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

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3 1 **Evidence of genetic monogamy in the lemur Indri (*Indri indri*).**

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5 2 ***A study based on paternity analysis and history of indri family groups in the Maromizaha***

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8 3 ***New Protected Area, Madagascar***

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35
36 13 **Abstract**

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39 14 Monogamy is a rare strategy among mammals but relatively common among primates.

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41
42 15 The study of evolution of monogamy in mammals and primates is lacking empirical studies

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45 16 that assess the relationship between a pair-living social organization and genetic

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48 17 monogamy. Sexual or genetic monogamy can only be assessed by performing molecular

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51 18 analyses and investigating rates of Extra-Pair Paternity (EPP). Studying the occurrence of

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54 19 EPP can provide valuable insights into reproductive strategies and their adaptive value.

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56
57 20 The indri is a pair-living primate that lives in stable groups. Their social units are composed

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59
60 21 of the reproductive pair and up to four more individuals, but extra-pair copulation (EPC)

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3 22 can occur. This raises the question of whether this event may or may not lead to EPP.
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5 23 Here, we investigated whether a pair-living social organization corresponds to genetic
6
7 24 monogamy in indris (*Indri indri*). We analyzed the paternity of 12 offspring from 7 pairs
8
9 25 using a set of six microsatellite loci on fecal samples (mean number of alleles 11.7 ± 1.8
10
11 26 (mean \pm SD). We found that in 92% of cases the genetic profile of the offspring matched
12
13 27 the paired male of the group for all the loci considered. In the only case of paternity
14
15 28 mismatch, the paternity assignment remained inconclusive. Our results show that in indri
16
17 29 genetic monogamy is the norm and support the hypothesis that pair-living social
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19 30 organization is associated with low EPP rate. Also, our results are in contrast with the
20
21 31 hypothesis of infertility as a reason to engage in EPC for this species.
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28 32 Keywords: Extra-Pair Copulation, Extra-Pair Paternity, primates, genetic monogamy, indri
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31

32 33 **Research Highlights**

- 34 34 • In 92% of cases, the paired male of the group did not have any locus mismatch
35
36 35 with the offspring. In the only case of paternity mismatch, we were not able to
37
38 36 assign the sire identity.
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40 37 • Our finding suggest that genetic monogamy is the norm in the indri, although EPC
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42 38 can occasionally occur.
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39 Introduction

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

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

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

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3 61 the group, respectively. EPP and EGP are interchangeable when considering pair-living
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5 62 monogamous species (Isvaran & Clutton-Brock, 2007), as in our study; we decided to
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8 63 apply the term EPP because we focus on the reproductive pair of the group.
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11 64 Birds are the typical example of a taxon in which more than 90% of species were
12
13 65 considered sexually monogamous because of their pair-living organization (Lack, 1968).
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16 66 However, molecular analysis have confirmed that EPP is widespread across birds (Griffith,
17
18 67 Owens & Thuman, 2002). Contrary to birds, pair living is very rare among mammals (3%)
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21 68 (Kleiman, 1977), and, when present, it is more frequently related to genetic monogamy
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23 69 (Lukas & Clutton-Brock, 2012, 2013). According to a study including 22 mammal species
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25
26 70 generally considered to have a monogamous mating system, the variation of EPP among
27
28 71 species seems to be related to the social structure, with pair-living species having the
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30
31 72 lowest rates of EPP (Cohas & Allainé, 2009). The strength of pair bonding has also been
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33 73 suggested to possibly play a role (Cohas & Allainé, 2009; Clutton-Brock & Isvaran, 2006).
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36 74 Among mammals, primates include the highest frequency of monogamous species
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38 75 (29%; Lukas & Clutton-Brock, 2013), usually organized in pair-living groups with immature
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40
41 76 or even mature offspring (Reichard & Boesch, 2003; Kappeler & van Schaik, 2002). Few
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44 77 studies have investigated the genetic paternity in putatively monogamous primates (not
45
46 78 necessarily pair-living as we defined it) and some of them show that EPP occurs, including
47
48 79 in tarsiers (*Tarsius lariang*, Driller, Perwitasari-Farajallah, Zischler & Merker, 2009), lemurs
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51 80 (*Hapalemur griseus alaotrensis*, Nievergelt, Mutschler, Feistner & Woodruff, 2002;
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53 81 *Cheirogaleus medius*, Fietz et al. 2000; *Phaner furcifer*, Schülke, Kappeler & Zischler, 2004),
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56 82 gibbons (*Hylobates lar*, Barelli et al. 2013; *Nomascus gabriellae*, Kenyon, Roos, Binh &
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3 83 Chivers, 2011). To date, Azara's night monkey (*Aotus azarae*) and Muller's Bornean gibbon
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5 84 (*Hylobates muelleri*) are the only two primate species in which no evidence of EPP was
6
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8 85 found; however, in the latter case, only four offspring were tested (reviewed in Lambert,
9
10 86 Sabol & Solomon, 2018).

