strength to conspecifics (Gamba et al., 2016). Furthermore, the individual-specific features of the indri song and acoustic similarities between fathers and sons can play a role in pair formation, dispersal and avoidance of inbreeding (Torti et al. 2017).

Using fecal samples collected in Maromizaha between 2009 and 2015, we obtained individual genetic fingerprints and examined the genetic relationships between sires and offspring. In the presence of an observed EPC (Bonadonna et al., 2014), we hypothesized that the indri population we studied would show a certain degree of EPP during successive years and across different groups. Previous studies on gibbons (*H. lar)*, demonstrated that EPC may frequently occur after group encounters (Reichard, 1995). A more recent work on the same population studied by Reichard has found an EPP rate of 9.5% on a total of 42 offspring (Bartlett, Light & Brockelman, 2016). Our prediction is that we would find a limited occurrence of EPP in indris, which present a pair-living social organization where EPC occurs.

Methods

Study Site

We conducted the study on a wild population of indris (*I. indri*) in the New Protected Area (NAP) of Maromizaha (18°56’49’’ S – 48°27’53’’ E). We accessed the forest from the

village of Anevoka (on the RN2), 147 km east of Antananarivo in the Alaotra Mangoro Region (Province of Toamasina), central-eastern Madagascar. Maromizaha (1880 ha) is a primary and secondary mid-altitude (800-1200m) evergreen rainforest, and it is part of the ecological corridor of Ankeniheny-Zahamena (Fig. 1). The study included an area of approximately 140 ha of continuous rainforest.

Subjects and Sample Collection

Nine indri groups have been habituated to human presence and have been the subject of ongoing etho-ecological studies in Maromizaha since 2009. Seven out of nine habituated groups were included in this study (Fig. 1).

We updated group composition and demographic records year by year, identifying each individual using natural marks. The population density in the study area was 27.7 $\pm$ 4.0 (mean $\pm$ SD) individuals and 8.4 $\pm$ 1.0 (mean $\pm$ SD) groups per km^2, comparable to other populations inhabiting different sites (Bonadonna et al. 2017; Pollock 1975).

Each group was composed of a reproductive pair plus one to three individuals. These additional indris are usually the pair's offspring; however, a case of immigration of an individual into another group was observed in another forest (GB and VT, unpublished data) (Table 1). We never observed copulation attempts outside the mating season, which ranges between December and February (Pollock 1975, GB VT pers. obs.). The reproductive pairs remained stable over time, except for one takeover in 2015, after the death of the injured resident male (CDG, pers. obs.) (Table 1).

For DNA analyses, we included 26 individuals from seven groups: seven parental pairs and 12 offspring. Unfortunately, not all the individuals that were part of a group during

our study were included in DNA analyses, either because we did not have samples available, or because DNA extraction did not succeed (Table 1).

Between 2011 and 2015, we obtained at least two fecal samples for each animal, each corresponding to a different defecation. We collected fecal samples immediately after defecation to avoid individual misidentification during the sampling process. Feces were put in 20ml labeled tubes filled with RNAlater® Ambion (Nsubuga et al. 2004) wearing disposable gloves and stored at room temperature in the field and at 4°C once transferred to the lab. We conducted all the genetic analyses at the New York University Molecular Anthropology Laboratory.

DNA Extraction

Genomic DNA was extracted from feces using the QIAamp DNA® Stool Mini Kit (Qiagen®, Hilden, Germany). To maximize the amount of DNA extracted we slightly modified the manufacturer's protocol (QIAamp DNA Stoll Handbook 04/2010): we used 300 mg stool instead of 180-220 mg; we added 35 µL of proteinase K rather than 25 µL and incubated at 70°C for 30 minutes, instead of 10 minutes, during the DNA purification phase. We then applied 75 µL Buffer AE on the QIAamp membrane rather than 200 µL for the first DNA elution and incubate the spin column with Buffer AE at room temperature for 15 minutes instead of one minute. We used the same QIAamp membrane to obtain a second DNA elution applying 50 µL of Buffer AE and incubate for 15 minutes at room temperature.

