Development and characterization of a multiplex panel of microsatellite markers for the Reunion free-tailed bat *Mormopterus francoismoutoui*

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The ecology and conservation status of many island-restricted bats remain largely unexplored. The free-tailed bat *Mormopterus francoismoutoui* is a small insectivorous tropical bat, endemic to Reunion Island (Indian Ocean). Despite being widely distributed on the island, the fine-scale genetic structure and evolutionary ecology of M. francoismoutoui remains under-investigated, and therefore its ecology is poorly known. Here, we used Illumina paired-end sequencing to develop microsatellite markers for M. francoismoutoui , based on the genotyping of 31 individuals from distinct locations all over the island. We selected and described 12 polymorphic microsatellite loci with high levels of heterozygosity, which provide novel molecular markers for future genetic population-level studies of *M. francoismoutoui*.

1 **Development and characterization of a multiplex panel of microsatellite**

2 **markers for the Reunion free-tailed bat *Mormopterus francoismoutoui***

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14 **Abstract**

15 The ecology and conservation status of many island-restricted bats remain largely unexplored.

16 The free-tailed bat *Mormopterus francoismoutoui* is a small insectivorous tropical bat, endemic

17 to Reunion Island (Indian Ocean). Despite being widely distributed on the island, the fine-scale

18 genetic structure and evolutionary ecology of *M. francoismoutoui* remains under-investigated,

19 and therefore its ecology is poorly known. Here, we used Illumina paired-end sequencing to

20 develop microsatellite markers for *M. francoismoutoui*, based on the genotyping of 31

21 individuals from distinct locations all over the island. We selected and described 12 polymorphic

22 microsatellite loci with high levels of heterozygosity, which provide novel molecular markers for

23 future genetic population-level studies of *M. francoismoutoui*.

24

25 Subjects: Ecology, Conservation, Biology, Genetics, Molecular Biology

26 Keywords: microsatellites, bats, *Mormopterus francoismoutoui*, Illumina, Reunion Island

INTRODUCTION

About a quarter of over 1,300 currently known bat species are endemic to islands (Conenna et al., 2017), in which they sometimes represent the only indigenous mammals. These island-restricted bats play important roles in insular ecosystems through seed dispersal, pollination, and pest control (Aziz et al., 2017; Chen et al., 2017; Kemp et al., 2019). They are also significantly more threatened than continental bat species because of a limited resilience to the combined effects of natural disturbances, typical of island ecosystems, and anthropogenic threats such as urbanization or the development of intensive agriculture (Jones et al., 2009). Yet, scientific knowledge of the species biology and conservation status of these island-restricted bats is scarce.

The free-tailed bat *Mormopterus francoismoutoui* (Goodman et al., 2008; Figure 1a) is a small insectivorous tropical bat endemic to Reunion Island, a volcanic island located in the Indian Ocean (Mascarene Archipelago) (Figure 1b), which has emerged de novo about 3 million years ago (Cadet, 1980). This small territory (2512 km²) is shaped by a very steep mountainous landscape and a great diversity of habitats, which have suffered from the clearing of natural forest, agricultural expansion and urbanization during the 350 years of human colonization (Lagabrielle et al., 2009). Despite such a fragmented landscape, *M. francoismoutoui* is broadly distributed on the island (Moutou, 1982; Barataud & Giosa, 2013), and forms monospecific colonies that range from a few hundreds to several dozen thousands of individuals (Dietrich et al., 2015). This species occupies caves and crevices along with cliff faces but also roosts in a variety of anthropogenic settings, such as buildings, bridges and picnic kiosques (Goodman et al., 2008). This proximity with human populations is often considered as a nuisance due to the considerable amount of excrement and their musky smell. As a result, several colonies have been

50   displaced from public spaces (Augros et al., 2015). However, *M. francoismoutoui* is not

51   considered at serious threat of population decline (Least Concerned, IUCN 2019) but it is

52   protected by French law.

53

54   To date, only one study has used molecular markers to evaluate the genetic diversity of *M.*

55   *francoismoutoui*. Goodman et al. (2008) sequenced the mitochondrial D-loop and 2 nuclear

56   introns (fibrinogen and thyrotrophin encoding genes) from 31 bats sampled in 10 colonies. They

57   found a high genetic diversity using the D-loop marker (28 haplotypes and 2.91% sequence

58   divergence), but only limited genetic structure and no apparent association with geography.

