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1 **Highly polymorphic microsatellite markers for the assessment of male reproductive skew**  
2 **and genetic variation in Critically Endangered crested macaques (*Macaca nigra*)**

3 Antje Engelhardt<sup>1,2\*</sup>, Laura Muniz<sup>3,4</sup>, Dyah Perwitasari-Farajallah<sup>5,6</sup>, Anja Widdig<sup>3,4,7\*</sup>

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6 <sup>1</sup>Junior Research Group of Primate Sexual Selection, German Primate Center, Germany

7 <sup>2</sup>Courant Research Center Evolution of Social Behavior, Georg-August-University, Germany

8 <sup>3</sup>Junior Research Group of Primate Kin Selection, Department of Primatology, Max-Planck  
9 Institute for Evolutionary Anthropology, Germany

10 <sup>4</sup>Research Group of Behavioural Ecology, Institute of Biology, University of Leipzig, Germany

11 <sup>5</sup>Primate Research Centre, Bogor Agricultural University, Indonesia

12 <sup>6</sup>Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural  
13 University, Indonesia

14 <sup>7</sup>German Center for Integrative Biodiversity Research, Germany

15

16 \*Corresponding authors:

17 -Antje Engelhardt, School of Natural Sciences and Psychology, Liverpool John Moores  
18 University, Byrom Street, Liverpool L3 3AF, United Kingdom, email:  
19 [A.Engelhardt@ljmu.ac.uk](mailto:A.Engelhardt@ljmu.ac.uk); phone: +44 151231 2434

20 **and**

21 -Anja Widdig, Junior Research Group of Primate Kin Selection, Department of Primatology,  
22 Max-Planck Institute for Evolutionary Anthropology, Deutscher Platz 6, D-04103 Leipzig,  
23 Germany, email: [anja.widdig@eva.mpg.de](mailto:anja.widdig@eva.mpg.de); phone: +49 341 9736 707

24 **Abstract**

25 Genetic analyses based on non-invasively collected samples have become an important tool for  
26 evolutionary biology and conservation. Crested macaques (*Macaca nigra*), endemic to  
27 Sulawesi, Indonesia, are important for our understanding of primate evolution as Sulawesi  
28 macaques represent an exceptional example of primate adaptive radiation. Crested macaques  
29 are also Critically Endangered. However, to date we know very little about their genetics. The  
30 aim of our study was to find and validate microsatellite markers useful for evolutionary,  
31 conservation and other genetic studies on wild crested macaques. Using faecal samples of 176  
32 wild macaques living in the Tangkoko Reserve, Sulawesi, we identified 12 polymorphic  
33 microsatellite loci through cross-species PCR amplification with later modification of some of  
34 these primers. We tested their suitability by investigating and exploring patterns of paternity,  
35 observed heterozygosity and evidence for inbreeding. We assigned paternity to 63 of 65 infants  
36 with high confidence. Among cases with solved paternity, we found no evidence of extra-group  
37 paternity and natal breeding. We found a relatively steep male reproductive skew B index of  
38  $0.330 \pm 0.267$ ; mean  $\pm$  SD) and mean alpha paternity of 65% per year with large variation across  
39 groups and years (29-100%). Finally, we detected an excess in observed heterozygosity and no  
40 evidence of inbreeding across our three study groups, with an observed heterozygosity of  
41  $0.766 \pm 0.059$  and expected heterozygosity of  $0.708 \pm 0.059$ , and an inbreeding coefficient of -  
42  $0.082 \pm 0.035$ . Our results indicate that the selected markers are useful for genetic studies on  
43 wild crested macaques, and possible also other Sulawesi and closely related macaques. They  
44 further suggest that the Tangkoko population of crested macaques is still genetically variable  
45 despite its small size, isolation and the species' reproductive patterns. This gives us hope that  
46 other endangered primate species living in small, isolated populations may also retain a healthy  
47 gene pool, at least in the short term.

48

49 **Keywords:** microsatellite markers, *Macaca nigra*, Sulawesi, conservation, paternity,  
50 reproductive skew, genetic variation, inbreeding, heterozygosity

51 Conflict of Interest: The authors declare that they have no conflict of interest

52

## 53 **Introduction**

54 The development of genetic analyses has revolutionized various fields in the medical and life  
55 sciences. More recently, genetic analyses based on naturally dropped animal waste such as fur,  
56 feathers and faeces have created new opportunities for studies of wildlife under natural  
57 conditions, particularly endangered and/or elusive species, and other species in which capturing  
58 constitutes an ethical problem (e.g. Waits & Paetkau 2005). Potential applications of genetic  
59 analyses for field studies include examining the occurrence, distribution and history of species  
60 (e.g. Hewitt 2000; Leonard 2008; Ram et al. 2015), investigating taxonomic relationships and  
61 speciation (e.g. Tosi et al. 2004), assessing hybridization (e.g. Roos et al. 2011; Charpentier et  
62 al. 2012; Godinho et al. 2015), determining the level of heterozygosity, gene flow and the risk  
63 of inbreeding depression of isolated populations (Luikart et al. 1998; Nürnberg et al. 1998;  
64 Widdig et al. 2004; Knief et al. 2015; Ram et al. 2015; Widdig et al. 2017), monitoring  
65 population developments and movements (e.g. Nowak et al. 2014), identifying species (Harms  
66 et al. 2015), and studying reproductive patterns (Widdig et al. 2004; Engelhardt et al. 2006;  
67 Syručková et al. 2015) and kin relationships in groups and populations (e.g. van Horn et al.  
68 2008; Montague et al. 2014). Hence, studies of evolutionary biology, biogeography and  
69 behavioural ecology greatly benefit from the availability of genetic analyses based on non-  
70 invasively collected samples, as does conservation management (Schwartz et al. 2007). The  
71 genetic markers used in such studies often need to be specified for the species in question,  
72 although the same markers can be used for closely related species.