11
12
13 87 The occurrence of EPP has consequences on individuals' reproductive success. Several
14
15 88 non-exclusive hypotheses have been proposed to explain the presence of EPP, such as to
16
17 89 guard against infertility of the social mate, to increase the genetic diversity of the
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19 90 offspring with consequences at population level, to provide "good genes" for the
20
21 91 offspring, and to gain direct benefit given by access to resources (Birkhead & Møller, 1992;
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23 92 Griffith et al., 2002; Møller & Thornhill, 1998). However, there is still a lack of empirical
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25 93 data to support these hypotheses (Griffith et al., 2002; Akçay & Roughgarden, 2007),
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27 94 especially among primates.

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33 95 The occurrence of EPP is directly related to the possibility of engagement in Extra-Pair
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35 96 Copulation (EPC). A social context with easy access to potential mates within and between
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37 97 groups increases the probability of EPCs and, as a consequence, can lead to higher rates of
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39 98 EPP (Cohas & Allainé, 2009). In pair-living species, the opportunities of engaging in EPC are
40
41 99 lower because of more efficient control of the partner over potential mates. In territorial
42
43 100 species, the overlap between territories and the rate of intergroup encounters can
44
45 101 influence the chances of being in contact and interacting with potential mates, as
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47 102 suggested in gibbons (*Hylobates lar*, Reichard & Sommer, 1997).

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51 103 It seems that there is a relationship between genetic monogamy and the strength of
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53 104 the pair bond (Huck, Fernandez-Duque, Babb, Schurr, 2014), and recent reviews proposed
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3 105 the hypothesis that social organization and mate guarding are the main factors influencing
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5 106 a monogamous mating system in primates (Lambert, Sabol & Solomon, 2018, Reichard
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7 107 2018). In this study, we investigate the genetic relationship among reproductive pairs,
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9 108 potential sires, and offspring to explore whether pair living is associated with genetic
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11 109 monogamy in the indris (*Indri indri*).
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16 110 Bonadonna and colleagues (2014) reported the first observation of EPC in indris
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18 111 between a neighboring paired female and a paired male within the same population of
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20 112 this study. This event raised the question of whether EPC may lead to EPP; our study aims
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22 113 to address this question with subsequent eventual reconsideration of the monogamous
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24 114 mating system of this species at the genetic level.
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29 115 The indri is a diurnal lemur living in family groups composed of up to six individuals,
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31 116 usually consisting of the reproductive pair and their offspring (Pollock, 1975, Bonadonna
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33 117 et al., 2014). The pair bonding in indris is remarkable. The reproductive pair is the basal
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35 118 unit of a group (Pollock, 1975) and we have records of individuals being together for at
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37 119 least a decade (unpublished data).
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42 120 This species can be considered a slow breeder. The gestation period reported for
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44 121 indris is 157 days (Godfrey, Samonds, Jungers, Sutherlan, Irwin, 2004) and another study
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46 122 reported 176 days (Weir, 2014) with interbirth intervals of two to three years (Weir,
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48 123 2014), females give births to one offspring at a time between May and July, and
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50 124 individuals presumably become potentially reproductive at the age of three, when they
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52 125 start singing (unpublished data). Group members tend to be cohesive, and usually the
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54 126 individuals of the reproductive pair stay within a distance between 0 and 13 m from each
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3 127 other (Pollock, 1975). Each group occupies a stable and exclusive territory and intergroup
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5 128 encounters are very rare (Bonadonna et al. 2017). The indri is one of the few singing
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8 129 primates and, besides having a function in signaling territory occupancy and defense (Torti
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10 130 et al., 2013), the song also plays an important role in the mating and social system of this
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12 131 species. The temporal and spectral characteristics of the song can signal the pair-bond
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14 132 strength to conspecifics (Gamba et al., 2016). Furthermore, the individual-specific features
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16 133 of the indri song and acoustic similarities between fathers and sons can play a role in pair
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18 134 formation, dispersal and avoidance of inbreeding (Torti et al. 2017).
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23 135 Using fecal samples collected in Maromizaha between 2009 and 2015, we obtained
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25 136 individual genetic fingerprints and examined the genetic relationships between sires and
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27 137 offspring. In the presence of an observed EPC (Bonadonna et al., 2014), we hypothesized
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29 138 that the indri population we studied would show a certain degree of EPP during successive
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31 139 years and across different groups. Previous studies on gibbons (*H. lar*), demonstrated that
32
33 140 EPC may frequently occur after group encounters (Reichard, 1995). A more recent work
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35 141 on the same population studied by Reichard has found an EPP rate of 9.5% on a total of 42
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37 142 offspring (Bartlett, Light & Brockelman, 2016). Our prediction is that we would find a
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39 143 limited occurrence of EPP in indris, which present a pair-living social organization where
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41 144 EPC occurs.
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49 145 **Methods**

50 51 52 146 *Study Site*

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55 147 We conducted the study on a wild population of indris (*I. indri*) in the New Protected
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57 148 Area (NAP) of Maromizaha (18°56'49" S – 48°27'53" E). We accessed the forest from the
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3 149 village of Anevoka (on the RN2), 147 km east of Antananarivo in the Alaotra Mangoro
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5
6 150 Region (Province of Toamasina), central-eastern Madagascar. Maromizaha (1880 ha) is a
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8 151 primary and secondary mid-altitude (800-1200m) evergreen rainforest, and it is part of
9
10 152 the ecological corridor of Ankeniheny-Zahamena (Fig. 1). The study included an area of
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12
13 153 approximately 140 ha of continuous rainforest.