For the samples collected in the field during 2014, DNA purification was conducted using the automated robotic workstation QIAcube HT supported by the software

QIAxtractor 4.17.1 (Qiagen®) setting the protocol for QXT Liquid DNA V1. The preparation of the samples required a bath at 70°C for at least five minutes of the 2.0 mL tubes containing 300 mg of smashed feces and 1.6 mL of Buffer ASL. Afterwards, tubes were centrifuged at maximum speed (13000 RPM) for 10 minutes. 200 µL of supernatant were then transferred to separate wells of the QIAextractor lysis plate before starting the run. At the end of the run, we obtained 70 µL of DNA elution for each sample. We stored the extracted DNA at 4° C for immediate use.

Microsatellite Genotyping

We initially identified a set of 10 microsatellite marker loci as potentially variable in indris (Zaonarivelo et al. 2007). Polymorphism of each locus was tested using monolocus PCRs. Four loci failed to provide amplification products, and we therefore chose to use the six loci among the ten tested that provided good quality amplification products for multiplex PCRs (Table 2). The 5' end forward primer of each locus was labeled with a fluorescent dye (FAM, HEX) to analyze simultaneously loci of similar allelic size. PCR amplification was carried out in 10 µL reaction volume containing 2 µL DNA template, 5 µL Multiplex PCR Master Mix (Qiagen®), 0.1 µL of each primer, and two µL RNase-free water. The cycle conditions included a pre-incubation step at 95 °C for 15 min. We then performed 50 cycles with denaturation at 94 °C for 30 s, annealing at 54°C or 60 °C (depending on the locus, Table 2) for 90 s. The first extension phase was at 72 °C for 60 s; the final extension phase was at 60 °C for 30 min.

We separated PCR products by electrophoresis using a 48 capillary ABI 3730 DNA Analyzer (Applied Biosystems) for allele size estimates. We mixed 1 µL of PCR product with

6.85 µL HiDi formamide (Applied Biosystems) and 0.15 µL Genescan 500-ROX size standard (Applied Biosystems). We carried out automated allele calling using the GENEMAPPER 3.7 software (Applied Biosystems). We subsequently confirmed by eye all the allele calls and checked for consistency across replicate PCRs of the same sample or from the same individual. To minimize possible genotyping errors due to allelic dropout, we repeated independent PCRs for each locus depending on whether the individual resulted in being heterozygote (minimum three replicates) or homozygote (minimum five replicates) for a certain locus.

We used the software CERVUS 3.0 to calculate observed and expected heterozygosity to test deviation from Hardy-Weinberg equilibrium (HWE), and to estimate null allele frequency for each locus.

Paternity test and assignment

We identified 14 paired individuals (7 males, 7 females), and 12 potential offspring. We included the reproductive males of the neighboring groups as potential sires for each potential offspring. Unpaired neighboring males exceeding the age of 3 at the time of habituation were also included as potential sires because: (i) we could not exclude that they had potentially mated with a female in the study population before or during the sampling period; and (ii) the youngest indri we observed forming a group was 3 and half years old. We also considered the presence of potential sires from the unsampled neighboring groups. In the simulation, we included one unsampled potential sire for each neighboring non-habituated group.

Ten out of the 12 offspring were born during the 2009-2015 study period in Maromizaha; two were already present in the groups at the beginning of the habituation, and we could not know whether they were born in the group or immigrated. We considered these individuals as both potential offspring and potential sires. For these two indris, all the reproductive males were also included as potential fathers. For the single case of offspring and social father mismatch, we included each individual qualified as potential sire as a candidate father. The average number of potential sires for the 12 offspring was 7.3 ± 2.2 (mean ± SD) and the potential sires sampled were 4.1 ± 2.2 (mean ± SD).

Based on microsatellite genotypes, we ran parentage analyses using CERVUS 3.0 (Kalinowski, Taper, & Marshall, 2007). This program compares likelihood ratios (LOD scores) of all candidate fathers, assigning or excluding paternity to the most likely parent using statistical criteria generated by computer simulation. A true parent has a positive LOD score; on the contrary, a negative LOD score indicates that the potential father is unrelated to the offspring. The program takes genotyping errors and the presence of close relatives into account (Marshall et al. 1998; Jones & Ardren, 2003); we applied a mistyping rate of 0.01 This procedure ensures the running of blind analyses.