73 Genetic markers are not yet available but would be very important for the Sulawesi macaques.  
74 The seven species of macaques on the island of Sulawesi (*Macaca brunnescens*, *M. hecki*, *M.*  
75 *maurus*, *M. nigra*, *M. nigrescens*, *M. ochreata*, *M. tonkeana*), the main island of the Wallacea  
76 biodiversity hotspot, are an important group for our understanding of primate evolution.  
77 Endemic to the island, they are a prominent example of primate adaptive radiation and  
78 speciation in relation to the processes of geological change and colonization of new areas  
79 (Groves et al. 1980). All seven species live in different habitats with only narrow overlapping  
80 contact zones, in which interbreeding occurs (Fooden 1982; Evans et al. 2003). Furthermore,  
81 Sulawesi macaques are the only macaques classified as extremely socially tolerant with high  
82 conciliatory tendencies and low degrees of power asymmetries (Thierry et al. 2000; Thierry  
83 2004). Few studies have investigated Sulawesi macaques in the wild because their habitat is  
84 very difficult to access. However, the rainforests of Sulawesi are now more accessible, and the  
85 infrastructure on Sulawesi has improved, facilitating studies of Sulawesi wildlife. However,  
86 with these developments, the natural habitat of the macaques is shrinking and fragmented, and  
87 heavily exploited by humans. As a result, all seven Sulawesi macaques are in danger of  
88 extinction to various degrees (IUCN 2016). Given the precarious situation and geographic  
89 isolation of Sulawesi macaques, genetic studies on these species are important not only for our  
90 understanding of primate evolution (Evans et al. 1999, 2003), but also for their conservation  
91 management.

92 Crested macaques, *M. nigra*, are only found on the northern tip of Sulawesi. Habitat degradation  
93 and bushmeat hunting have brought this species to the edge of extinction, with the largest  
94 remaining population of less than 2000 animals seemingly occurring in Tangkoko Reserve  
95 (Palacios et al. 2012; Melfi 2010). There are at least two reasons why we need genetic studies  
96 of crested macaques. First, crested macaques are of particular interest for better understanding  
97 primate evolution since the species possesses features not found in any of the other Sulawesi  
98 macaques. For example, other Sulawesi macaques live in groups of up to 40 animals, while

99 crested macaques live in large groups sometimes containing over 100 individuals (Riley 2010;  
100 Marty et al. 2015). Despite the large group size, crested macaques seem to be an extreme case  
101 in terms of male-male reproductive competition with males fighting fiercely for dominance  
102 (Marty 2015) and dominant males able to monopolize matings with fertile females (Engelhardt  
103 et al. in revision). The male hierarchy, particularly the first three ranks, is so important that it is  
104 clearly signalled in the occurrence and structure of loud calls (Neumann et al. 2010). Based on  
105 these observations, we can expect male reproductive skew in favour of dominant males as  
106 observed in other primates (reviewed in Widdig 2013), meaning that many infants sired during  
107 a male's tenure will share paternal genes. At the same time, the male hierarchy in crested  
108 macaques is highly dynamic (Neumann et al. 2011), with high takeover rates resulting in a mean  
109 alpha tenure of only 12 months (Marty et al. 2015), so infants born in different years often have  
110 different fathers. However, the genetic consequences of male reproductive strategies at the  
111 population level remain unclear as no study has investigated male reproduction in crested  
112 macaques using genetic data. High reproductive skew may result in lower genetic variation as  
113 only few, top-ranking males pass on their genes to the next generation; however, the high  
114 takeover rate in alpha male position may counteract the effect of reproductive monopolisation  
115 and contribute to the maintenance of genetic variation in the population.

116 The second reasons why we need genetic studies of crested macaques is that they are the most  
117 threatened Sulawesi macaques, and are Critically Endangered (IUCN 2016). Genetic studies of  
118 crested macaques are limited to mitochondrial and autosomal DNA phylogeny (Evans et al.  
119 1999, 2003). The degree of gene flow and the risk of inbreeding depression remain unclear for  
120 the remaining populations of crested macaques. Furthermore, many animals, rescued from  
121 illegal captivity and currently held in sanctuaries, await release into the wild. We cannot  
122 determine the genetic value of these individuals for wild populations until genetic evaluations  
123 are feasible. It is important to detect hybrids amongst these rescued individuals to avoid  
124 releasing them into hybrid-free populations. Finally, we need to understand the genetic variation

125 in the largest population remaining in its natural distribution range, Tangkoko. This information  
126 is highly relevant to conservation management. However, we still lack genetic markers useful  
127 for such analyses in crested macaques.

128 The first aim of this study was to identify highly polymorphic microsatellite (short tandem  
129 repeats or STR) markers for reliable genotyping in crested macaques. Testing primers originally  
130 designed for other, usually closely related species (cross-species amplification) is often the  
131 cheapest and fastest way to define a set of useful markers. Our second aim was to test the  
132 suitability of the selected markers. To do this, we determined marker polymorphism and checked  
133 for Hardy-Weinberg equilibrium and Mendelian inheritance between known mother-offspring  
134 pairs. Our third aim was to assign paternity to the Tangkoko animals and determine the degree  
135 of male reproductive skew (using the B index, Nonacs 2000, 2003) which we predicted to be  
136 high based on the observed mating skew (Engelhardt et al. in revision). We predicted a low  
137 degree of extra-group paternities and natal breeding, given that a few males monopolize all  
138 receptive females. As a final aim, we investigated whether this isolated population shows signs  
139 of loss of heterozygosity by comparing observed and expected heterozygosity, as well as  
140 evaluated estimates of inbreeding in this fragmented population.

141

## 142 **Methods**

### 143 Study population

144 We studied crested macaques at Tangkoko Reserve (1N 32'39'', 125E 12'42''), North  
145 Sulawesi, Indonesia. A recent study in the reserve estimated the population size to be less than  
146 2000 individuals (Palacios et al. 2012). Tangkoko Reserve borders another nature reserve,  
147 Duasudara Reserve, but is disconnected from all other forested areas in North Sulawesi. The  
148 number of crested macaques currently living in Duasudara Reserve is unknown, but preliminary

149 data suggest it to be very low (Palacios et al. 2012). However, there may be some genetic  
150 exchange between individuals in the two reserves.

151 As in other macaque species, female crested macaques stay in their natal groups for life, forming  
152 matriline, while males emigrate from their natal group. Males are fully grown when they  
153 emigrate and frequently challenge alpha males in another group when immigrating (Marty et  
154 al. 2015). Although females give birth year-round, they are moderately seasonal (Marty et al.  
155 2016) with an inter-birth interval of about 22 months (Marty et al. 2015).