59   However, because of their lower mutation rate compared to nuclear microsatellite markers,

60   mitochondrial markers may be poorly suitable to detect recent population changes (Wang, 2010),

61   such as those induced by landscape changes or the proliferation of anthropogenic roosting sites.

62   Therefore, microsatellite markers represent an ideal tool for inferring such questions (Cleary,

63   Waits & Hohenlohe, 2016), especially in the context of *M. francoismoutoui* facing recent habitat

64   fragmentation and agricultural expansion on Reunion Island.

65

66   The goal of this study was to use Illumina high-throughput sequencing to develop the first

67   microsatellite markers specifically for *M. francoismoutoui*, and to fully characterize these

68   markers using samples from all over the island. These markers could be used to quantity

69   population genetic structure and evaluate individual movements and dispersal strategies within

70   the mosaic-fragmented landscape of Reunion Island. Future studies can also use these markers to

71   increase understanding of mating behavior, relatedness and paternity, and therefore help

72   designing relevant conservation strategies.

73

MATERIALS AND METHODS

Field sampling

Two types of bat samples were used to develop the microsatellite markers: 1) previously-

extracted DNA from organs (pooled kidney, spleen and lung) of 12 bats that were collected in

two colonies as part of a previous study (DEAL permit 11/02/2013, Mélade et al., 2016), and 2)

wing punches specifically collected for this study from 31 bats in 13 colonies across the island

(Figure 1c, and Supplemental Information 1). For this, bats were captured using harp traps or

hand nets. A wing punch of the plagiopatagium was collected using a 2mm diameter circular

biopsy tool and stored in a sterile vial in an ice cooler in the field, prior to transfer to a -80°C

freezer. Bat capture, manipulation and release protocols were evaluated by the CYROI ethics

committee (n°114), approved by the French Ministry of Research (APAFIS#10140-

2017030119531267) and conducted under permits delivered by the DEAL

(DEAL/SEB/UBIO/2018-09).

DNA extraction and sequencing

Genomic DNA was extracted from the wing punches using the Qiagen Blood and Tissue Kit.

DNA samples (from organs and wing punches) were sequenced by the GENOSCREEN

platform, Lille, France (www.genoscreen.fr).The DNA quantity was assessed using the

Picogreen assay (Invitrogen, Carlsbad, CA, USA). Then, an equimolar pool of 1 to 5µg of

genomic DNA from the 12 bat organs was used for the production of microsatellite libraries, and

ran on one lane of an Illumina MiSeq Nano 2x300 v2 (Illumina Inc., San Diego, CA, USA).

95

96   *Design of primers and genotyping*

97   Data was de-multiplexed and quality-cleaned using Cutadapt v2.1 (Martin, 2011) and PRINSEQ

98   (Schmieder & Edwards, 2011). Sequences were then merged using Usearch and ran through the

99   QDD V.3.1 software (Meglécz et al., 2014). QDD treats all bioinformatics steps from raw

100   sequences until obtaining PCR primers including adapter/vector removal, detection of

101   microsatellites, detection of redundancy/possible mobile element association, selection of

102   sequences with target microsatellites and primer design by using BLAST, ClustalW and Primer3

103   softwares (Thompson, Higgins & Gibson, 1994; Johnson et al., 2008; Untergasser et al., 2012).

104   The first step to select primer pairs was done by keeping only perfect di/tri/tetra motives, with A

105   and B quality design (from internal parameters of QDD), and at least 20 bp between each primer

106   and microsatellite sequences, as recommended by QDD.

107

108   Among these high-quality loci, 24 (with the longest number of repeats) were then tested for

109   amplification on a limited number of samples (8 wing punches from distinct colonies,

110   Supplemental Information 1). Individual polymerase chain reactions (PCR) were performed in a

111   10µL reaction containing 0.5µL of template DNA (10 ng/µL,) 0.1µLof FastStartTaq DNA

112   polymerase (Roche, 5U/µL), 1µL of 10X Buffer, 0.24µL of DNTPs (10mM), 0.6µL of $MgCl_2$

113   (25mM) and 0.5µL of each primer (10µM). Reactions were amplified on a MJ Research PTC-

114   225 Tetrad Thermal Cycler and cycling conditions consisted of 10 min initial denaturation at

115   95°C, then 40 cycles at 95°C for 30s, 55°C for 30s, 72°C for 1min, and a final extension step at

116   72°C for 10 min. Amplification products (2µL) were analyzed on a QIAxcel (Qiagen) for the

117   biological validation of primers.