We set the confidence levels of paternity assignment at 90% (relaxed level) and 95% (strict level). The critical value corresponding to a strict or relaxed confidence level was given by the delta LOD score between the first and the second potential father, automatically calculated by CERVUS. We considered the assignment of the father given a known mother, to include the genotype of the mother when matching the genotype of

potential sires to offspring. This considers the case in which the parent pairs are mutually exclusive.

We assigned paternity in the cases of complete genotypes matching between the potential father and offspring. We excluded the paternity in cases of negative LOD scores and more than one allelic mismatch between the offspring and the potential father. We assigned EPP in cases of paternity exclusion for the social father; if the paternity was also excluded for all the other possible sampled sires, we defined the assignment of EPP as inconclusive.

Ethical standards

During the study, we did not have any physical contact with the animals. All fecal samples were collected from the ground immediately after defecation. The research was authorized by the "Ministère de l'Environnement et des Forêts" (MEF) of Madagascar. Research permits: N° 243/ 09/ MEF/ SG/ DGF/ DCB.SAP/ SLRSE, N° 118/ 10/ MEF/ SG/ DGF/ DCB.SAP/ SCBSE; N° 293/ 10/ MEF/ SG/ DGF/ DCB.SAP/ SCB, N° 274/ 11/ MEF/ SG/ DGF/ DCB.SAP/ SCB, N°245/12/MEF/SG/DGF/DCB.SAP/SCB, N°066/14/MEF/SG/DGF/DCB.SAP/ SCB. We adhered to applicable international, national, and/or institutional guidelines for the study on animals and non-human primates, including the American Society of Primatologist (ASP) Principle for the Ethical Treatment of non-human Primates, and the European Union directive guidelines for the study on animals and non-human primates (Directive 2010/63/EU). The study did not require an IACUC approval.

Results

Genotypes and Identity of Individuals

We included all the 26 individuals in the analyses. For three indris of the group 8MZ we failed to amplify the locus 67HDZ55, and for one of them (Jonah) also the locus 67HDZ62. We obtained confirmed genotype of 23 individuals for the locus 67HDZ55, 25 for the locus 67HDZ62, and 26 for all the four remaining loci (Table 3).

The number of alleles per locus varied between nine and 14, observed heterozygosity (HO) ranged from 0.840 to 0.962 and expected heterozygosity (HE) ranged between 0.814 and 0.928. None of the six loci showed significant deviation from the Hardy-Weinberg equilibrium (HWE) after Bonferroni correction (Table 3). Even if in some cases the presence of null allele frequency is close to the suggested threshold of 0.05, we included all the loci in the analyses because the level of heterozygosity observed was high and all known offspring-maternal pairs were free of possible homozygous mismatches for those loci. The combined non-exclusion probability of identity over six loci was 8.482×10^{-10}. Therefore, it is unlikely that the set of loci failed to differentiate between two randomly-selected individuals.

We found two cases of loci mismatch between mother and offspring. In one case (Locus 67HDZ55) the offspring resulted to be homozygous for this locus after six independent replicates (group 3MZ; genotypes: mother “Mena” 313-327; offspring “Blague” 330-330). In the other case (locus 67HDZ25), the mother was homozygous after eight independent replicates (group 4MZ; genotypes: mother “Eva” 224-224; offspring “Hendri” 226-228). In both cases the offspring matched with the mother for all the other

five loci, and all the six loci matched with the social father; therefore, we suggest that this incompatibility is related to the estimated error rate of 0.0733 and of 0.0728 for the loci 67HDZ55 and 67HDZ25, respectively. It is possible that known parent mismatches at one or more loci are due to the presence of null alleles (Kalinowski, Taper & Marshall, 2007), especially in the case of homozygous individuals; the high likelihood of parentage given by the other loci and the consistent match with both parents make it acceptable to consider the known mothers as the true parents. The same methodological approach would have been applied in the case of one locus mismatch with the fathers.

Paternity

Paternity based on CERVUS at a confidence level of 95% given a known mother was assigned to a total of 11 individuals out of 12 (92% against an expected assignment of 61% of the total offspring). We found a lower percentage of unassigned paternity than expected, 8% versus 39%. None of the assigned paternity presented any mismatch with the offspring for all the six loci considered, and the assigned fathers were consistently the reproductive males of the group (Table 4).