156 The Macaca Nigra Project observes three groups (R1, R2, PB) almost daily (R1 and R2 since  
157 2006 and PB since 2008 until present) collecting behavioural data including aggressive  
158 interactions and their outcomes through focal animal and ad libitum sampling (Altmann 1974).  
159 We also recorded births, deaths, and migration events. All adult individuals and sampled infants  
160 were individually recognised. During our study period, the home range of group R1 overlapped  
161 with that of R2 and PB. All three groups also overlapped with other, non-study groups. We  
162 individually recognised all adult individuals of the three groups as well as infants used for  
163 paternity analysis in this study. Group size ranged between 36 and >100 individuals across  
164 years.

165 We used the David's score (de Vries et al. 2006) to assess dominance rank on a matrix of  
166 proportions of wins calculated for each male-male dyad. We calculated David scores using the  
167 package "Steepness" (Leiva&de Vries 2011) in R (RTeam 2009). We used either hormonal  
168 data or data of sex skin swelling size to assess conception windows (for details see Higham et  
169 al. 2012). In addition, we combined demographic and hierarchy data to compute annual alpha  
170 tenure (A. Engelhardt, C. Neumann, P. Marty unpublished data).

171

172 Sample collection

173 We collected non-invasive faecal samples immediately after defecation from 176 individually  
174 recognized animals from all three groups from 2006 onwards. We collected up to three samples  
175 for each individual. Following the two-step alcohol-silica storage protocol (Nsubuga et al.  
176 2004), we placed 1-2 g from the surface of fresh faeces into a 50 ml plastic tube filled with 30  
177 ml of 99% ethanol for at least 24 hrs. Subsequently, we placed the sample in another tube filled  
178 with 30 ml of silica beads and stored it at room temperature until extraction. In a few cases, we  
179 collected ejaculates from males, which we stored in 98% ethanol at room temperature until  
180 extraction. We considered any adult males present or immigrating into our study groups during  
181 our study period as potential sires. We defined adult males as larger than fully grown females,  
182 with fully erupted canines and completely descended testes. We obtained DNA samples for 54  
183 of 56 potential sires (96%), including all adult males present in one of the three study groups  
184 since 2006. For one male, however, we only obtained one sample and the DNA obtained was  
185 of such low quality that it amplified successfully at only nine loci.

186 We also obtained faecal and blood samples during regular health checks of seven crested  
187 macaques (one of each per individual) from Dublin Zoo.

188

#### 189 DNA extraction

190 We extracted DNA from 100-150 mg of faeces with the GEN-IAL® all-tissue DNA extraction  
191 kit following the manufacturer's instructions with the exception that we eluted DNA in distilled  
192 water.

193

#### 194 Identification of polymorphic markers

##### 195 *a: Testing potential markers via cross-species amplification*

196 We tested 39 microsatellite loci previously described to be polymorphic in rhesus (*M. mulatta*),  
197 long-tailed (*M. fascicularis*) and Barbary (*M. sylvanus*) macaques (Nürnberg et al. 1998;  
198 Engelhardt et al. 2006; Brauch et al. 2008; Widdig et al. 2017) for allele amplification and

199 polymorphism with a set of nine different PCR conditions to increase the chances of successful  
200 cross-species amplification (cf. Moore et al. 1991) in crested macaques. For this, we combined  
201 three different magnesium salt concentrations (1.5 mM, 2.0 mM, 2.5 mM) with three different  
202 annealing temperatures (56, 58 and 60 °C or 51, 53 and 55 °C, depending on primer pair). In  
203 this step, we used a high quality pooled DNA sample (from blood) from the seven Dublin Zoo  
204 individuals. When we obtained a readable product for a primer pair, we selected the condition  
205 that yielded the highest concentration of the specific product and fewer stutters for individual  
206 genotyping and polymorphism check. We included the matching faecal and blood samples from  
207 the seven Dublin crested macaques to confirm that genotypes obtained from faecal samples  
208 matched those from blood samples. Finally, we tested Mendelian inheritance by individually  
209 amplifying DNAs from known mother-offspring pairs.

210

#### 211 *b: Genotyping and determination of alleles*

212 To genotype the 176 subjects, we used a two-step multiplex polymerase chain reaction (PCR)  
213 approach (modified from Arandjelovic et al. 2009). First, we amplified all loci in a multiplex  
214 approach using 4 µL of DNA extract (diluted 1:50 or 1:100), of 0.2 µL H<sub>2</sub>O, 2 µL 10x Master  
215 Taq Buffer with Mg<sup>2+</sup> (5PRIME®, 500 mM KCl, 100 mM Tris-HCl pH 8.3, 15 mM  
216 Mg(OAc)<sub>2</sub>), 2 µL 5x TaqMaster PCR Enhancer (5PRIME®), 0.8 µL dNTPs (10 mM), 1.2 µL  
217 MgCl (25 mM), 0.4 µL (10 pmol) of 12 unlabelled forward and reverse primers, respectively,  
218 and 0.2 µL 5PRIME® Taq DNA Polymerase (5 U/µL, Enzyme storage Buffer: 20 mM Tris·HCl  
219 pH 8.0, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 0.5% Tween®20, 0.5%  
220 Igepal®CA-630) in an Eppendorf® Master Cycler Gradient. We started with 2 min of  
221 denaturation at 94 °C, then ran 30 cycles of 20 sec denaturation at 94 °C, 30 sec of annealing at  
222 54 °C, 30 sec of elongation at 70 °C and ended with 10 min of final elongation at 70 °C.  
223 Following the multiplex approach, we ran singleplex PCRs to amplify one locus at a time using  
224 a similar protocol with specific annealing temperatures per primer pair (Table 1). Specifically,

225 we amplified 1  $\mu$ L of multiplex PCR with 13.7  $\mu$ L H<sub>2</sub>O, 2  $\mu$ L 10x Master Taq Buffer with  
226 Mg<sup>2+</sup> (5PRIME®, 500 mM KCl, 100 mM Tris-HCl pH 8.3, 15 mM Mg(OAc)<sub>2</sub>), 0.5  $\mu$ L 5x  
227 TaqMaster PCR Enhancer (5PRIME®), 0.8  $\mu$ L dNTPs (10 mM), 0.8  $\mu$ L MgCl (25 mM), 0.5  
228  $\mu$ L (10 pmol) of each primer labelled (HEX or FAM) forward and unlabelled reverse, and 0.2  
229  $\mu$ L 5PRIME® Taq DNA Polymerase (5U/ $\mu$ L, Enzyme storage Buffer: 20 mM Tris-HCl pH 8.0,  
230 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% Glycerol, 0.5% Tween®20, 0.5% Igepal®CA-  
231 630). We prepared singleplex PCR products for analysis by diluting PCR products between  
232 1:25 and 1:500, and mixing 1.5  $\mu$ L of diluted product into 14  $\mu$ L of Hi-Di Formamide buffer  
233 mixed with a size standard (HD400 from Applied Biosystems®). Finally, we ran amplicons on  
234 an ABI 3130xL sequencer and determined allele sizes with PeakScanner (Applied  
235 Biosystems®).

