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

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

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

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

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13 **Abstract**

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

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

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

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

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

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

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

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

22 can occur. This raises the question of whether this event may or may not lead to EPP.

23 Here, we investigated whether a pair-living social organization corresponds to genetic

24 monogamy in indris (*Indri indri*). We analyzed the paternity of 12 offspring from 7 pairs

25 using a set of six microsatellite loci on fecal samples (mean number of alleles 11.7 ± 1.8

26 (mean ± SD). We found that in 92% of cases the genetic profile of the offspring matched

27 the paired male of the group for all the loci considered. In the only case of paternity

28 mismatch, the paternity assignment remained inconclusive. Our results show that in indri

29 genetic monogamy is the norm and support the hypothesis that pair-living social

30 organization is associated with low EPP rate. Also, our results are in contrast with the

31 hypothesis of infertility as a reason to engage in EPC for this species.

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

33 **Research Highlights**

34 • In 92% of cases, the paired male of the group did not have any locus mismatch

35   with the offspring. In the only case of paternity mismatch, we were not able to

36   assign the sire identity.

37 • Our finding suggest that genetic monogamy is the norm in the indri, although EPC

38   can occasionally occur.

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

61 the group, respectively. EPP and EGP are interchangeable when considering pair-living

62 monogamous species (Isvaran & Clutton-Brock, 2007), as in our study; we decided to

63 apply the term EPP because we focus on the reproductive pair of the group.

64   Birds are the typical example of a taxon in which more than 90% of species were

65 considered sexually monogamous because of their pair-living organization (Lack, 1968).

66 However, molecular analysis have confirmed that EPP is widespread across birds (Griffith,

67 Owens & Thuman, 2002). Contrary to birds, pair living is very rare among mammals (3%)

68 (Kleiman, 1977), and, when present, it is more frequently related to genetic monogamy

69 (Lukas & Clutton-Brock, 2012, 2013). According to a study including 22 mammal species

70 generally considered to have a monogamous mating system, the variation of EPP among

71 species seems to be related to the social structure, with pair-living species having the

72 lowest rates of EPP (Cohas & Allainé, 2009). The strength of pair bonding has also been

73 suggested to possibly play a role (Cohas & Allainé, 2009; Clutton-Brock & Isvaran, 2006).

74   Among mammals, primates include the highest frequency of monogamous species

75 (29%; Lukas & Clutton-Brock, 2013), usually organized in pair-living groups with immature

76 or even mature offspring (Reichard & Boesch, 2003; Kappeler & van Schaik, 2002). Few

77 studies have investigated the genetic paternity in putatively monogamous primates (not

78 necessarily pair-living as we defined it) and some of them show that EPP occurs, including

79 in tarsiers (*Tarsius lariang*, Driller, Perwitasari-Farajallah, Zischler & Merker, 2009), lemurs

80 (*Hapalemur griseus alaotrensis,* Nievergelt, Mutschler, Feistner & Woodruff, 2002;

81 *Cheirogaleus medius*, Fietz et al. 2000; *Phaner furcifer*, Schülke, Kappeler & Zischler, 2004),

82 gibbons (*Hylobates lar*, Barelli et al. 2013; *Nomascus gabriellae*, Kenyon, Roos, Binh &

83   Chivers, 2011). To date, Azara's night monkey (*Aotus azarae*) and Muller's Bornean gibbon

84   (*Hylobates muelleri*) are the only two primate species in which no evidence of EPP was

85   found; however, in the latter case, only four offspring were tested (reviewed in Lambert,

86   Sabol & Solomon, 2018).

87   The occurrence of EPP has consequences on individuals' reproductive success. Several

88   non-exclusive hypotheses have been proposed to explain the presence of EPP, such as to

89   guard against infertility of the social mate, to increase the genetic diversity of the

90   offspring with consequences at population level, to provide "good genes" for the

91   offspring, and to gain direct benefit given by access to resources (Birkhead & Møller, 1992;

92   Griffith et al., 2002; Møller & Thornhill, 1998). However, there is still a lack of empirical

93   data to support these hypotheses (Griffith et al., 2002; Akçay & Roughgarden, 2007),

94   especially among primates.