16 154 *Subjects and Sample Collection*

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18 155 Nine indri groups have been habituated to human presence and have been the subject
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21 156 of ongoing etho-ecological studies in Maromizaha since 2009. Seven out of nine
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23 157 habituated groups were included in this study (Fig. 1).

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26 158 We updated group composition and demographic records year by year, identifying
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29 159 each individual using natural marks. The population density in the study area was $27.7 \pm$
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31 160 4.0 (mean \pm SD) individuals and 8.4 ± 1.0 (mean \pm SD) groups per km², comparable to
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33 161 other populations inhabiting different sites (Bonadonna et al. 2017; Pollock 1975).

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37 162 Each group was composed of a reproductive pair plus one to three individuals. These
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39 163 additional indris are usually the pair's offspring; however, a case of immigration of an
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42 164 individual into another group was observed in another forest (GB and VT, unpublished
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44 165 data) (Table 1). We never observed copulation attempts outside the mating season, which
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46 166 ranges between December and February (Pollock 1975, GB VT pers. obs.). The
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49 167 reproductive pairs remained stable over time, except for one takeover in 2015, after the
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51 168 death of the injured resident male (CDG, pers. obs.) (Table 1).

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55 169 For DNA analyses, we included 26 individuals from seven groups: seven parental pairs
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57 170 and 12 offspring. Unfortunately, not all the individuals that were part of a group during
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3 171 our study were included in DNA analyses, either because we did not have samples
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5 172 available, or because DNA extraction did not succeed (Table 1).
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9 173 Between 2011 and 2015, we obtained at least two fecal samples for each animal, each
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11 174 corresponding to a different defecation. We collected fecal samples immediately after
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13 175 defecation to avoid individual misidentification during the sampling process. Feces were
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15 176 put in 20ml labeled tubes filled with RNAlater® Ambion (Nsubuga et al. 2004) wearing
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17 177 disposable gloves and stored at room temperature in the field and at 4°C once transferred
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19 178 to the lab. We conducted all the genetic analyses at the New York University Molecular
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21 179 Anthropology Laboratory.
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25 26 180 *DNA Extraction* 27

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29 181 Genomic DNA was extracted from feces using the QIAamp DNA® Stool Mini Kit
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31 182 (Qiagen®, Hilden, Germany). To maximize the amount of DNA extracted we slightly
32
33 183 modified the manufacturer's protocol (QIAamp DNA Stoll Handbook 04/2010): we used
34
35 184 300 mg stool instead of 180-220 mg; we added 35 µL of proteinase K rather than 25 µL
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37 185 and incubated at 70°C for 30 minutes, instead of 10 minutes, during the DNA purification
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39 186 phase. We then applied 75 µL Buffer AE on the QIAamp membrane rather than 200 µL for
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41 187 the first DNA elution and incubate the spin column with Buffer AE at room temperature
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43 188 for 15 minutes instead of one minute. We used the same QIAamp membrane to obtain a
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45 189 second DNA elution applying 50 µL of Buffer AE and incubate for 15 minutes at room
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47 190 temperature.
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54 191 For the samples collected in the field during 2014, DNA purification was conducted
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56 192 using the automated robotic workstation QIAcube HT supported by the software
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3 193 QIAxtractor 4.17.1 (Qiagen®) setting the protocol for QXT Liquid DNA V1. The preparation
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5
6 194 of the samples required a bath at 70°C for at least five minutes of the 2.0 mL tubes
7
8 195 containing 300 mg of smashed feces and 1.6 mL of Buffer ASL. Afterwards, tubes were
9
10 196 centrifuged at maximum speed (13000 RPM) for 10 minutes. 200 µL of supernatant were
11
12 197 then transferred to separate wells of the QIAextractor lysis plate before starting the run.
13
14 198 At the end of the run, we obtained 70 µL of DNA elution for each sample. We stored the
15
16 199 extracted DNA at 4° C for immediate use.
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21 *Microsatellite Genotyping*