236 We analysed the samples in two laboratories (German Primate Center and Max-Planck Institute  
237 for Evolutionary Anthropology), with the identical protocols and equipment. We compared five  
238 individuals genotyped in both laboratories on the 12 markers and found genotype inconsistency  
239 in 2 of the 118 alleles, giving an error rate of 0.016.

240

#### 241 *c: Modification of markers*

242 Many of the tested primer pairs produced unspecific products, typically detected as three or  
243 more differently sized amplicons resulting from the simultaneous amplification of two or more  
244 loci (Smith et al. 2000). Since only 7 markers repeatedly produced up to two alleles per  
245 individual, we modified specific primers for crested macaques for the other five identified  
246 markers (Table 1). For this, we located sequences closer to the repetitive sequence than the  
247 respective original primers. We then generated ligation of PCR products of the specific  
248 microsatellites into plasmid vector pCR®2.1-TOPO® with the TOPO TA Cloning®Kit  
249 (INVITROGEN, Carlsbad, USA) followed by colony hybridisation as described in Takenaka  
250 et al. (1993). We isolated plasmids containing the specific repeats from *E. coli* using the

251 QIAprep Spin Miniprep Kit (Qiagen). Next, we conducted fluorescent sequencing with the  
252 Autocycle Sequence Kit Big Dye in the ABI Prism 3100 sequencer (Applied Biosystems, Foster  
253 City, USA). Finally, we synthesised the selected primer sequences with Thermo Hybaid, Ulm,  
254 Germany (Table 1). There may be further additional suitable markers among those we tested,  
255 particularly if they are optimised for the species.

256

#### 257 *d: Final marker selection*

258 We selected the 12 best markers using the following criteria: 1) we preferred markers with tetra-  
259 repeats over di-repeats, 2) amplification success at least 50%, 3) markers that were polymorphic  
260 with at least 3 alleles) and 4) markers with reliable allele size scoring (no or few  
261 stutters/multiple peaks). As faecal samples contain only a small amount of DNA and a high  
262 level of allelic dropouts (Bayes et al. 2000), we genotyped three independent faecal samples for  
263 each individual if available. Based on previous studies (Engelhardt et al. 2006; Brauch et al.  
264 2008), we accepted a heterozygous genotype only if two different samples of the same  
265 individual showed the same result in at least four amplifications; likewise, we accepted a  
266 homozygous genotype if it was consistent in at least six amplifications (Taberlet et al. 1996). If  
267 we identified a third allele during analysis, we doubled the number of amplifications.

268

#### 269 *Testing the suitability of selected markers*

##### 270 *a: Polymorphic information content and Hardy-Weinberg equilibrium*

271 To investigate the suitability of our markers, we first calculated the polymorphic information  
272 content (PIC), an estimate of the discriminating power of markers (ranging from 0-1, from no  
273 allelic variation to only new alleles) (Botstein et al. 1980). We also tested markers for deviation  
274 from Hardy-Weinberg equilibrium (HWE). We considered that deviation from the HWE would  
275 indicate genotyping problems, such as segregating null alleles or incorrectly distinguished  
276 alleles.

277

278 *b: Assessment of Mendelian inheritance*

279 We investigated whether behavioural mothers (known from behavioural observations, i.e.  
280 association and nursing) were also the genetic mothers by testing Mendelian inheritance for 65  
281 mother-offspring pairs through genotype matching using the 12 best markers (including the 5  
282 specifically designed for crested macaques).

283

284 *Investigating paternity distribution*

285 *a: Paternity determination*

286 We used the 65 mother-offspring pairs in paternity analysis. Our paternity dataset included all  
287 offspring born into the three groups between 2006 and 2011 that we could sample. Following  
288 a conservative approach, we assigned paternity only when exclusion and likelihood calculations  
289 revealed the same father (cf. Widdig et al. 2017). In our exclusion method, we assigned  
290 paternity to the male who had no mismatches with a given mother-offspring pair across all loci  
291 while all other potential sires mismatched the offspring at two or more loci (strict exclusion).  
292 We also assigned paternity to the male with no mismatches with a given mother-offspring pair  
293 across all loci while one or more males mismatched the offspring at one locus only (relaxed  
294 exclusion). We used the program FINDSIRE ([https://www.uni-kiel.de/medinfo/  
295 mitarbeiter/krawczak/download/](https://www.uni-kiel.de/medinfo/mitarbeiter/krawczak/download/)) to establish paternity exclusion. We used the same set of  
296 males (i.e., all potential sires) to calculate likelihood-odds (LOD) scores and confidence levels  
297 and confirm sires using likelihood analyses in CERVUS 3.0. We used the following parameters  
298 in CERVUS: simulated offspring: 100; number of candidate fathers: 56; proportion of candidate  
299 fathers sampled: 0.96; proportion of loci typed: 0.99; proportion of loci mistyped: 0.01;  
300 minimum number of typed loci: 10. To assess the proportion of extra-group paternities, we  
301 checked whether the assigned sire was a member of the infant's birth group at the time of  
302 infant's conception using demographic and hormonal data (A. Engelhardt, unpublished data).

303 Given the delay in natal dispersal, we also investigated whether the assigned sire was natal to  
304 the birth group of the infant to detect cases of natal breeding using demographic data (A.  
305 Engelhardt, unpublished data).