95   The occurrence of EPP is directly related to the possibility of engagement in Extra-Pair

96   Copulation (EPC). A social context with easy access to potential mates within and between

97   groups increases the probability of EPCs and, as a consequence, can lead to higher rates of

98   EPP (Cohas & Allainé, 2009). In pair-living species, the opportunities of engaging in EPC are

99   lower because of more efficient control of the partner over potential mates. In territorial

100   species, the overlap between territories and the rate of intergroup encounters can

101   influence the chances of being in contact and interacting with potential mates, as

102   suggested in gibbons (*Hylobates lar,* Reichard & Sommer, 1997).

103   It seems that there is a relationship between genetic monogamy and the strength of

104   the pair bond (Huck, Fernandez-Duque, Babb, Schurr, 2014), and recent reviews proposed

105   the hypothesis that social organization and mate guarding are the main factors influencing

106   a monogamous mating system in primates (Lambert, Sabol & Solomon, 2018, Reichard

107   2018). In this study, we investigate the genetic relationship among reproductive pairs,

108   potential sires, and offspring to explore whether pair living is associated with genetic

109   monogamy in the indris (*Indri indri*).

110   Bonadonna and colleagues (2014) reported the first observation of EPC in indris

111   between a neighboring paired female and a paired male within the same population of

112   this study. This event raised the question of whether EPC may lead to EPP; our study aims

113   to address this question with subsequent eventual reconsideration of the monogamous

114   mating system of this species at the genetic level.

115   The indri is a diurnal lemur living in family groups composed of up to six individuals,

116   usually consisting of the reproductive pair and their offspring (Pollock, 1975, Bonadonna

117   et al., 2014). The pair bonding in indris is remarkable. The reproductive pair is the basal

118   unit of a group (Pollock, 1975) and we have records of individuals being together for at

119   least a decade (unpublished data).

120   This species can be considered a slow breeder. The gestation period reported for

121   indris is 157 days (Godfrey, Samonds, Jungers, Sutherlan, Irwin, 2004) and another study

122   reported 176 days (Weir, 2014) with interbirth intervals of two to three years (Weir,

123   2014), females give births to one offspring at a time between May and July, and

124   individuals presumably become potentially reproductive at the age of three, when they

125   start singing (unpublished data). Group members tend to be cohesive, and usually the

126   individuals of the reproductive pair stay within a distance between 0 and 13 m from each

127   other (Pollock, 1975). Each group occupies a stable and exclusive territory and intergroup

128   encounters are very rare (Bonadonna et al. 2017). The indri is one of the few singing

129   primates and, besides having a function in signaling territory occupancy and defense (Torti

130   et al., 2013), the song also plays an important role in the mating and social system of this

131   species. The temporal and spectral characteristics of the song can signal the pair-bond

132   strength to conspecifics (Gamba et al., 2016). Furthermore, the individual-specific features

133   of the indri song and acoustic similarities between fathers and sons can play a role in pair

134   formation, dispersal and avoidance of inbreeding (Torti et al. 2017).

135   Using fecal samples collected in Maromizaha between 2009 and 2015, we obtained

136   individual genetic fingerprints and examined the genetic relationships between sires and

137   offspring. In the presence of an observed EPC (Bonadonna et al., 2014), we hypothesized

138   that the indri population we studied would show a certain degree of EPP during successive

139   years and across different groups. Previous studies on gibbons (*H. lar)*, demonstrated that

140   EPC may frequently occur after group encounters (Reichard, 1995). A more recent work

141   on the same population studied by Reichard has found an EPP rate of 9.5% on a total of 42

142   offspring (Bartlett, Light & Brockelman, 2016). Our prediction is that we would find a

143   limited occurrence of EPP in indris, which present a pair-living social organization where

144   EPC occurs.

Methods

146   *Study Site*

147   We conducted the study on a wild population of indris (*I. indri*) in the New Protected

148   Area (NAP) of Maromizaha (18°56′49′′ S – 48°27′53′′ E). We accessed the forest from the

149   village of Anevoka (on the RN2), 147 km east of Antananarivo in the Alaotra Mangoro

150   Region (Province of Toamasina), central-eastern Madagascar. Maromizaha (1880 ha) is a

151   primary and secondary mid-altitude (800-1200m) evergreen rainforest, and it is part of

152   the ecological corridor of Ankeniheny-Zahamena (Fig. 1). The study included an area of

153   approximately 140 ha of continuous rainforest.