Only one out of 12 offspring tested (Tsiky, group 6MZ) showed a mismatch with the social father (Zokybe, group 6MZ) for all the six loci, and a negative LOD score. We have also found that the LOD score was negative for all the potential fathers tested. However, the reproductive female (Befotsy, group 6MZ) had no mismatch with the offspring (Table 4). Tsiky was born in the summer of 2010, the year after the habituation of the group; Zokybe was the paired male of the group at the time of habituation (in September 2009) and was the paired male at the time of the reproductive season (starting in December

2009); Tsiky was born in the summer of 2010, the first birth season after the habituation of the group; therefore, we can consider the mismatch as a case of EPP with inconclusive paternity assignment. This evidence indicates an EPP rate of 8% in our sample.

The most closely related individual to the indri with unassigned paternity was Hendri, a potential reproductive male of the neighboring group 4MZ, having two loci mismatches even when considering the genotype of the mother. However, it is unlikely that Hendri can be the father: Hendri and Tsiky are heterozygotes for all the loci considered, eliminating the possibility of allelic dropout at the mismatching loci; furthermore, the LOD score was negative, excluding Hendri as a potential sire for Tsiky.

It must be noted that an unsampled individual was in the group 6MZ during the year of conception and left the group the year after the birth of Tsiky (Table 1). If the male that left the group was one of the offspring, we would exclude the possibility that he was a potential sire, and we would hypothesize that the sire would be an unknown male. Unfortunately, our samples did not allow us to test this hypothesis, leaving the paternity assignment inconclusive.

The male (Emilio) involved in the EPC observed in 2011 (Bonadonna et al. 2014) with the female of the group 3MZ (Mena) was not the sire of the individual (Blague) born after the EPC; in fact, it was the resident and paired male of the group 3MZ (Ratsy) that was found to be Blague's father (Table 4).

Discussion

Our study investigates the genetic relationship between offspring, social parents and potential sires in indris, within the same population where an EPC was observed (Bonadonna et al. 2014). The results we presented in this paper provide new insights into the mating system of this pair-living primate.

The paternity analysis revealed that all genetic profiles of the offspring matched with the social fathers, except for one case. Because in this case the paternity remained inconclusive, we cannot exclude our hypothesis that EPC may lead to EPP in indris, but we have to weigh this phenomenon across our study groups. In fact, we confirmed genetic monogamy for 11 out of the 12 cases we tested. The 8% mismatch with the paired males we found in indris is lower than the high rate of EPP found by Fietz and colleagues (2000) in the nocturnal dwarf lemur (*C. medius*, 44%), but comparable with the study of Barelli et al. (2013) reporting rates varying from 8.5% to 10% for white handed gibbons (*H. lar*).

Our results agree with the low rate of EPP in monogamous and constantly associated pairs predicted by van Schaik & Kappeler (2003) and Clutton-Brock & Isvaran (2006). Our study also supports the hypothesis of Cohas & Allainé (2009), who pointed out the important role of pair-living social organization in ensuring paternity exclusivity, in addition to pair-bonding strength and mate guarding.

White-handed gibbons share with indris several characteristics: they usually have a stable reproductive pair per group, and they show exclusive territoriality (Brockelman et al., 1998). They also share the emission of loud songs that, in both species, have a role in

signaling pair-bonding strength (Geissmann & Orgendilger 2000; Gamba et al., 2016). Long distance calls can also broadcast information to potential mates (Torti et al., 2013, 2017; Gamba et al., 2016). Each group exchanges information about individuals' sex, status, and genetic relatedness throughout the emission of loud songs way beyond the limit of a territory (Torti et al., 2017). In this way, groups are not isolated units and individuals can communicate with one another without having visual or physical contact (Giacoma et al., 2010; Gamba et al., 2016; Bonadonna et al. 2017). Such a system would allow regulating inter-group dynamics including the possibility of engaging in EPC (Bonadonna et al., 2014), and therefore providing a certain degree of flexibility in the mating system of this species.

The takeover of paired individuals is rare and, according to our observations, the case reported for the group 3MZ in Maromizaha was the result of a conflict between males (CDG, pers. obs.). We suggest that both pair-bonding strength and social structure play a role in the flexibility of a monogamous mating system, as we found in indris.