306

307 *b: Degree of male reproductive skew*

308 We determined the degree of male reproductive skew using Nonacs' B Index (Nonacs 2000,  
309 2003) with Skew Calculator 2003 (<http://www.eeb.ucla.edu/Faculty/Nonacs/PI.htm>). Positive  
310 values of the B index suggest that the skew is higher than expected, while negative values  
311 suggest that reproduction is more equally distributed than expected (Kutsukake & Nunn 2006).  
312 Furthermore, an index close to 0 indicates a random distribution of paternities across potential  
313 sires, whereas values close to 1 suggest a high monopolization of reproduction by a single male.  
314 The advantage of the B index is that it can incorporate the total number of days adult males  
315 spent in a given group per year. We included information on group membership in the skew  
316 calculation based on demographic data. The program also computes 95% confidence intervals  
317 (CI) with the width of the confidence interval revealing the precision of the estimates. If the CI  
318 includes zero, then the distribution of paternity among group males is not significantly different  
319 from random.

320 As our sampling effort was not consistent across the study period, the skew analysis includes  
321 only years and groups in which we sampled at least 45% of offspring born  
322 (mean±SD=66.8%±28.6%). Therefore, we restricted the skew analysis to offspring born  
323 between 2007 and 2009 in R1 and R2 and born in 2009 in PB, giving 51 offspring with solved  
324 paternity. Although crested macaques are only moderately seasonal, we calculated the annual  
325 skew per group and year. Ideally, we should determine the degree of skew in successful  
326 conceptions during each alpha tenure, however, the number of offspring conceived per alpha  
327 tenure was low due to the typically short tenure (mean 12 months; see Marty et al. 2015).

328

329 Assessing genetic variation and inbreeding

330 For each of the selected markers, we computed standard population genetic parameters of  
331 genetic variation within a population. First, we calculated the expected heterozygosity ( $H_e$ ),  
332 defined as the probability that an individual in a population is heterozygous at a given locus.  
333 Second, we determined the observed heterozygosity ( $H_o$ ) by counting the frequency of  
334 heterozygous individuals per locus. If the observed heterozygosity is lower than expected, this  
335 indicates inbreeding, while a higher than expected heterozygosity suggests a mixture of two  
336 previously isolated populations (Hartl & Clark 1997). Furthermore, we determined inbreeding  
337 coefficients (FIS), where positive values indicate a deficit of heterozygosity (i.e., inbreeding)  
338 while negative values indicate an excess of heterozygosity (Hedrick 2000). We conducted all  
339 calculations (including PIC and HWE) in CERVUS 3.0 (Kalinowski et al. 2007) except the  
340 Wright F statistics (FIS), which we computed in FSTAT (version 2.9.3.) (Goudet 2001).

341

342 **Ethical note**

343 Research complied with protocols approved by the Indonesian Institute for Science and  
344 Technology (RISTEK) and the Indonesian Ministry of Forestry (PHKA) and adhered to the  
345 legal requirements of Indonesia and Germany. We received permits to collect samples and  
346 export DNA extracts from the Indonesian Ministry of Forestry. Furthermore, we carried out our  
347 research in compliance with the animal care regulations and the principles of the American  
348 Society of Primatologists and the German Primate Center for the ethical treatment of non-  
349 human primates. We collected faecal samples from wild and captive individuals non-invasively  
350 after the animals left the site without disturbing, threatening or harming them in their natural  
351 behaviour, and obtained blood samples as part of the regular health check.

352

353 **Results**

354 Identification of polymorphic markers

355 Overall, 31 % (12/39) of the markers we tested were suitable for investigating the crested  
356 macaque population at Tangkoko. These included 10 tetra-nucleotide and 2 di-nucleotide loci  
357 (Table 1) with 4-9 alleles per locus (Table 2). We typed 176 individuals at  $12 \pm 0.3$  (mean  $\pm$  SD)  
358 loci (Table 2).

359

360 Testing the suitability of selected markers

361 *a: Polymorphic information content and Hardy-Weinberg equilibrium*

362 The PIC ranged 0.538 - 0.790 with a mean of  $0.658 \pm 0.075$  (mean  $\pm$  SD) (Table 2) suggesting  
363 our markers had high discriminating power. We detected no significant deviation from Hardy-  
364 Weinberg or evidence of null alleles (Table 1).

365

366 *b: Mendelian inheritance*

367 We confirmed all 65 maternities (assigned by behavioural observations) through genotype  
368 matching (65 pairs \* 10-12 loci) with one mismatch in one mother-offspring pair.

369

370 Investigating paternity distribution

371 *a: Paternity determination*

372 Our dataset included 65 offspring for which we could solve 63 paternities (97%). In 40 cases,  
373 we excluded all males on at least two loci, except for the assigned sire, who matched the  
374 offspring-mother pair at all loci (strict exclusion). In 14 cases, the assigned sire had no  
375 mismatch with the respective mother-offspring pair, but we excluded the next candidate sire at  
376 only one locus (relaxed exclusion). In 8 further cases, the assigned sire had one mismatch with  
377 the given infant, while the next likely sires had at least two mismatches (best match). In one  
378 case, two males matched the infant-mother pair at all loci (tie) and both males were also present

379 in the group around the conception of the infant. In this case, we accepted the male assigned by  
380 CERVUS (Kalinowski et al. 2007) as the sire. In all cases, CERVUS supported the sires  
381 assigned based on exclusion rules (95% confidence level, see Supplement for an overview of  
382 genotypes and trios). In the remaining two cases, we did not assign paternity because the  
383 exclusion and likelihood approach did not reveal the same father. We found no evidence of  
384 extra-group paternity or natal breeding in the solved paternity cases.

385

#### 386 *b: Degree of male reproductive skew*

387 Although 18 males sired the 63 infants investigated, the mean male reproductive skew per group  
388 and year as assessed by the B index was relatively high (mean±SD: 0.330±0.267, range: 0.021  
389 to 0.672). The B index was significantly different from a random distribution across groups and  
390 years (e.g., very high for all years in group R2), except for two of three years in group R1 (Table  
391 3). A posteriori analysis showed that the sex ratio (m/f) was negatively related to the B index;  
392 a female biased sex ratio significantly increased the B index (Spearman rho=-0.857, N=7,  
393 p=0.014) (Table 3). Finally, the mean proportion of alpha paternity was 65% per year with high  
394 variation across groups (29-100%).