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23 201 We initially identified a set of 10 microsatellite marker loci as potentially variable in
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25 202 indris (Zaonarivelo et al. 2007). Polymorphism of each locus was tested using monocus
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27 203 PCRs. Four loci failed to provide amplification products, and we therefore chose to use the
28
29 204 six loci among the ten tested that provided good quality amplification products for
30
31 205 multiplex PCRs (Table 2). The 5' end forward primer of each locus was labeled with a
32
33 206 fluorescent dye (FAM, HEX) to analyze simultaneously loci of similar allelic size. PCR
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35 207 amplification was carried out in 10 µL reaction volume containing 2 µL DNA template, 5 µL
36
37 208 Multiplex PCR Master Mix (Qiagen®), 0.1 µL of each primer, and two µL RNase-free water.
38
39 209 The cycle conditions included a pre-incubation step at 95 °C for 15 min. We then
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41 210 performed 50 cycles with denaturation at 94 °C for 30 s, annealing at 54°C or 60 °C
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43 211 (depending on the locus, Table 2) for 90 s. The first extension phase was at 72 °C for 60 s;
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45 212 the final extension phase was at 60 °C for 30 min.
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53 213 We separated PCR products by electrophoresis using a 48 capillary ABI 3730 DNA
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55 214 Analyzer (Applied Biosystems) for allele size estimates. We mixed 1 µL of PCR product with
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3 215 6.85 μ L HiDi formamide (Applied Biosystems) and 0.15 μ L Genescan 500-ROX size
4
5
6 216 standard (Applied Biosystems). We carried out automated allele calling using the
7
8 217 GENEMAPPER 3.7 software (Applied Biosystems). We subsequently confirmed by eye all
9
10 218 the allele calls and checked for consistency across replicate PCRs of the same sample or
11
12
13 219 from the same individual. To minimize possible genotyping errors due to allelic dropout,
14
15 220 we repeated independent PCRs for each locus depending on whether the individual
16
17 221 resulted in being heterozygote (minimum three replicates) or homozygote (minimum five
18
19 222 replicates) for a certain locus.
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23 223 We used the software CERVUS 3.0 to calculate observed and expected heterozygosity
24
25 224 to test deviation from Hardy-Weinberg equilibrium (HWE), and to estimate null allele
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27 225 frequency for each locus.
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31 226 *Paternity test and assignment*

32
33 227 We identified 14 paired individuals (7 males, 7 females), and 12 potential offspring.
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35 228 We included the reproductive males of the neighboring groups as potential sires for each
36
37 229 potential offspring. Unpaired neighboring males exceeding the age of 3 at the time of
38
39 230 habituation were also included as potential sires because: (i) we could not exclude that
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41 231 they had potentially mated with a female in the study population before or during the
42
43 232 sampling period; and (ii) the youngest indri we observed forming a group was 3 and half
44
45 233 years old. We also considered the presence of potential sires from the unsampled
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47 234 neighboring groups. In the simulation, we included one unsampled potential sire for each
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49 235 neighboring non-habituated group.
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3 236 Ten out of the 12 offspring were born during the 2009-2015 study period in
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6 237 Maromizaha; two were already present in the groups at the beginning of the habituation,
7
8 238 and we could not know whether they were born in the group or immigrated. We
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10 239 considered these individuals as both potential offspring and potential sires. For these two
11
12 240 indris, all the reproductive males were also included as potential fathers. For the single
13
14 241 case of offspring and social father mismatch, we included each individual qualified as
15
16 242 potential sire as a candidate father. The average number of potential sires for the 12
17
18 243 offspring was 7.3 ± 2.2 (mean \pm SD) and the potential sires sampled were 4.1 ± 2.2 (mean
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23 244 \pm SD).

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

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

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3 258 potential sires to offspring. This considers the case in which the parent pairs are mutually
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6 259 exclusive.

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9 260 We assigned paternity in the cases of complete genotypes matching between the
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11 261 potential father and offspring. We excluded the paternity in cases of negative LOD scores
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13 262 and more than one allelic mismatch between the offspring and the potential father. We
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16 263 assigned EPP in cases of paternity exclusion for the social father; if the paternity was also
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18 264 excluded for all the other possible sampled sires, we defined the assignment of EPP as
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21 265 inconclusive.

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24 266 *Ethical standards*

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27 267 During the study, we did not have any physical contact with the animals. All fecal samples
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29 268 were collected from the ground immediately after defecation. The research was
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31 269 authorized by the “Ministère de l’Environnement et des Forêts” (MEF) of Madagascar.
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33
34 270 Research permits: N° 243/ 09/ MEF/ SG/ DGF/ DCB.SAP/ SLRSE, N° 118/ 10/ MEF/ SG/
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36 271 DGF/ DCB.SAP/ SCBSE; N° 293/ 10/ MEF/ SG/ DGF/ DCB.SAP/ SCB, N° 274/ 11/ MEF/ SG/
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38 272 DGF/ DCB.SAP/ SCB, N°245/12/MEF/SG/DGF/DCB.SAP/SCB,
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41 273 N°066/14/MEF/SG/DGF/DCB.SAP/ SCB. We adhered to applicable international, national,
42
43 274 and/or institutional guidelines for the study on animals and non-human primates,
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45
46 275 including the American Society of Primatologist (ASP) Principle for the Ethical Treatment
47
48 276 of non-human Primates, and the European Union directive guidelines for the study on
49
50
51 277 animals and non-human primates (Directive 2010/63/EU). The study did not require an
52
53
54 278 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