154   *Subjects and Sample Collection*

155   Nine indri groups have been habituated to human presence and have been the subject

156   of ongoing etho-ecological studies in Maromizaha since 2009. Seven out of nine

157   habituated groups were included in this study (Fig. 1).

158   We updated group composition and demographic records year by year, identifying

159   each individual using natural marks. The population density in the study area was 27.7 $\pm$

160   4.0 (mean $\pm$ SD) individuals and 8.4 $\pm$ 1.0 (mean $\pm$ SD) groups per km^2, comparable to

161   other populations inhabiting different sites (Bonadonna et al. 2017; Pollock 1975).

162   Each group was composed of a reproductive pair plus one to three individuals. These

163   additional indris are usually the pair's offspring; however, a case of immigration of an

164   individual into another group was observed in another forest (GB and VT, unpublished

165   data) (Table 1). We never observed copulation attempts outside the mating season, which

166   ranges between December and February (Pollock 1975, GB VT pers. obs.). The

167   reproductive pairs remained stable over time, except for one takeover in 2015, after the

168   death of the injured resident male (CDG, pers. obs.) (Table 1).

169   For DNA analyses, we included 26 individuals from seven groups: seven parental pairs

170   and 12 offspring. Unfortunately, not all the individuals that were part of a group during

171   our study were included in DNA analyses, either because we did not have samples

172   available, or because DNA extraction did not succeed (Table 1).

173   Between 2011 and 2015, we obtained at least two fecal samples for each animal, each

174   corresponding to a different defecation. We collected fecal samples immediately after

175   defecation to avoid individual misidentification during the sampling process. Feces were

176   put in 20ml labeled tubes filled with RNAlater® Ambion (Nsubuga et al. 2004) wearing

177   disposable gloves and stored at room temperature in the field and at 4°C once transferred

178   to the lab. We conducted all the genetic analyses at the New York University Molecular

179   Anthropology Laboratory.

180   *DNA Extraction*

181   Genomic DNA was extracted from feces using the QIAamp DNA® Stool Mini Kit

182   (Qiagen®, Hilden, Germany). To maximize the amount of DNA extracted we slightly

183   modified the manufacturer's protocol (QIAamp DNA Stoll Handbook 04/2010): we used

184   300 mg stool instead of 180-220 mg; we added 35 µL of proteinase K rather than 25 µL

185   and incubated at 70°C for 30 minutes, instead of 10 minutes, during the DNA purification

186   phase. We then applied 75 µL Buffer AE on the QIAamp membrane rather than 200 µL for

187   the first DNA elution and incubate the spin column with Buffer AE at room temperature

188   for 15 minutes instead of one minute. We used the same QIAamp membrane to obtain a

189   second DNA elution applying 50 µL of Buffer AE and incubate for 15 minutes at room

190   temperature.

191   For the samples collected in the field during 2014, DNA purification was conducted

192   using the automated robotic workstation QIAcube HT supported by the software

193 QIAxtractor 4.17.1 (Qiagen®) setting the protocol for QXT Liquid DNA V1. The preparation

194 of the samples required a bath at 70°C for at least five minutes of the 2.0 mL tubes

195 containing 300 mg of smashed feces and 1.6 mL of Buffer ASL. Afterwards, tubes were

196 centrifuged at maximum speed (13000 RPM) for 10 minutes. 200 µL of supernatant were

197 then transferred to separate wells of the QIAextractor lysis plate before starting the run.

198 At the end of the run, we obtained 70 µL of DNA elution for each sample. We stored the

199 extracted DNA at 4° C for immediate use.

200 *Microsatellite Genotyping*

201   We initially identified a set of 10 microsatellite marker loci as potentially variable in

202 indris (Zaonarivelo et al. 2007). Polymorphism of each locus was tested using monolocus

203 PCRs. Four loci failed to provide amplification products, and we therefore chose to use the

204 six loci among the ten tested that provided good quality amplification products for

205 multiplex PCRs (Table 2). The 5' end forward primer of each locus was labeled with a

206 fluorescent dye (FAM, HEX) to analyze simultaneously loci of similar allelic size. PCR

207 amplification was carried out in 10 µL reaction volume containing 2 µL DNA template, 5 µL

208 Multiplex PCR Master Mix (Qiagen®), 0.1 µL of each primer, and two µL RNase-free water.

209 The cycle conditions included a pre-incubation step at 95 °C for 15 min. We then

210 performed 50 cycles with denaturation at 94 °C for 30 s, annealing at 54°C or 60 °C

211 (depending on the locus, Table 2) for 90 s. The first extension phase was at 72 °C for 60 s;

212 the final extension phase was at 60 °C for 30 min.