118

119   Then, fluorescent labeled forward primers were synthesized (Applied Biosystems) and used with

120   unlabeled reverse primers in a polymorphism analysis performed on a larger set of samples (23

121   new wing punches from 13 colonies, Supplemental Information 1). We used the same

122   amplification reactions, except that 1μL of two-fold diluted DNA and between 0.08μL and

123   0.25μL of each primer (20μM) were used (Table 1). Cycling conditions were the same as for the

124   development step. Amplification products (1μL) were separated on Applied Biosystems 3730XL

125   Analyzer with GeneScan 500LIZ (Applied Biosystems) internal size standard. The results of the

126   microsatellite profiles were examined using GeneMapper 5 (Applied Biosystems) and peaks

127   were scored by hand. Primer pairs were selected for further analyses when (1) they produced

128   amplicons for all 23 individuals, (2) they did not amplify non-specific fragments, and (3) they

129   revealed length polymorphism (i.e. at least three different allele sizes). For the final selected loci,

130   we genotyped eight additional samples (those tested with the QIAxcel) to reach a total of 31

131   genotyped samples. These loci were then multiplexed in three reactions using seven samples

132   (Supplemental Information 1) and the same PCR conditions as above.

133

134   *Statistical analysis*

135   Linkage disequilibrium for each pair of loci was tested using GENEPOP 4.2 (Rousset, 2008) and

136   significance was assessed after a sequential Bonferroni correction. The frequency of null alleles

137   was computed based on the method of Brookfield et al. (1996). We then assessed the number of

138   alleles per locus (N_A), the observed (H_O) and the expected (H_E) heterozygosity per locus, the F_{IS}

139   for each locus as well as the global $F_{IS,}$ using the R package Adegenet (Jombart, 2008). We

140   tested whether loci were in Hardy-Weinberg Equilibrium by using 1000 Monte Carlo

141   permutations, as implemented by the function hw.test in pegas R package (Paradis, 2010).

142

143 **RESULTS AND DISCUSSION**

144 We obtained a total of 1 104 709 sequences from the Illumina run, merged in 58 302 contigs.

145 After the QDD process, 2 378 microsatellite loci were identified, 313 suitable primer pairs were

146 retained and 24 high-quality loci were selected and tested for polymorphism in 23 individuals.

147 Twelve of them showed proper amplification and high level of polymorphism in the 31

148 genotyped individuals and were multiplexed in 3 distinct reactions. Despite the presence of

149 stutter bands for some loci, none of them impaired the recognition or differentiation between

150 homozygotes and heterozygotes. Primer sequences, the size range of amplification product and

151 multiplex assignment for each of the twelve microsatellite loci are presented in Table 1.

152

153 No evidence of linkage disequilibrium was found in the analyzed loci after sequential Bonferroni

154 correction: only 1 pairwise locus combination showed a significant probability of linkage

155 disequilibrium at $p < 0.05$ (MF_loc05-MF_loc11: $p = 0.033$). The percentage of null alleles was

156 low and ranged from 0–6.7%. Global F_{IS} value was 0.0092, suggesting no heterozygous

157 deficiency. Tests for concordance with Hardy-Weinberg equilibrium revealed a marginal

158 deviation in MF_loc07 only (Table 1).

159

160 The level of genetic variability was high across loci. Indeed, we found high levels of allelic

161 richness, with an average of 9.6 alleles per locus. Calculation of allele frequencies across the 31

162 analyzed individuals revealed that MF_loc28 was the only locus with a single allele displaying a

163 frequency greater than 0.6 (Figure 2). The expected heterozygosity (H_E) per locus ranged from

164 0.450 to 0.928, and the observed heterozygosity (H_O) ranged from 0.359 to 0.962 (Table 1).