It has been suggested that once a monogamous mating system is established, low rates of EPP are related to intensive male care (Huck et al., 2014). In the indri, males cover the primary role in territorial defense (Pollock 1975), which can be considered an indirect form of parental care (Brockelman, 1975; Kleiman, 1977). The territorial defense is part of the resource defense strategy: the male can guard the access to the female, and both the female and offspring have access to resources (Clutton-Brock, 1991; Møller & Thornhill, 1998). This idea agrees with the hypothesis that parental care is an indirect evolutionary consequence of mate guarding (Huck et al., 2014). There is no more direct form of

parental care by males in indris apart from the fact that they may occasionally transfer infants from a branch to another while the mother is feeding (Torti, pers. comm.).

As suggested by Bonadonna and colleagues (2014), EPC can be a female strategy to enhance male guarding. Given the fact that the EPC reported for indris has been observed at the beginning of the mating season, it could have increased the male's guarding behavior and also reduced the chances of EPP. An enhanced guarding of the paired male would also improve resource monopolization, especially during the mating season (Brotherton & Komers, 2003).

We can also indirectly assume that EPP and the occurrence of EPCs can be underestimated. The direct and indirect benefits for females adopting differential reproductive strategies in indris are not easily identifiable. Interestingly, the genetic profile of the individual born after the breeding season in which we observed the EPC suggests that the female successfully reproduced with her social partner during the same year. Thus, our findings suggest that the female was not likely to engage in EPC to avoid partner infertility (Palombit, 1994; Brotherton & Komers, 2003), as hypothesized for white-handed gibbons (in which two out of three EPPs were found in the same pair-living group, Barelli et al., 2013).

Our results excluded that the current paired male of the group 6MZ (Zokybe) sired the oldest individual of the offspring (Tsiky), representing the only case of EPP in our study, but he did not present any loci mismatch with the individual born in 2014 (Hira). We consider two possible scenarios to explain our results. In the first scenario, the genetic father of the individual with unassigned paternity might have preceded a male takeover,

which would explain the mismatch of the paired male as the sire of the offspring. Partner take-over in the indri can occur. This fact would configure a forced "divorce" as reported for brown titi monkeys (*Callicebus brunneus*; Lawrence, 2007), and owl monkeys (*Aotus azarae;* Fernandez-Duque & Huck, 2013), and it agrees with the serial monogamy model proposed by Fernandez-Duque & Huck (2013). This first scenario seems unlikely because Zokybe was reported as the paired male during the reproductive season that preceded the birth of Tsiky despite the changes in the group composition. In the second, and more likely scenario, there was no takeover of the reproductive male because Zokybe was the paired-male of the female at the time of conception. This scenario is in accordance with our hypothesis that partner infertility is not a primary cause of EPC and EPP in indris because Zokybe was likely the father of the younger offspring (no evidence of allelic mismatch).

Although we were not able to assign the paternity to an individual, based on our results, we can draw some conclusion about the mating system in indris. According to our findings and observations, individuals that reach maturity can either leave or remain in the group. We found that two resident males (group 4MZ and 8MZ), both over the reproductive age, were the offspring of the reproductive pairs. Furthermore, we never observed a copulation event between a mother and her potential reproductive offspring, and finally we did not find any case of offspring sired by an older brother. Therefore, we did not find any evidence to support the hypothesis that adults prior dispersal are likely candidates for siring offspring.

The floating of individuals without a territory, delayed dispersal and immigration have an important role in the dynamics of the monogamous mating system in pair-living

primates (Porter, Grote, Fernandez-Duque & Di Fiore, 2017; Jacobs, Frankel, Rice, Kiefer & Bradley, 2018). Unfortunately, there are few studies and data available on the dispersal pattern and dynamics of the social organization in the indri. The role of these factors in the social and mating system of this species need further investigation. However, even if in one case we could not assign the paternity, in all the other cases we found no loci mismatches between social fathers and offspring. In addition, we did not find any case of ambiguity among potential fathers, making our results consistent.

Genetic analyses can reveal a level of plasticity in the mating system of species considered monogamous (Díaz-Muñoz & Bales 2016). Although our study is limited to a relatively small sample size, it is indicative that genetic monogamy seems to be the norm in indris, with an EPP rate comparable to other pair-living monogamous primates. Furthermore, it is unlikely that a female would reproduce with another male in the group other than her partner. This situation would exclude that the social organization of indris may change from pair-living to polyandrous as it has been found in primates with a similar behavioral ecology, such as the gibbons (Barelli, Heistermann, Boesch, Reichard, 2008).