213   We separated PCR products by electrophoresis using a 48 capillary ABI 3730 DNA

214 Analyzer (Applied Biosystems) for allele size estimates. We mixed 1 µL of PCR product with

215 6.85 µL HiDi formamide (Applied Biosystems) and 0.15 µL Genescan 500-ROX size

216 standard (Applied Biosystems). We carried out automated allele calling using the

217 GENEMAPPER 3.7 software (Applied Biosystems). We subsequently confirmed by eye all

218 the allele calls and checked for consistency across replicate PCRs of the same sample or

219 from the same individual. To minimize possible genotyping errors due to allelic dropout,

220 we repeated independent PCRs for each locus depending on whether the individual

221 resulted in being heterozygote (minimum three replicates) or homozygote (minimum five

222 replicates) for a certain locus.

223   We used the software CERVUS 3.0 to calculate observed and expected heterozygosity

224 to test deviation from Hardy-Weinberg equilibrium (HWE), and to estimate null allele

225 frequency for each locus.

226 *Paternity test and assignment*

227   We identified 14 paired individuals (7 males, 7 females), and 12 potential offspring.

228 We included the reproductive males of the neighboring groups as potential sires for each

229 potential offspring. Unpaired neighboring males exceeding the age of 3 at the time of

230 habituation were also included as potential sires because: (i) we could not exclude that

231 they had potentially mated with a female in the study population before or during the

232 sampling period; and (ii) the youngest indri we observed forming a group was 3 and half

233 years old. We also considered the presence of potential sires from the unsampled

234 neighboring groups. In the simulation, we included one unsampled potential sire for each

235 neighboring non-habituated group.

236   Ten out of the 12 offspring were born during the 2009-2015 study period in

237 Maromizaha; two were already present in the groups at the beginning of the habituation,

238 and we could not know whether they were born in the group or immigrated. We

239 considered these individuals as both potential offspring and potential sires. For these two

240 indris, all the reproductive males were also included as potential fathers. For the single

241 case of offspring and social father mismatch, we included each individual qualified as

242 potential sire as a candidate father. The average number of potential sires for the 12

243 offspring was 7.3 $\pm$ 2.2 (mean $\pm$ SD) and the potential sires sampled were 4.1 $\pm$ 2.2 (mean

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

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

259 exclusive.

260   We assigned paternity in the cases of complete genotypes matching between the

261 potential father and offspring. We excluded the paternity in cases of negative LOD scores

262 and more than one allelic mismatch between the offspring and the potential father. We

263 assigned EPP in cases of paternity exclusion for the social father; if the paternity was also

264 excluded for all the other possible sampled sires, we defined the assignment of EPP as

265 inconclusive.

266 *Ethical standards*

267 During the study, we did not have any physical contact with the animals. All fecal samples

268 were collected from the ground immediately after defecation. The research was

269 authorized by the "Ministère de l'Environnement et des Forêts" (MEF) of Madagascar.

270 Research permits: N° 243/ 09/ MEF/ SG/ DGF/ DCB.SAP/ SLRSE, N° 118/ 10/ MEF/ SG/

271 DGF/ DCB.SAP/ SCBSE; N° 293/ 10/ MEF/ SG/ DGF/ DCB.SAP/ SCB, N° 274/ 11/ MEF/ SG/

272 DGF/ DCB.SAP/ SCB, N°245/12/MEF/SG/DGF/DCB.SAP/SCB,

273 N°066/14/MEF/SG/DGF/DCB.SAP/ SCB. We adhered to applicable international, national,

274 and/or institutional guidelines for the study on animals and non-human primates,

275 including the American Society of Primatologist (ASP) Principle for the Ethical Treatment

276 of non-human Primates, and the European Union directive guidelines for the study on

277 animals and non-human primates (Directive 2010/63/EU). The study did not require an

278 IACUC approval.

279 **Results**

280 *Genotypes and Identity of Individuals*

281   We included all the 26 individuals in the analyses. For three indris of the group 8MZ

282 we failed to amplify the locus 67HDZ55, and for one of them (Jonah) also the locus

283 67HDZ62. We obtained confirmed genotype of 23 individuals for the locus 67HDZ55, 25

284 for the locus 67HDZ62, and 26 for all the four remaining loci (Table 3).