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3 301 five loci, and all the six loci matched with the social father; therefore, we suggest that this
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5 302 incompatibility is related to the estimated error rate of 0.0733 and of 0.0728 for the loci
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8 303 67HDZ55 and 67HDZ25, respectively. It is possible that known parent mismatches at one
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10 304 or more loci are due to the presence of null alleles (Kalinowski, Taper & Marshall, 2007),
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12 305 especially in the case of homozygous individuals; the high likelihood of parentage given by
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14 306 the other loci and the consistent match with both parents make it acceptable to consider
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16 307 the known mothers as the true parents. The same methodological approach would have
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18 308 been applied in the case of one locus mismatch with the fathers.
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24 309 *Paternity*

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26 310 Paternity based on CERVUS at a confidence level of 95% given a known mother was
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28 311 assigned to a total of 11 individuals out of 12 (92% against an expected assignment of 61%
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30 312 of the total offspring). We found a lower percentage of unassigned paternity than
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32 313 expected, 8% versus 39%. None of the assigned paternity presented any mismatch with
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34 314 the offspring for all the six loci considered, and the assigned fathers were consistently the
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36 315 reproductive males of the group (Table 4).
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41 316 Only one out of 12 offspring tested (Tsiky, group 6MZ) showed a mismatch with the
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43 317 social father (Zokybe, group 6MZ) for all the six loci, and a negative LOD score. We have
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45 318 also found that the LOD score was negative for all the potential fathers tested. However,
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47 319 the reproductive female (Befotsy, group 6MZ) had no mismatch with the offspring (Table
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49 320 4). Tsiky was born in the summer of 2010, the year after the habituation of the group;
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51 321 Zokybe was the paired male of the group at the time of habituation (in September 2009)
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53 322 and was the paired male at the time of the reproductive season (starting in December
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3 323 2009); Tsiky was born in the summer of 2010, the first birth season after the habituation
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5 324 of the group; therefore, we can consider the mismatch as a case of EPP with inconclusive
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8 325 paternity assignment. This evidence indicates an EPP rate of 8% in our sample.
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11 326 The most closely related individual to the indri with unassigned paternity was Hendri,
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13 327 a potential reproductive male of the neighboring group 4MZ, having two loci mismatches
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16 328 even when considering the genotype of the mother. However, it is unlikely that Hendri
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18 329 can be the father: Hendri and Tsiky are heterozygotes for all the loci considered,
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21 330 eliminating the possibility of allelic dropout at the mismatching loci; furthermore, the LOD
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23 331 score was negative, excluding Hendri as a potential sire for Tsiky.
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26 332 It must be noted that an unsampled individual was in the group 6MZ during the year
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28 333 of conception and left the group the year after the birth of Tsiky (Table 1). If the male that
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30 334 left the group was one of the offspring, we would exclude the possibility that he was a
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32 335 potential sire, and we would hypothesize that the sire would be an unknown male.
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34 336 Unfortunately, our samples did not allow us to test this hypothesis, leaving the paternity
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36 337 assignment inconclusive.
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41 338 The male (Emilio) involved in the EPC observed in 2011 (Bonadonna et al. 2014) with
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43 339 the female of the group 3MZ (Mena) was not the sire of the individual (Blague) born after
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45 340 the EPC; in fact, it was the resident and paired male of the group 3MZ (Ratsy) that was
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47 341 found to be Blague's father (Table 4).
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3 342 **Discussion**
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6 343 Our study investigates the genetic relationship between offspring, social parents and
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9 344 potential sires in indris, within the same population where an EPC was observed
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11 345 (Bonadonna et al. 2014). The results we presented in this paper provide new insights into
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14 346 the mating system of this pair-living primate.
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17 347 The paternity analysis revealed that all genetic profiles of the offspring matched with
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19 348 the social fathers, except for one case. Because in this case the paternity remained
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21
22 349 inconclusive, we cannot exclude our hypothesis that EPC may lead to EPP in indris, but we
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24 350 have to weigh this phenomenon across our study groups. In fact, we confirmed genetic
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27 351 monogamy for 11 out of the 12 cases we tested. The 8% mismatch with the paired males
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29 352 we found in indris is lower than the high rate of EPP found by Fietz and colleagues (2000)
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31 353 in the nocturnal dwarf lemur (*C. medius*, 44%), but comparable with the study of Barelli et
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34 354 al. (2013) reporting rates varying from 8.5% to 10% for white handed gibbons (*H. lar*).