In conclusion, our study contributes to the scanty literature of genetic studies of wild monogamous, pair-living primates. We found that the model of pair-living social organization fits this species although it might not be consistently associated with genetic monogamy despite the low EPP rate. However, our results were not fully conclusive about paternity assignment, requiring further investigations on the genetic structure of the population, and the dispersal dynamics. Future studies should focus on providing further

insight into the mechanisms involved in the maintenance of socially monogamous systems and their genetic variability.

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Table 1 Annual group composition of the seven indri groups, since the habituation to 2015, including name and sex of the individuals. Gray cells: presence of an individual in the group. Italic individuals: genetic data not available. Bold names: offspring born in the group during the study period.

Group	Individual	Sex	2009	2010	2011	2012	2013	2014	2015
1MZ	Bevolo (R)	Female							
	Jery (R)	Male							
	Fotsy (>3†)	*Male*							
	Maintso	Female							
	Berthy	Female							
	Akora	*Unknown*							
2MZ	Soa (R)	Female							
	Max (R)	Male							
	Kinga	*Unknown*							
	Fanihy	Female							
	Afo	*Unknown*							
3MZ	Mena (R)	Female			EPC‡				
	Ratsy (R)	Male							
	Mahagaga (R)	*Male*							
	Tsara (>3†)	*Male*							
	Zandry	Female							
	Blague	Male							
	Faly	*Female*							
	Laro	*Unknown*							
	Tonga (>3†)	*Female*							
4MZ	Eva (R)	Female							
	Koto (R)	Male							
	Hendri (>3†)	Male							
	Gibet	Male							
	Nic	*Unknown*							
6MZ	Befotsy (R)	Female							
	Zokybe (R)	Male							
	Marco (>3†)	Male							
	Guido (>3†)	Male							
	Tsiky	Male							
	Hira	Unknown							
8MZ	Bemasoandro (R)	Female							
	Jonah (R)	Male							
	Cesar (>3)	Male							
	Zafy	Male							
	Mika	*Unknown*							

9MZ	Sissie (R)	Female		
	Emilio (R)	Male	EPC‡	
	Njaka	*Male*		
	Ovy	Male		
	Dosy	*Unknown*		

654

655 **Table 2** Microsatellite Loci Applied with Respective Primers, Annealing Temperatures and Number of PCR (Torti et al. 2017)

656

Locus	Forward primer	Reverse Primer	Repeat motif	Annealing temp. (°C)	Number of PCR cycles	Size range (bp)
67HDZ25	GGACCCTAATTCA AATATCACCTC	GGCATTTCTACT CCAGGTTGG	(CA)16	54	50	218-253
67HDZ62	AGCCCTTTCTCTC AATGCC	CCTTCTTTGTTAT CTTTCTGCATC	(GT)21	54	50	203-217
67HDZ18	GGACTGGTAGAT TTCTGGGTTTAG	CAGCCACTCCAA TGCAAAG	(CA)7C(CA)15	60	50	164-190
67HDZ55	TCAGGAGTTGGG ACCAGGG	ATGAAGGGATG GAGGTGGG	(GT)18	60	50	312-334
67HDZ180	TCCCCTCCTCAGT CATTTCTC	CGTGAAGCTCGT GTGTATGG	(CA)17	60	50	113-136
67HDZ39	CAGAGCCAGGGT TCAAATTC	TTGTCTTTTCTGC CACTGTAGG	(CA)11	60	50	148-162

Table 3 Per Locus Summary of six Microsatellites Markers for *Indri indri*

Locus	# Individuals	# Alleles	Observed Heterozygosity	Expected Heterozygosity	Hardy Weinberg Equilibrium p-value	Allele Null Probability
67HDZ25	26	13	0.923	0.876	0.1090	-0.0487
67HDZ62	25	9	0.840	0.814	0.4591	-0.0290
67HDZ18	26	11	0.962	0.883	0.8488	-0.0529
67HDZ55	23	14	0.913	0.928	0.7909	-0.0042
67HDZ180	26	11	0.846	0.876	0.8869	0.0091
67HDZ39	26	12	1.000	0.908	0.1046	-0.0589

Table 4 Paternity analyses results of 12 offspring and respective known and potential parents of seven indri groups. For the potential fathers we included the number of loci mismatch and the sign of the LOD score (positive or negative). . We indicated if the paternity was assigned to the paired male or remained inconclusive, we specified if the male involved in the extra pair-copulation was excluded as sire.