285   The number of alleles per locus varied between nine and 14, observed heterozygosity

286 (HO) ranged from 0.840 to 0.962 and expected heterozygosity (HE) ranged between 0.814

287 and 0.928. None of the six loci showed significant deviation from the Hardy-Weinberg

288 equilibrium (HWE) after Bonferroni correction (Table 3). Even if in some cases the

289 presence of null allele frequency is close to the suggested threshold of 0.05, we included

290 all the loci in the analyses because the level of heterozygosity observed was high and all

291 known offspring-maternal pairs were free of possible homozygous mismatches for those

292 loci. The combined non-exclusion probability of identity over six loci was 8.482 x 10^{-10}.

293 Therefore, it is unlikely that the set of loci failed to differentiate between two randomly-

294 selected individuals.

295   We found two cases of loci mismatch between mother and offspring. In one case

296 (Locus 67HDZ55) the offspring resulted to be homozygous for this locus after six

297 independent replicates (group 3MZ; genotypes: mother "Mena" 313-327; offspring

298 "Blague" 330-330). In the other case (locus 67HDZ25), the mother was homozygous after

299 eight independent replicates (group 4MZ; genotypes: mother "Eva" 224-224; offspring

300 "Hendri" 226-228). In both cases the offspring matched with the mother for all the other

301   five loci, and all the six loci matched with the social father; therefore, we suggest that this

302   incompatibility is related to the estimated error rate of 0.0733 and of 0.0728 for the loci

303   67HDZ55 and 67HDZ25, respectively. It is possible that known parent mismatches at one

304   or more loci are due to the presence of null alleles (Kalinowski, Taper & Marshall, 2007),

305   especially in the case of homozygous individuals; the high likelihood of parentage given by

306   the other loci and the consistent match with both parents make it acceptable to consider

307   the known mothers as the true parents. The same methodological approach would have

308   been applied in the case of one locus mismatch with the fathers.

309   *Paternity*

310   Paternity based on CERVUS at a confidence level of 95% given a known mother was

311   assigned to a total of 11 individuals out of 12 (92% against an expected assignment of 61%

312   of the total offspring). We found a lower percentage of unassigned paternity than

313   expected, 8% versus 39%. None of the assigned paternity presented any mismatch with

314   the offspring for all the six loci considered, and the assigned fathers were consistently the

315   reproductive males of the group (Table 4).

316   Only one out of 12 offspring tested (Tsiky, group 6MZ) showed a mismatch with the

317   social father (Zokybe, group 6MZ) for all the six loci, and a negative LOD score. We have

318   also found that the LOD score was negative for all the potential fathers tested. However,

319   the reproductive female (Befotsy, group 6MZ) had no mismatch with the offspring (Table

320   4). Tsiky was born in the summer of 2010, the year after the habituation of the group;

321   Zokybe was the paired male of the group at the time of habituation (in September 2009)

322   and was the paired male at the time of the reproductive season (starting in December

323 2009); Tsiky was born in the summer of 2010, the first birth season after the habituation

324 of the group; therefore, we can consider the mismatch as a case of EPP with inconclusive

325 paternity assignment. This evidence indicates an EPP rate of 8% in our sample.

326   The most closely related individual to the indri with unassigned paternity was Hendri,

327 a potential reproductive male of the neighboring group 4MZ, having two loci mismatches

328 even when considering the genotype of the mother. However, it is unlikely that Hendri

329 can be the father: Hendri and Tsiky are heterozygotes for all the loci considered,

330 eliminating the possibility of allelic dropout at the mismatching loci; furthermore, the LOD

331 score was negative, excluding Hendri as a potential sire for Tsiky.

332   It must be noted that an unsampled individual was in the group 6MZ during the year

333 of conception and left the group the year after the birth of Tsiky (Table 1). If the male that

334 left the group was one of the offspring, we would exclude the possibility that he was a

335 potential sire, and we would hypothesize that the sire would be an unknown male.

336 Unfortunately, our samples did not allow us to test this hypothesis, leaving the paternity

337 assignment inconclusive.