395

#### 396 Assessing genetic variation and inbreeding

397 The observed heterozygosity ( $H_o$ ) ranged from 0.665 to 0.856, and expected heterozygosity  
398 ( $H_e$ ) from 0.613 to 0.818 (Table 2). The mean observed heterozygosity  
399 (mean±SD=0.766±0.059) was greater than the mean expected heterozygosity  
400 (mean±SD=0.708±0.059) (Table 2) suggesting no risk of inbreeding at this point in time in our  
401 study groups (see Hartl & Clark, 1997, for comparison). In other words, while we expected  
402 around 70% of individuals to be heterozygous at a given locus under random mating conditions,  
403 on average approximately 76% of individuals were heterozygous. Similarly, the mean FIS  
404 across the three groups was -0.082±0.035 (mean±SD) with FIS consistently below zero for all

405 12 polymorphic loci, indicating an excess of observed heterozygosity (see Hedrick, 2000, for  
406 comparison). In other words, individuals were less related than expected under random mating.  
407 Finally, we found no major differences between groups in terms of number of alleles per locus  
408 and degree of heterozygosity (Table 2), suggesting comparable estimates of genetic variability  
409 despite different group size, degree of skew and duration of alpha tenure.

410

## 411 **Discussion**

412 Our results show that the 12 selected microsatellite markers provide reliable information on  
413 individual genotypes in crested macaques and are useful for various applications in field studies  
414 on this species. Specifically, they provided high confidence in paternity assignment, a relatively  
415 high level of polymorphic information content and genetic variation (assessed by  
416 heterozygosity and inbreeding coefficients) and a high accuracy of allele characterization (i.e.,  
417 low occurrence or absence of mutations). Furthermore, they mainly comprise tetra-nucleotide  
418 repeats, which are usually easier to analyse and thus enhance the reliability of genotyping.  
419 Altogether, the selected markers fulfil important genetic and technical criteria that are critical  
420 for the precision and efficacy of high-throughput genotyping (Butler et al. 2001).

421 We report highly polymorphic markers in Sulawesi macaques. Although we used primers  
422 formerly applied to other macaque species, several markers did not generate satisfying PCR  
423 products. We thus modified specific primers for crested macaques that produced much more  
424 reliable amplification results. However, given that Sulawesi macaques split from their common  
425 ancestor with southern pig-tailed macaques from Borneo (*M. nemestrina*) only in the early to  
426 middle Pleistocene (Fooden 1969; Evans et al. 1999), most, if not all, of the loci used in this  
427 study are likely informative in the other Sulawesi macaque species too. With the validated  
428 markers and improved primers, we thus provide an important tool for conservation management  
429 to assess gene flow, heterozygosity and inbreeding depression of small and/or isolated

430 populations across the whole island. Furthermore, with this set of markers, we will be able to  
431 conduct more detailed studies of population genetics, sexual selection, behaviour and  
432 sociobiology, including parentage data. We encourage the application of the selected markers  
433 to other Sulawesi macaque species.

434 We assigned paternity to 97% of offspring sampled with 95% confidence, demonstrating the  
435 high analytical power of the marker set and its usefulness for studies of sexual selection and  
436 reproductive success. Although we cannot draw conclusions for the two offspring with  
437 unsolved paternity, all cases of solved paternity show no indication of extra-group paternity and  
438 natal breeding. This is interesting, given that male crested macaques do not disperse until they  
439 fully developed, and their competitive ability is sufficient for challenging alpha males in non-  
440 natal groups (Marty et al. 2015). Furthermore, groups are large enough for unrelated potential  
441 mates to coexist in the natal group. It thus seems that male crested macaques need to migrate  
442 and successfully take over the alpha position to reproduce (Marty et al. 2016). It is also  
443 surprising that we found no extra-group paternity. Adjacent groups meet frequently and groups  
444 are too large and the vegetation is too dense for males to oversee the whole group. This suggests  
445 that females ready to conceive are either well mate-guarded during inter-group encounters, or  
446 refrain from mating with non-group males. More detailed behavioural observations during  
447 intergroup encounters are needed to show which of these two explanations hold true for crested  
448 macaques.

449 As predicted from mating observations, we found a skew in male reproduction towards alpha  
450 males. The mean alpha paternity was 65% and ranged 29-100% across years and groups.  
451 Similarly, the degree of skew varied considerably across groups. Notably, our study on crested  
452 macaques found the highest B index reported so far for any primate (maximum: 0.672, mean:  
453 0.330). In a study of free-ranging rhesus macaques, the skew in one large group varied 0.049-  
454 0.106 across six consecutive years (Widdig et al. 2004) and in one small group, the mean B

455 index was 0.084 over two consecutive years (Dubuc et al. 2011). In wild Assamese macaques  
456 (*M. assamensis*), the mean B index was only 0.087 over six years in one group, with the alpha  
457 share of paternity limited to 29% (Sukmak et al. 2014).

458 Takeover rates had a negative effect on reproductive skew. The largest group, R1, generally  
459 had a lower skew and was subject to frequent alpha takeovers (i.e., the male hierarchy was  
460 dynamic), while group R2 showed skew values as high as 0.672, but had fewer takeovers (i.e.,  
461 extended alpha tenure). These data are in line with results from species with extraordinary long  
462 alpha tenures, such as capuchin monkeys (*Cebus capucinus*), with an observed B index  
463 calculated across eight alpha tenure periods varying from -0.125 to 0.473 (mean: 0.274) (Muniz  
464 et al. 2010). Similarly, mountain gorillas (*Gorilla beringei beringei*) showed B indices between  
465 0.337-0.432 in four groups containing multiple males of long tenure (Bradley et al. 2005). It is  
466 surprising, however, that the skew in R2 study group was higher than in the gorilla study, where  
467 a single male usually monopolizes all reproduction in his group. Skew calculations across these  
468 three studies are comparable as they were calculated over the timeframe of alpha male tenure  
469 typical for each species. In other words, for crested macaques with their extraordinary short  
470 alpha tenure we computed annual skew per group, while in the two other species with long  
471 tenure, skew was computed over multiple years of alpha tenure per group. One potential reason  
472 for the comparatively large skew in crested macaques is that male crested macaques need to  
473 maximize their reproductive effort in a short timeframe. Hence, alpha tenure length might affect  
474 the inter-specific variation in reproductive skew. However, our study might also provide a  
475 potential explanation for the intra-specific variation in skew. A more female biased sex ratio  
476 significantly increased the B index which suggests that when more females are available, there  
477 is more room for a few males to successfully monopolize receptive females, in contrast to when  
478 more male competitors are present. This supports the hypothesis that enhanced male  
479 monopolization, among other factors, results in higher degree of reproductive skew (Ostner et  
480 al. 2008; Gogarten & Koenig 2012).