165 These results are in line with the findings from Goodman et al. (2008) who suggested elevated

166 genetic diversity in the *M. francoismoutoui* population. Based on the analysis of mitochondrial

167 DNA (D-loop), they indeed found a high level of haplotype diversity which was similar to what

168 is found in the closely related species, *Mormopterus jugularis*, inhabiting the much more bigger

169 island of Madagascar (Ratrimomanarivo et al., 2009).

170

171 **CONCLUSIONS**

172 In this study, we developed and validated 12 polymorphic microsatellite markers for the Reunion

173 free-tailed bat *M. francoismoutoui*. These markers will facilitate further investigations on

174 population genetics, social structure and behavioral ecology of this bat species. This research can

175 be used to help to develop conservation and management plans for this understudied species. To

176 our knowledge, the markers developed here also represent the first microsatellite markers

177 available for the bat genus *Mormopterus* and may probably be used in closely related species

178 (e.g. *M. acetabulosus* from Mauritius and *M. jugularis* from Madagascar), thus helping clarifying

179 the biogeographic patterns and evolutionary history of these Afro-Malagasy species in islands of

180 the western Indian Ocean.

181

182 **ACKNOWLEDGEMENTS**

183 We are grateful to Stéphane Augros and Eco-Med Océan Indien for the help in identifying bat

184 colonies and for the supporting fieldwork provided by Yann Gomard, Steve M. Goodman, Axel

185 Hoarau, Léa Joffrin, Erwan Lagadec, Camille Lebarbenchon, Beza Ramasindrazana, Céline Toty

186 and David A. Wilkinson. We also thank Serena Dool and an anonymous reviewer for their

187 constructive comments on a previous version of this manuscript.

188

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and

any reference numbers):

Bat capture, manipulation and release were evaluated by the CYROI ethics committee

(n°114) and approved by the French Ministry of Research (APAFIS#10140-

2017030119531267).

196

Field Study Permissions

The following information was supplied relating to field study approvals (i.e., approving body

and reference numbers):

Direction de l'Environnement, de l'Aménagement et du Logement (DEAL Réunion),

permit number 11/02/2013 and DEAL/SEB/UBIO/2018-09.

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251

252   **Figure Legends**

253

254   **Figure 1. (a) Illustration of *M. francoismoutoui* and (b, c) location of bat colonies in**

255   **Reunion Island used for microsatellite development.** Orange and green circles correspond to

256   colonies where bat organs and wing punches have been collected, respectively. Photo credit:

257   Jean-Marc Le Deun. Maps were created with PanMap and licensed under CC BY 3.0 Unported

258   (Grobe, Diepenbroek & Siems, 2003).

259

260

261   **Figure 2. Baseline allele frequencies for all loci, averaged across all 31 analyzed individuals**

262   **of *M. francoismoutoui*.**

Figure 1

Figure 1. (a) Illustration of *M. francoismoutoui* and (b, c) location of bat colonies in Reunion Island used for microsatellite development.

Orange and green circles correspond to colonies where bat organs and wing punches have been collected, respectively. Photo credit: Jean-Marc Le Deun. Maps were created with PanMap and licensed under CC BY 3.0 Unported (Grobe, Diepenbroek & Siems, 2003).
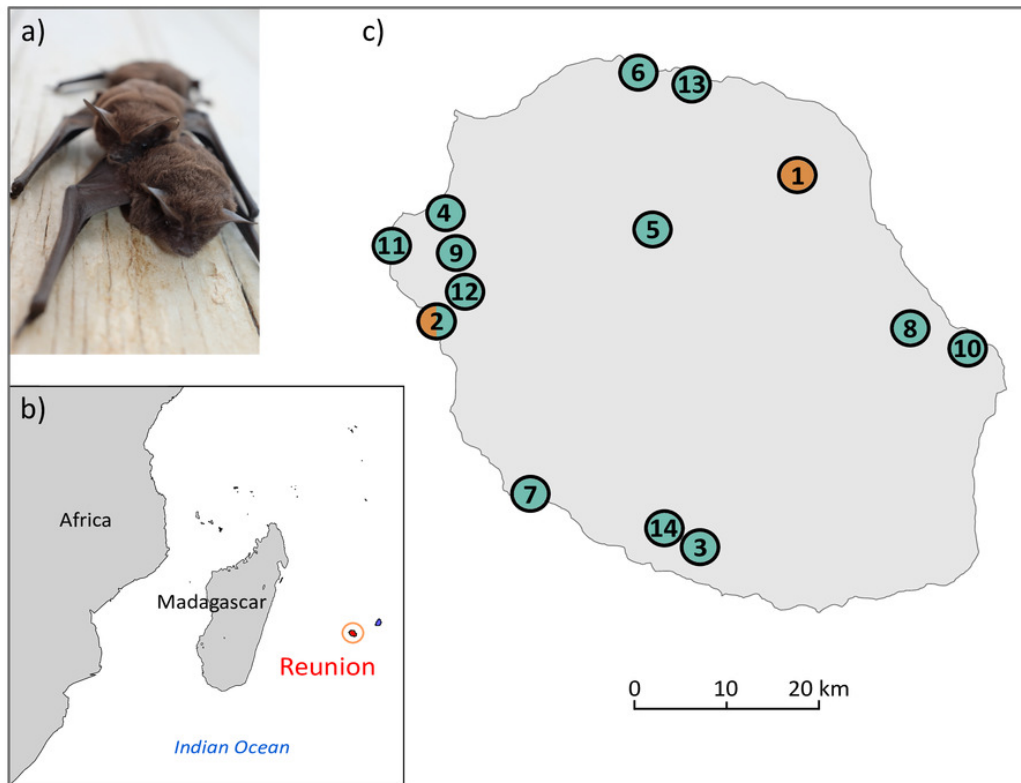
Figure 2