338   The male (Emilio) involved in the EPC observed in 2011 (Bonadonna et al. 2014) with

339 the female of the group 3MZ (Mena) was not the sire of the individual (Blague) born after

340 the EPC; in fact, it was the resident and paired male of the group 3MZ (Ratsy) that was

341 found to be Blague's father (Table 4).

342   **Discussion**

343   Our study investigates the genetic relationship between offspring, social parents and

344   potential sires in indris, within the same population where an EPC was observed

345   (Bonadonna et al. 2014). The results we presented in this paper provide new insights into

346   the mating system of this pair-living primate.

347   The paternity analysis revealed that all genetic profiles of the offspring matched with

348   the social fathers, except for one case. Because in this case the paternity remained

349   inconclusive, we cannot exclude our hypothesis that EPC may lead to EPP in indris, but we

350   have to weigh this phenomenon across our study groups. In fact, we confirmed genetic

351   monogamy for 11 out of the 12 cases we tested. The 8% mismatch with the paired males

352   we found in indris is lower than the high rate of EPP found by Fietz and colleagues (2000)

353   in the nocturnal dwarf lemur (*C. medius*, 44%), but comparable with the study of Barelli et

354   al. (2013) reporting rates varying from 8.5% to 10% for white handed gibbons (*H. lar*).

355   Our results agree with the low rate of EPP in monogamous and constantly associated

356   pairs predicted by van Schaik & Kappeler (2003) and Clutton-Brock & Isvaran (2006). Our

357   study also supports the hypothesis of Cohas & Allainé (2009), who pointed out the

358   important role of pair-living social organization in ensuring paternity exclusivity, in

359   addition to pair-bonding strength and mate guarding.

360   White-handed gibbons share with indris several characteristics: they usually have a

361   stable reproductive pair per group, and they show exclusive territoriality (Brockelman et

362   al., 1998). They also share the emission of loud songs that, in both species, have a role in

363   signaling pair-bonding strength (Geissmann & Orgendilger 2000; Gamba et al., 2016). Long

364   distance calls can also broadcast information to potential mates (Torti et al., 2013, 2017;

365   Gamba et al., 2016). Each group exchanges information about individuals' sex, status, and

366   genetic relatedness throughout the emission of loud songs way beyond the limit of a

367   territory (Torti et al., 2017). In this way, groups are not isolated units and individuals can

368   communicate with one another without having visual or physical contact (Giacoma et al.,

369   2010; Gamba et al., 2016; Bonadonna et al. 2017). Such a system would allow regulating

370   inter-group dynamics including the possibility of engaging in EPC (Bonadonna et al., 2014),

371   and therefore providing a certain degree of flexibility in the mating system of this species.

372   The takeover of paired individuals is rare and, according to our observations, the case

373   reported for the group 3MZ in Maromizaha was the result of a conflict between males

374   (CDG, pers. obs.). We suggest that both pair-bonding strength and social structure play a

375   role in the flexibility of a monogamous mating system, as we found in indris.

376   It has been suggested that once a monogamous mating system is established, low

377   rates of EPP are related to intensive male care (Huck et al., 2014). In the indri, males cover

378   the primary role in territorial defense (Pollock 1975), which can be considered an indirect

379   form of parental care (Brockelman, 1975; Kleiman, 1977). The territorial defense is part of

380   the resource defense strategy: the male can guard the access to the female, and both the

381   female and offspring have access to resources (Clutton-Brock, 1991; Møller & Thornhill,

382   1998). This idea agrees with the hypothesis that parental care is an indirect evolutionary

383   consequence of mate guarding (Huck et al., 2014). There is no more direct form of

384   parental care by males in indris apart from the fact that they may occasionally transfer

385   infants from a branch to another while the mother is feeding (Torti, pers. comm.).

386   As suggested by Bonadonna and colleagues (2014), EPC can be a female strategy to

387   enhance male guarding. Given the fact that the EPC reported for indris has been observed

388   at the beginning of the mating season, it could have increased the male's guarding

389   behavior and also reduced the chances of EPP. An enhanced guarding of the paired male

390   would also improve resource monopolization, especially during the mating season

391   (Brotherton & Komers, 2003).