481 The high degree of male reproductive skew observed in our study animals did not translate into  
482 lower genetic variation in the population than we would expected under random mating. This  
483 is interesting given that only a few dominant males pass their genes into the next generation.  
484 Most likely, the high rates of alpha male takeover reported for this population counterbalance  
485 this effect. We need more detailed data on genetic variation in relation to tenure length to  
486 understand this process better.

487 Our study animals reflect a geographically isolated population of a Critically Endangered  
488 species, but our analysis indicates no recent threat of considerable loss of heterozygosis and/or  
489 of inbreeding depression in the study population. Compared to studies of other macaque  
490 species, mainly using different markers (e.g. *M. mulatta*, Bercovitch and Nürnberg 1997; *M.*  
491 *sinica*, Keane et al. 1997; *M. sylvanus*, Kümmerli and Martin 2005; *M. fuscata*, Inoue and  
492 Takenata 2008; *M. assamensis*, Sukmak et al. 2014), our markers were highly polymorphic.  
493 Despite the small population size, it is possible that males migrate in and out of the Tangkoko  
494 population, contributing to the genetic variability observed.

495 In contrast to our results, we found no polymorphism in a set of mtDNA markers in another  
496 study using a subset of the individuals included here (i.e., 12 females and 4 non-natal males  
497 from two groups) (A. Engelhardt, unpublished data). This could indicate that the population of  
498 Tangkoko may already be inbred or stems from one single matriline. To determine the degree  
499 of inbreeding in crested macaques at Tangkoko more precisely, we will need extended studies  
500 over a broader range of groups. Furthermore, we need studies investigating the links between  
501 reproductive patterns, genetic variation and population demography over time to expand our  
502 understanding of viability of threatened populations in the wild.

503 In conclusion, we provide genetic markers useful for studies on the conservation management  
504 and evolutionary biology of crested macaques, and likely of Sulawesi macaques in general.  
505 Parentage analysis of these species can contribute insights to the relationship between social

506 style, reproductive patterns and relatedness among macaque species (Schülke & Ostner 2008).  
507 The fact that the Tangkoko population of crested macaques is still genetically variable despite  
508 its small size, isolation and the species' reproductive patterns gives hope that other endangered  
509 primate species living in small, isolated populations may also retain a healthy gene pool, at least  
510 in the short term. However, while the population in Tangkoko does not seem to be suffering  
511 from genetic depletion, other isolated populations of crested macaques might. With the  
512 described markers at hand, we will now be able to assess and manage genetic variation across  
513 all populations of crested macaques scattered over North Sulawesi.

514

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529

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Table 1: Characterization of 12 primer pairs for amplifying polymorphic microsatellite loci in crested macaques with PCR conditions, deviation from Hardy-Weinberg equilibrium and estimated null allele frequency. F indicates forward primers and R indicates reverse primers.

Locus	Repeat pattern	Length of PCR product Zoo [bp]	Length of PCR product Tangkoko [bp]	Annealing temperature [°C]	Hardy-Weinberg deviation <sup>a</sup>	Estimated null allele frequency	Primer sequence (5'-3') (including modified primers)	Reference
D1S548	Tetra	181-201	185-209	58	n.s.	-0.0394	F: GAACTCATTGGCAAAGGAA R: GCCTCTTTGTTGCAGTGATT	Lathuilliere and Menard 2001
D3S1768	Tetra	129-137	129-157	58	n.s.	-0.046	F: GGTTGCTGCCAAAGATTAGA R: AACTACATGATTCTAGCACA	Lathuilliere and Menard 2001
D5S1457	Tetra	123, 127, 131	123-139	60	n.s.	-0.0609	F: TAGGTTCTGGGCATGTCTG R: TTGCTTGGCACACTTCAGG	Bayes et al. 2000
D6S493*	Tetra	261-269**	139-159***	58	n.s.	-0.0374	F: GCAACAGTTTATGCTAAAGC R: TTCCATGGCAGAAATTGTTT	Nürnberg et al., 1998
D6S501*	Tetra	163-179**	129-145***	58	n.s.	-0.0345	F: GCTGGAAACTGATAAGGGCT R: CTTTATCTTTAATATAGGATTATTGG	Lathuilliere and Menard 2001
D7S2204	Tetra	171-247	220-268	58	n.s.	-0.0579	F: TCATGACAAAACAGAAATTAAGTG R: AGTAAATGGAATTGCTTGTTACC	Lathuilliere and Menard 2001
D10S1432	Tetra	137-145	132-148	58	n.s.	-0.0773	F: CAGTGGACACTAAACACAATCC R: TAGATTATCTAAATGGTGGATTTCC	Lathuilliere and Menard 2001
D11S925	Di	205-221	179-237	60	n.s.	-0.0379	F: GAACCAAGGTCGTAAGTCC R: TAGACCATTATGGGGGCAAA	Lathuilliere and Menard 2001
D12S67*	Tetra	135,177-193**	159-185***	58	n.s.	-0.0262	F: GCAACAGTTTATGCTAAAGC R: TGTTGTTCAAGGGTCAAATG	Nürnberg et al., 1998
D13S765*	Tetra	220,224,232**	137-165***	58	n.s.	-0.0512	F: TGTAACCTTACTTCAAATGGCTCA R: ATTTACCTAACATTTACCCATC	Zhang et al. 2001
D14S255*	Di	173-185**	91-113***	60	n.s.	-0.0142	F: AGCTTCCAATACCTCACCAA R: CTCTTAGTGGTCATTCTCAC	Nürnberg et al., 1998
D18S536	Tetra	144-152	144-164	58	n.s.	-0.0491	F: ATTACTACTGGTGTAGTCCT R: CACAGTTGTGTGAGCCAGT	Kümmerli and Martin 2005