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37 355 Our results agree with the low rate of EPP in monogamous and constantly associated
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39 356 pairs predicted by van Schaik & Kappeler (2003) and Clutton-Brock & Isvaran (2006). Our
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42 357 study also supports the hypothesis of Cohas & Allainé (2009), who pointed out the
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44 358 important role of pair-living social organization in ensuring paternity exclusivity, in
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47 359 addition to pair-bonding strength and mate guarding.
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50 360 White-handed gibbons share with indris several characteristics: they usually have a
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52 361 stable reproductive pair per group, and they show exclusive territoriality (Brockelman et
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55 362 al., 1998). They also share the emission of loud songs that, in both species, have a role in
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3 363 signaling pair-bonding strength (Geissmann & Orgendilger 2000; Gamba et al., 2016). Long
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5 364 distance calls can also broadcast information to potential mates (Torti et al., 2013, 2017;
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8 365 Gamba et al., 2016). Each group exchanges information about individuals' sex, status, and
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10 366 genetic relatedness throughout the emission of loud songs way beyond the limit of a
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12 367 territory (Torti et al., 2017). In this way, groups are not isolated units and individuals can
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14 368 communicate with one another without having visual or physical contact (Giacoma et al.,
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16 369 2010; Gamba et al., 2016; Bonadonna et al. 2017). Such a system would allow regulating
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18 370 inter-group dynamics including the possibility of engaging in EPC (Bonadonna et al., 2014),
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20 371 and therefore providing a certain degree of flexibility in the mating system of this species.
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26 372 The takeover of paired individuals is rare and, according to our observations, the case
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28 373 reported for the group 3MZ in Maromizaha was the result of a conflict between males
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30 374 (CDG, pers. obs.). We suggest that both pair-bonding strength and social structure play a
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32 375 role in the flexibility of a monogamous mating system, as we found in indris.
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36 376 It has been suggested that once a monogamous mating system is established, low
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38 377 rates of EPP are related to intensive male care (Huck et al., 2014). In the indri, males cover
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40 378 the primary role in territorial defense (Pollock 1975), which can be considered an indirect
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42 379 form of parental care (Brockelman, 1975; Kleiman, 1977). The territorial defense is part of
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44 380 the resource defense strategy: the male can guard the access to the female, and both the
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46 381 female and offspring have access to resources (Clutton-Brock, 1991; Møller & Thornhill,
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48 382 1998). This idea agrees with the hypothesis that parental care is an indirect evolutionary
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50 383 consequence of mate guarding (Huck et al., 2014). There is no more direct form of
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3 384 parental care by males in indris apart from the fact that they may occasionally transfer
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5 385 infants from a branch to another while the mother is feeding (Torti, pers. comm.).
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9 386 As suggested by Bonadonna and colleagues (2014), EPC can be a female strategy to
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11 387 enhance male guarding. Given the fact that the EPC reported for indris has been observed
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13 388 at the beginning of the mating season, it could have increased the male's guarding
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15 389 behavior and also reduced the chances of EPP. An enhanced guarding of the paired male
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17 390 would also improve resource monopolization, especially during the mating season
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19 391 (Brotherton & Komers, 2003).
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24 392 We can also indirectly assume that EPP and the occurrence of EPCs can be
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26 393 underestimated. The direct and indirect benefits for females adopting differential
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28 394 reproductive strategies in indris are not easily identifiable. Interestingly, the genetic
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30 395 profile of the individual born after the breeding season in which we observed the EPC
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32 396 suggests that the female successfully reproduced with her social partner during the same
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34 397 year. Thus, our findings suggest that the female was not likely to engage in EPC to avoid
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36 398 partner infertility (Palombit, 1994; Brotherton & Komers, 2003), as hypothesized for
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38 399 white-handed gibbons (in which two out of three EPPs were found in the same pair-living
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40 400 group, Barelli et al., 2013).
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46 401 Our results excluded that the current paired male of the group 6MZ (Zokybe) sired the
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48 402 oldest individual of the offspring (Tsiky), representing the only case of EPP in our study,
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50 403 but he did not present any loci mismatch with the individual born in 2014 (Hira). We
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52 404 consider two possible scenarios to explain our results. In the first scenario, the genetic
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54 405 father of the individual with unassigned paternity might have preceded a male takeover,
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3 406 which would explain the mismatch of the paired male as the sire of the offspring. Partner