392   We can also indirectly assume that EPP and the occurrence of EPCs can be

393   underestimated. The direct and indirect benefits for females adopting differential

394   reproductive strategies in indris are not easily identifiable. Interestingly, the genetic

395   profile of the individual born after the breeding season in which we observed the EPC

396   suggests that the female successfully reproduced with her social partner during the same

397   year. Thus, our findings suggest that the female was not likely to engage in EPC to avoid

398   partner infertility (Palombit, 1994; Brotherton & Komers, 2003), as hypothesized for

399   white-handed gibbons (in which two out of three EPPs were found in the same pair-living

400   group, Barelli et al., 2013).

401   Our results excluded that the current paired male of the group 6MZ (Zokybe) sired the

402   oldest individual of the offspring (Tsiky), representing the only case of EPP in our study,

403   but he did not present any loci mismatch with the individual born in 2014 (Hira). We

404   consider two possible scenarios to explain our results. In the first scenario, the genetic

405   father of the individual with unassigned paternity might have preceded a male takeover,

406   which would explain the mismatch of the paired male as the sire of the offspring. Partner

407   take-over in the indri can occur. This fact would configure a forced "divorce" as reported

408   for brown titi monkeys (*Callicebus brunneus*; Lawrence, 2007), and owl monkeys (*Aotus*

409   *azarae;* Fernandez-Duque & Huck, 2013), and it agrees with the serial monogamy model

410   proposed by Fernandez-Duque & Huck (2013). This first scenario seems unlikely because

411   Zokybe was reported as the paired male during the reproductive season that preceded the

412   birth of Tsiky despite the changes in the group composition. In the second, and more likely

413   scenario, there was no takeover of the reproductive male because Zokybe was the paired-

414   male of the female at the time of conception. This scenario is in accordance with our

415   hypothesis that partner infertility is not a primary cause of EPC and EPP in indris because

416   Zokybe was likely the father of the younger offspring (no evidence of allelic mismatch).

417   Although we were not able to assign the paternity to an individual, based on our

418   results, we can draw some conclusion about the mating system in indris. According to our

419   findings and observations, individuals that reach maturity can either leave or remain in

420   the group. We found that two resident males (group 4MZ and 8MZ), both over the

421   reproductive age, were the offspring of the reproductive pairs. Furthermore, we never

422   observed a copulation event between a mother and her potential reproductive offspring,

423   and finally we did not find any case of offspring sired by an older brother. Therefore, we

424   did not find any evidence to support the hypothesis that adults prior dispersal are likely

425   candidates for siring offspring.

426   The floating of individuals without a territory, delayed dispersal and immigration have

427   an important role in the dynamics of the monogamous mating system in pair-living

428   primates (Porter, Grote, Fernandez-Duque & Di Fiore, 2017; Jacobs, Frankel, Rice, Kiefer &

429   Bradley, 2018). Unfortunately, there are few studies and data available on the dispersal

430   pattern and dynamics of the social organization in the indri. The role of these factors in

431   the social and mating system of this species need further investigation. However, even if

432   in one case we could not assign the paternity, in all the other cases we found no loci

433   mismatches between social fathers and offspring. In addition, we did not find any case of

434   ambiguity among potential fathers, making our results consistent.

435   Genetic analyses can reveal a level of plasticity in the mating system of species

436   considered monogamous (Díaz-Muñoz & Bales 2016). Although our study is limited to a

437   relatively small sample size, it is indicative that genetic monogamy seems to be the norm

438   in indris, with an EPP rate comparable to other pair-living monogamous primates.

439   Furthermore, it is unlikely that a female would reproduce with another male in the group

440   other than her partner. This situation would exclude that the social organization of indris

441   may change from pair-living to polyandrous as it has been found in primates with a similar

442   behavioral ecology, such as the gibbons (Barelli, Heistermann, Boesch, Reichard, 2008).

443   In conclusion, our study contributes to the scanty literature of genetic studies of wild

444   monogamous, pair-living primates. We found that the model of pair-living social

445   organization fits this species although it might not be consistently associated with genetic

446   monogamy despite the low EPP rate. However, our results were not fully conclusive about

447   paternity assignment, requiring further investigations on the genetic structure of the

448   population, and the dispersal dynamics. Future studies should focus on providing further

449 insight into the mechanisms involved in the maintenance of socially monogamous systems

450 and their genetic variability.