<sup>a</sup>n.s.=no significant deviation

\*primers of this marker were modified to be specific to crested macaques

\*\*before primer modification

\*\*\*after primer modification

Table 2: Number of alleles, observed and expected heterozygosity, polymorphic information content and inbreeding coefficient for twelve selected markers overall (all) and per group (R1, R2, PB), with the mean and standard deviation (SD) across all markers. The analysis is based on 176 crested macaques from three groups in the Tangkoko population in North Sulawesi, Indonesia

Locus	Number of alleles				Observed heterozygosity				Expected heterozygosity				Polymorphic information content				Inbreeding coefficient			
	all	R1	R2	PB	all	R1	R2	PB	all	R1	R2	PB	all	R1	R2	PB	all	R1	R2	PB
D1s548	6	5	6	5	0.784	0.726	0.833	0.881	0.736	0.726	0.765	0.736	0.697	0.681	0.725	0.690	-0.065	0.000	-0.090	-0.199
D3s1768	7	7	6	6	0.851	0.855	0.881	0.833	0.781	0.757	0.776	0.768	0.744	0.713	0.734	0.721	-0.089	-0.131	-0.137	-0.086
D5s1457	6	5	5	5	0.727	0.714	0.717	0.714	0.649	0.674	0.645	0.609	0.589	0.613	0.581	0.541	-0.121	-0.060	-0.112	-0.175
D6s493	5	4	5	3	0.688	0.683	0.627	0.780	0.643	0.648	0.614	0.658	0.579	0.580	0.553	0.577	-0.070	-0.054	-0.021	-0.190
D6s501	5	4	5	4	0.727	0.679	0.783	0.714	0.682	0.675	0.692	0.669	0.614	0.602	0.621	0.598	-0.067	-0.006	-0.133	-0.068
D7s2204	6	6	6	6	0.805	0.831	0.817	0.756	0.724	0.727	0.69	0.721	0.674	0.673	0.633	0.668	-0.112	-0.144	-0.185	-0.049
D10s1432	4	4	4	4	0.710	0.690	0.833	0.548	0.613	0.615	0.628	0.567	0.538	0.542	0.545	0.476	-0.159	-0.124	-0.332	0.035
D11s925	9	9	8	9	0.792	0.805	0.746	0.810	0.748	0.754	0.731	0.758	0.725	0.731	0.701	0.714	-0.059	-0.068	-0.020	-0.069
D12s67	9	9	8	7	0.856	0.869	0.879	0.762	0.818	0.825	0.779	0.806	0.790	0.796	0.735	0.768	-0.047	-0.054	-0.130	0.055
D13s765	7	7	7	6	0.795	0.762	0.800	0.810	0.727	0.691	0.703	0.762	0.693	0.655	0.656	0.713	-0.095	-0.104	-0.140	-0.063
D14s255	3	3	3	3	0.665	0.774	0.550	0.619	0.651	0.669	0.601	0.626	0.575	0.591	0.529	0.537	-0.021	-0.158	0.085	0.011
D18s536	6	6	5	5	0.787	0.771	0.767	0.805	0.723	0.711	0.705	0.702	0.672	0.655	0.651	0.635	-0.089	-0.085	-0.089	-0.149
<b>Mean</b>	<b>6.1</b>	<b>5.8</b>	<b>5.7</b>	<b>5.3</b>	<b>0.766</b>	<b>0.763</b>	<b>0.769</b>	<b>0.753</b>	<b>0.708</b>	<b>0.706</b>	<b>0.694</b>	<b>0.699</b>	<b>0.658</b>	<b>0.653</b>	<b>0.639</b>	<b>0.637</b>	<b>-0.082</b>	<b>-0.082</b>	<b>-0.109</b>	<b>-0.079</b>
<b>SD</b>	<b>1.7</b>	<b>1.9</b>	<b>1.4</b>	<b>1.6</b>	<b>0.059</b>	<b>0.064</b>	<b>0.095</b>	<b>0.089</b>	<b>0.059</b>	<b>0.054</b>	<b>0.059</b>	<b>0.070</b>	<b>0.075</b>	<b>0.069</b>	<b>0.072</b>	<b>0.087</b>	<b>0.035</b>	<b>0.049</b>	<b>0.097</b>	<b>0.082</b>

Table 3: Degree of male reproductive skew in three groups of crested macaques at Tangkoko Reserve, Indonesia, 2007-2009. We provide the number of potential group sires, number of group sires, number of adult females, number of determined paternities, proportion of alpha-male paternity, proportion of alpha-male tenure across the year, the observed B value, the lower and upper confidence interval (each 0.95%) together with the P value that the observed B value is due to chance (significant values in bold). The B index incorporates male residency in days per group and year. This analysis includes a total of 51 offspring.

Group and year	Number of potential group sires	Number of group sires	Number of adult females	Number of determined paternities	Proportion of alpha-male paternity [%]	Proportion of alpha-male tenure across the year [%]	Observed B index	P level	Lower confidence interval	Upper confidence interval
R1 2007	15	4	20	9	55.56	73.15	0.179	<b>0.001</b>	0.033	0.455
R1 2008	20	2	21	3	33.33	73.42	0.139	0.165	-0.303	0.562
R1 2009	21	5	25	7	28.57	97.26	0.021	0.250	-0.133	0.289
R2 2007	14	3	18	9	77.78	18.38	0.527	<b>0.000</b>	0.192	0.865
R2 2008	7	1	19	7	100.00	100.00	0.672	<b>0.000</b>	0.214	0.672
R2 2009	10	1	20	9	100.00	100.00	0.621	<b>0.000</b>	0.251	0.621
PB 2009	16	3	17	7	57.14	31.51	0.153	<b>0.016</b>	0.016	0.506
<b>Mean</b>	<b>14.7</b>	<b>2.7</b>	<b>20.0</b>	<b>7.3</b>	<b>64.63</b>	<b>70.53</b>	<b>0.330</b>			
<b>SD</b>	<b>5.0</b>	<b>1.5</b>	<b>2.6</b>	<b>2.1</b>	<b>29.14</b>	<b>33.44</b>	<b>0.267</b>			