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6 407 take-over in the indri can occur. This fact would configure a forced “divorce” as reported
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8 408 for brown titi monkeys (*Callicebus brunneus*; Lawrence, 2007), and owl monkeys (*Aotus*
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10 409 *azarae*; Fernandez-Duque & Huck, 2013), and it agrees with the serial monogamy model
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12 410 proposed by Fernandez-Duque & Huck (2013). This first scenario seems unlikely because
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15 411 Zokybe was reported as the paired male during the reproductive season that preceded the
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18 412 birth of Tsiky despite the changes in the group composition. In the second, and more likely
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20 413 scenario, there was no takeover of the reproductive male because Zokybe was the paired-
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22 414 male of the female at the time of conception. This scenario is in accordance with our
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25 415 hypothesis that partner infertility is not a primary cause of EPC and EPP in indris because
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27 416 Zokybe was likely the father of the younger offspring (no evidence of allelic mismatch).
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31 417 Although we were not able to assign the paternity to an individual, based on our
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33 418 results, we can draw some conclusion about the mating system in indris. According to our
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35 419 findings and observations, individuals that reach maturity can either leave or remain in
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38 420 the group. We found that two resident males (group 4MZ and 8MZ), both over the
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41 421 reproductive age, were the offspring of the reproductive pairs. Furthermore, we never
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43 422 observed a copulation event between a mother and her potential reproductive offspring,
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45 423 and finally we did not find any case of offspring sired by an older brother. Therefore, we
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48 424 did not find any evidence to support the hypothesis that adults prior dispersal are likely
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50 425 candidates for siring offspring.
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53 426 The floating of individuals without a territory, delayed dispersal and immigration have
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56 427 an important role in the dynamics of the monogamous mating system in pair-living
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3 428 primates (Porter, Grote, Fernandez-Duque & Di Fiore, 2017; Jacobs, Frankel, Rice, Kiefer &
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5 429 Bradley, 2018). Unfortunately, there are few studies and data available on the dispersal
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8 430 pattern and dynamics of the social organization in the indri. The role of these factors in
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10 431 the social and mating system of this species need further investigation. However, even if
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12 432 in one case we could not assign the paternity, in all the other cases we found no loci
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14 433 mismatches between social fathers and offspring. In addition, we did not find any case of
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16 434 ambiguity among potential fathers, making our results consistent.
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21 435 Genetic analyses can reveal a level of plasticity in the mating system of species
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23 436 considered monogamous (Díaz-Muñoz & Bales 2016). Although our study is limited to a
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25 437 relatively small sample size, it is indicative that genetic monogamy seems to be the norm
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27 438 in indris, with an EPP rate comparable to other pair-living monogamous primates.
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29 439 Furthermore, it is unlikely that a female would reproduce with another male in the group
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31 440 other than her partner. This situation would exclude that the social organization of indris
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33 441 may change from pair-living to polyandrous as it has been found in primates with a similar
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35 442 behavioral ecology, such as the gibbons (Barelli, Heistermann, Boesch, Reichard, 2008).
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41 443 In conclusion, our study contributes to the scanty literature of genetic studies of wild
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43 444 monogamous, pair-living primates. We found that the model of pair-living social
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45 445 organization fits this species although it might not be consistently associated with genetic
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47 446 monogamy despite the low EPP rate. However, our results were not fully conclusive about
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49 447 paternity assignment, requiring further investigations on the genetic structure of the
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51 448 population, and the dispersal dynamics. Future studies should focus on providing further
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3 449 insight into the mechanisms involved in the maintenance of socially monogamous systems
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5 450 and their genetic variability.
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11
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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							
	<i>Fotsy (>3[†])</i>	<i>Male</i>							
	Maintso	Female							
	Berthy	Female							
	Akora	<i>Unknown</i>							
2MZ	Soa (R)	Female							
	Max (R)	Male							
	Kinga	<i>Unknown</i>							
	Fanihy	Female							
	Afo	<i>Unknown</i>							
3MZ	Mena (R)	Female			EPC [‡]				
	Ratsy (R)	Male							
	<i>Mahagaga (R)</i>	<i>Male</i>							
	<i>Tsara (>3[†])</i>	<i>Male</i>							
	Zandry	Female							
	Blague	Male							
	Faly	<i>Female</i>							
	Laro	<i>Unknown</i>							
<i>Tonga (>3[†])</i>	<i>Female</i>								
4MZ	Eva (R)	Female							
	Koto (R)	Male							
	Hendri (>3 [†])	Male							
	Gibet	Male							
	Nic	<i>Unknown</i>							
6MZ	Befotsy (R)	Female							
	Zokybe (R)	Male							
	<i>Marco (>3[†])</i>	<i>Male</i>							
	<i>Guido (>3[†])</i>	<i>Male</i>							
	Tsiky	Male							
	Hira	<i>Unknown</i>							
8MZ	Bemasoandro (R)	Female							
	Jonah (R)	Male							
	Cesar (>3)	Male							
	Zafy	Male							
	Mika	<i>Unknown</i>							