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650 **Table 1** Annual group composition of the seven indri groups, since the habituation to

651 2015, including name and sex of the individuals. Gray cells: presence of an individual in

652 the group. Italic individuals: genetic data not available. Bold names: offspring born in the

653 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

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

					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%

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.

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

663   Study site located in Center-Eastern Madagascar (A), in the Maromizaha forest,

664   accessible from the village Anevoka (B). The multipurpose center (18°58'34'' S –

665   48°27'53'' E) was used as base-camp (C). Box C is showing the spatial distribution of

666   the indri groups included in the genetic analysis (solid green polygons), and the

667   schematic representation of unsampled neighboring groups (gray striped polygons,

668   GX1 to GX7). The uppercase names within each studied group are the social fathers;

669   the lowercase names are the offspring genotyped. "*" denotes the only individual that

670   resulted with inconclusive paternity assignment. Original satellite images (Google Earth

671   - Image © 2018 CNES / Airbus; downloaded on June 27, 2018) have been graphically

672   simplified and adjusted with GIMP 2.10.2. Map of the indri territories created with

673   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.

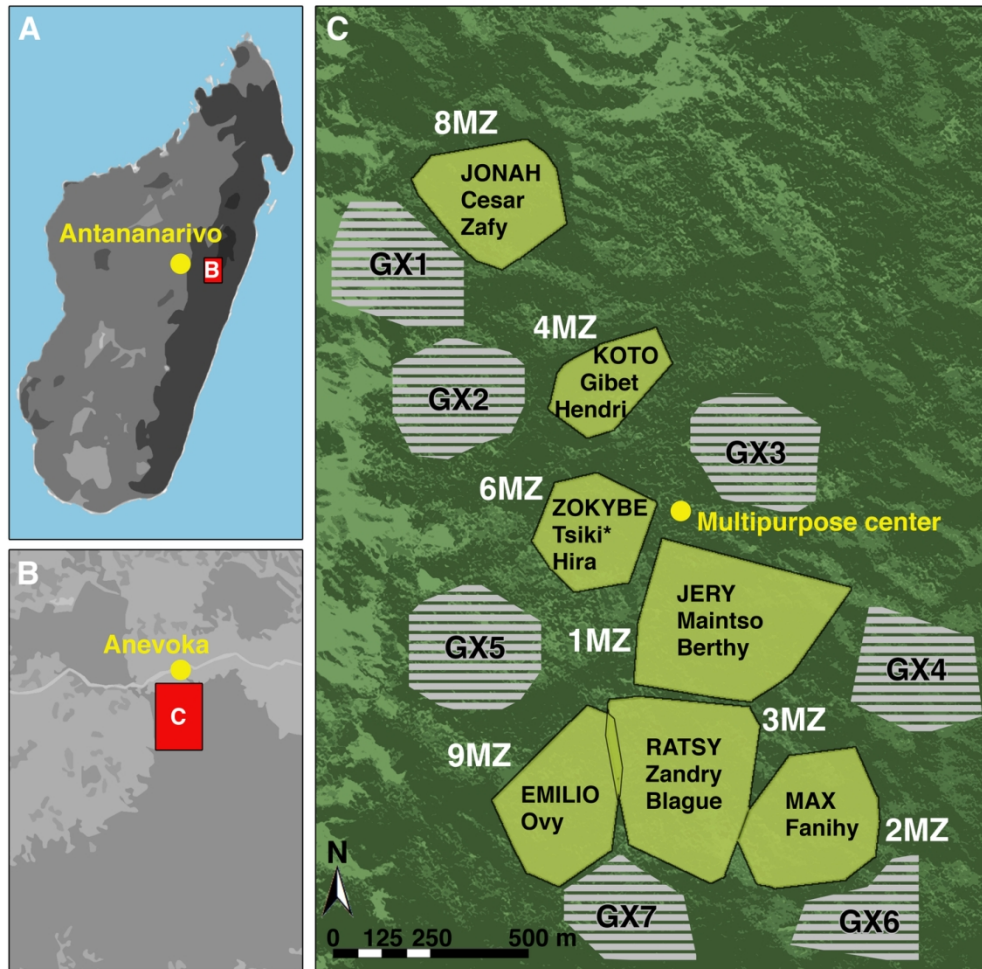- Our finding suggest that genetic monogamy is the norm in indri, although EPC can occasionally occur.

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).

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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.

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