				Potential Sires Sampled		Assigned Paternity		
Group	Y of Birth	Offspring	Mother (# mismatches)	Paired Male (# mismatches, LOD score sign)	Neighboring Male(s) Genotyped (# mismatches, LOD score sign)	Paired Male	EPC Male†	Inconclusive Assignment
1MZ	2010	Maintso	Bevolo (0)	Jery (0, +)	Ratsy (6, -), Zokybe (6, -), Cesar (4, -)	x		
1MZ	2012	Berthy	Bevolo (0)	Jery (0, +)	Ratsy (5, -), Zokybe (6, -)	x		
2MZ	2012	Fanihy	Soa (0)	Max (0, +)	Ratsy (4, -)	x		
3MZ	2010	Zandry	Mena (0)	Ratsy (0, +)	Cesar (3, -), Jery (4, -), Max (4, -), Emilio (5, -)	x		
3MZ	2012	Blague	Mena (1)	Ratsy (0, +)	Jery (5, -), Max (5, -), Emilio (6, -)	x	excluded	
4MZ	N/A	Hendri	Eva (1)	Koto (0, +)	Jery (3, -), Emilio (4, -), Max (4, -), Jonah (4, -), Zokybe (5, -), Cesar (5, -), Ratsy (6, -)	x		
4MZ	2012	Gibet	Eva (0)	Koto (0, +)	Hendri (4, -), Zokybe (6, -)	x		
6MZ	2010	Tsiky	Befotsy (0)	Zokybe (6, -)	Hendri (2, -), Ratsy (4, -), Cesar(4, -), Koto (5, -), Jery (5, -), Max (5, -), Emilio (6, -)			x
6MZ	2014	Hira	Befotsy (0)	Zokybe (0, +)	Koto (2, -), Hendri (3, -), Jery (5, -)	x		
8MZ	N/A	Cesar	Bemasoandro (0)	Joanh (0, +)	Jery (2, -), Ratsy (2, -), Max (2, -), Zokybe (3, -), Koto(4, -) Emilio (4, -), Hendri (5, -)	x		
8MZ	2012	Zafy	Bemasoandro (0)	Joanh (0, +)	Cesar (3, -)	x		
9MZ	2013	Ovy	Sissie (0)	Emilio (0, +)	Ratsy (6, -)	x		
Percentage of paternity assignement						92%	0%	8%

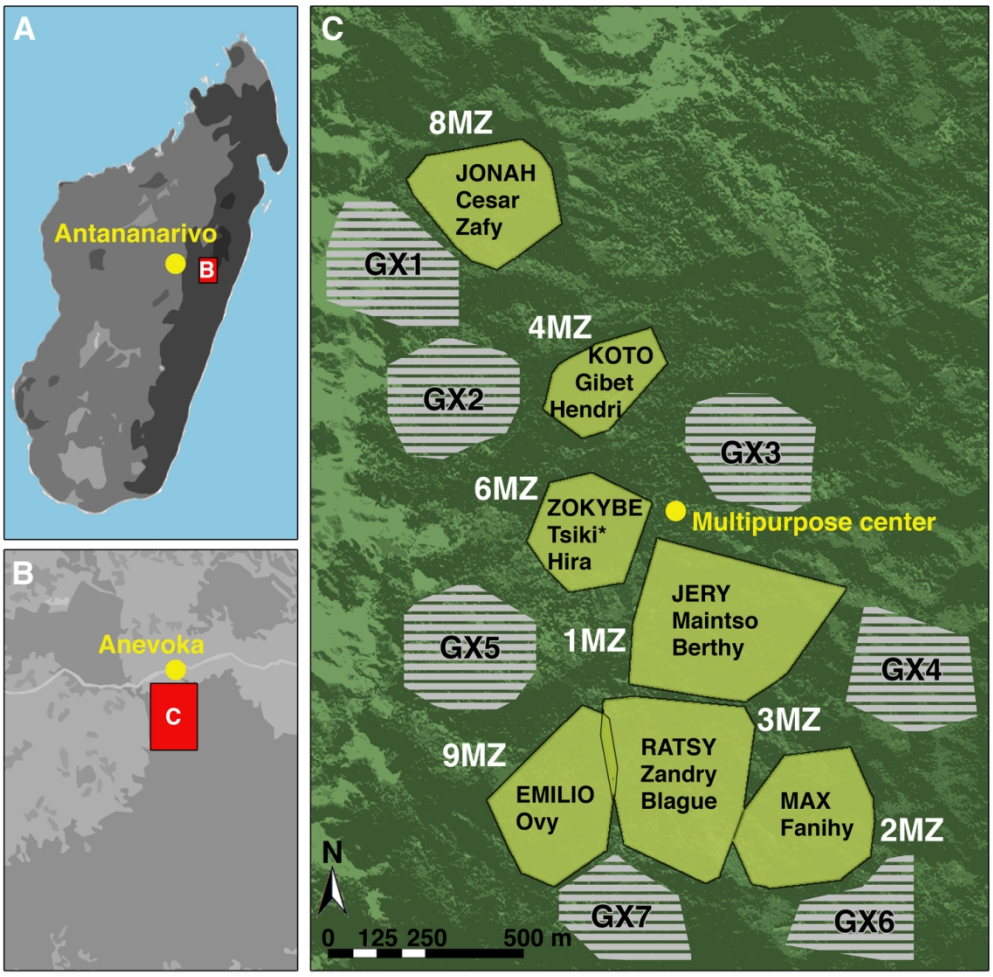
† EPC male: There is only one case of EPC reported in indris (Bonadonna et al. 2014). Emilio is the male that participated in the EPC event observed during the mating season that preceded the birth of Blague.