9MZ	Sissie (R)	Female		
	Emilio (R)	Male		EPC [‡]
	Njaka	<i>Male</i>		
	Ovy	Male		
	Dosy	<i>Unknown</i>		

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

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Locus	Forward primer	Reverse Primer	Repeat motif	Annealing temp. (°C)	Number of PCR cycles	Size range (bp)
67HDZ25	GGACCCTAATTCA AATATCACCTC	GGCATTCTACT CCAGTTGG	(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 CATTCTC	CGTGAAGCTCGT GTGTATGG	(CA)17	60	50	113-136
67HDZ39	CAGAGCCAGGGT TCAAATTC	TTGTCTTTCTGC CACTGTAGG	(CA)11	60	50	148-162

657 **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

658 **Table 4** Paternity analyses results of 12 offspring and respective known and potential parents of seven indri groups. For the potential
 659 fathers we included the number of loci mismatch and the sign of the LOD score (positive or negative). . We indicated if the paternity was

Group	Y of Birth	Offspring	Mother (# mismatches)	Potential Sires Sampled		Assigned Paternity		
				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%

660 assigned to the paired male or remained inconclusive, we specified if the male involved in the extra pair-copulation was excluded as sire.

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† 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.

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3 662 **Figure 1.** Study site and indri groups included in the study
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5 663 Study site located in Center-Eastern Madagascar (A), in the Maromizaha forest,
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7 664 accessible from the village Anevoka (B). The multipurpose center (18°58'34" S –
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9 665 48°27'53" E) was used as base-camp (C). Box C is showing the spatial distribution of
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11 666 the indri groups included in the genetic analysis (solid green polygons), and the
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13 667 schematic representation of unsampled neighboring groups (gray striped polygons,
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15 668 GX1 to GX7). The uppercase names within each studied group are the social fathers;
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17 669 the lowercase names are the offspring genotyped. "*" denotes the only individual that
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19 670 resulted with inconclusive paternity assignment. Original satellite images (Google Earth
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21 671 - Image © 2018 CNES / Airbus; downloaded on June 27, 2018) have been graphically
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23 672 simplified and adjusted with GIMP 2.10.2. Map of the indri territories created with
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25 673 ArcGIS® 10.2 (ESRI).
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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 findings suggest that genetic monogamy is the norm in indri, although EPC can occasionally occur.

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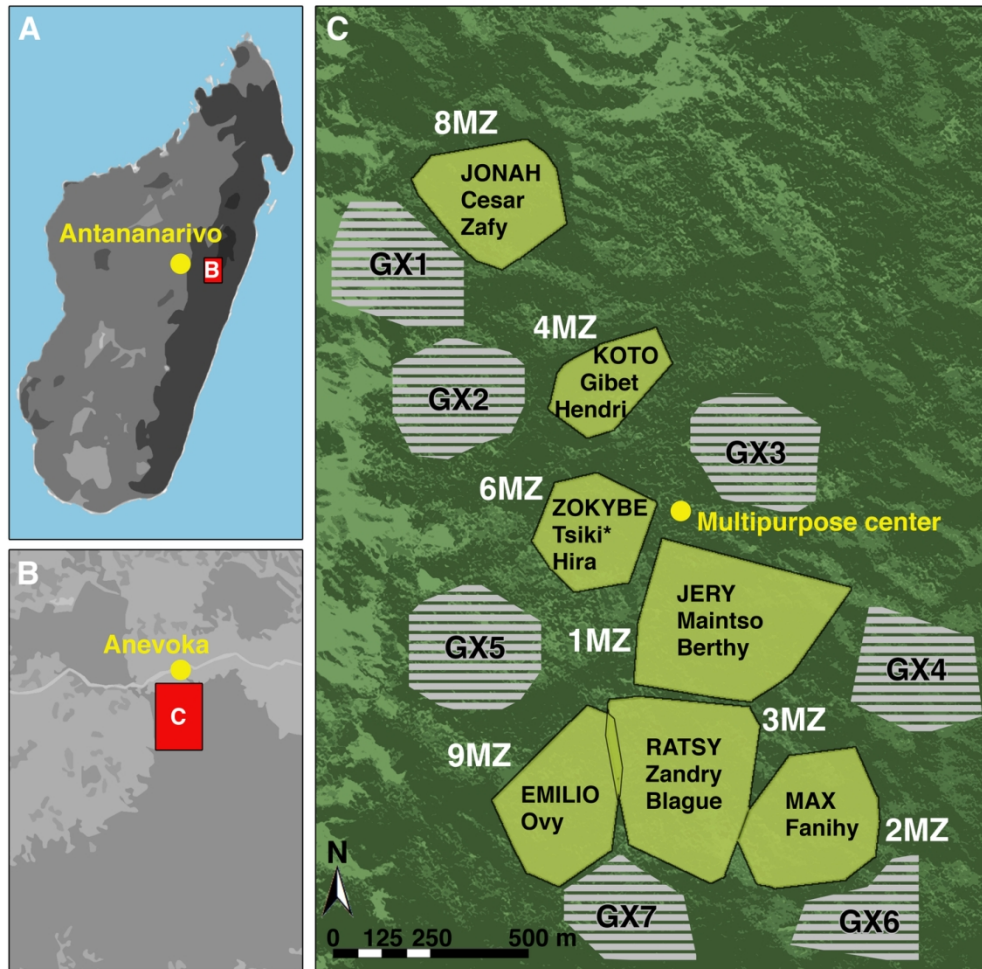


Figure 1. Study site and indri groups included in the study

Study site located in Center-Eastern Madagascar (A), in the Maromizaha forest, accessible from the village Anevoka (B). The multipurpose center ($18^{\circ}58'34''$ S – $48^{\circ}27'53''$ 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)

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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)