Figure 1. Study site and indri groups included in the study

Study site located in Center-Eastern Madagascar (A), in the Maromizaha forest, accessible from the village Anevoka (B). The multipurpose center (18°58'34'' S – 48°27'53'' E) was used as base-camp (C). Box C is showing the spatial distribution of the indri groups included in the genetic analysis (solid green polygons), and the schematic representation of unsampled neighboring groups (gray striped polygons, GX1 to GX7). The uppercase names within each studied group are the social fathers; the lowercase names are the offspring genotyped. "*" denotes the only individual that resulted with inconclusive paternity assignment. Original satellite images (Google Earth - Image © 2018 CNES / Airbus; downloaded on June 27, 2018) have been graphically simplified and adjusted with GIMP 2.10.2. Map of the indri territories created with ArcGIS® 10.2 (ESRI).

Research Highlights

- In 92% of cases, the paired male of the group did not have any loci mismatch with the offspring. In the only case of paternity mismatch, we were not able to assign the sire identity.
- Our finding suggest that genetic monogamy is the norm in indri, although EPC can occasionally occur.



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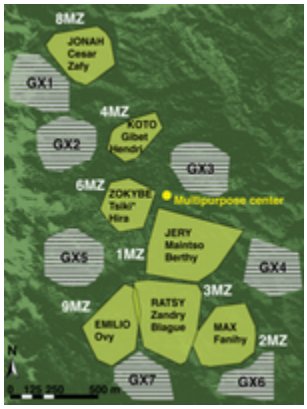
Study site located in Center-Eastern Madagascar (A), in the Maromizaha forest, accessible from the village Anevoka (B). The multipurpose center (18°58'34'' S – 48°27'53'' E) was used as base-camp (C). Box C is showing the spatial distribution of the indri groups included in the genetic analysis (solid green polygons), and the schematic representation of unsampled neighboring groups (gray striped polygons, GX1 to GX7). The uppercase names within each studied group are the social fathers; the lowercase names are the offspring genotyped. "*" denotes the only individual that resulted with inconclusive paternity assignment. Original satellite images (Google Earth - Image © 2018 CNES / Airbus; downloaded on June 27, 2018) have been graphically simplified and adjusted with GIMP 2.10.2. Map of the indri territories created with ArcGIS® 10.2 (ESRI).

127x124mm (300 x 300 DPI)



Spatial distribution of the indri groups included in the genetic and paternity analyses (solid green polygons), and the schematic representation of unsampled neighboring groups (gray striped polygons, GX1 to GX7). The uppercase names within each studied group are the social fathers; the lowercase names are the offspring tested. "*" denotes the only individual we found with inconclusive paternity assignment.

12x16mm (300 x 300 DPI)