Figure 2. Baseline allele frequencies for all loci, averaged across all 31 analyzed individuals of *M. francoismoutoui*.
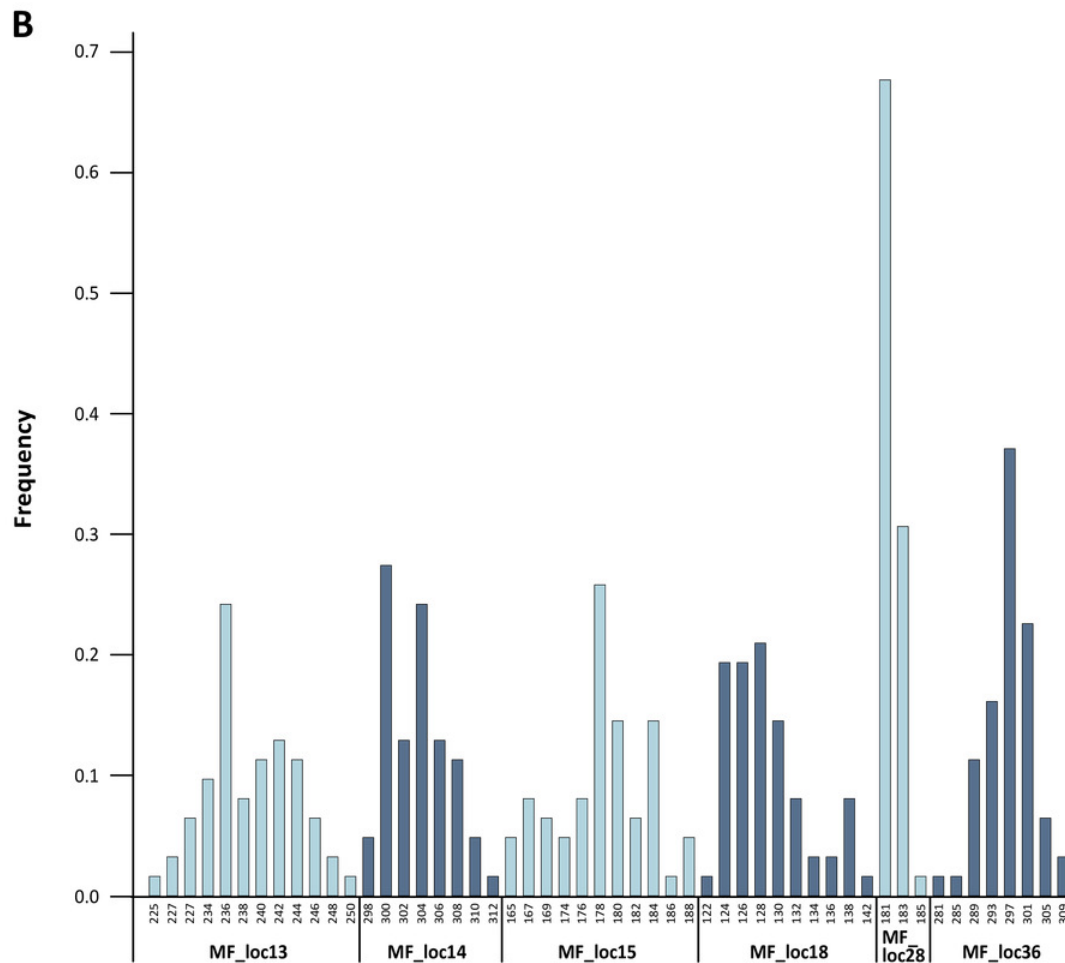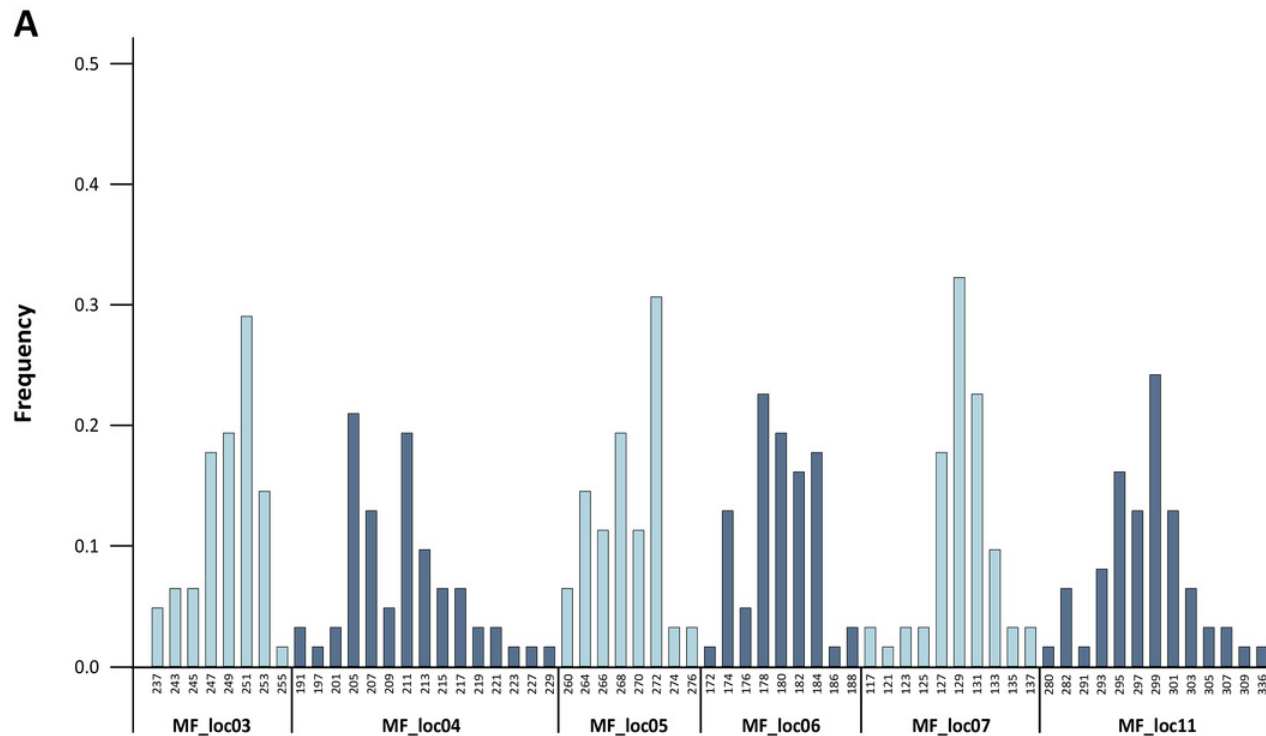
Table 1. Characteristics of the 12 microsatellite loci developed for *M. francoismoutoui*.

Fluorescent labels attached to forward primers are indicated in brackets.

1 **Table 1. Characteristics of the 12 microsatellite loci developed for *M. francoismoutoui*.**

2 Fluorescent labels attached to forward primers are indicated in brackets.

Locus name	Repeat motif	Primer sequences (5'-3')	GB	MP	Range (bp)	NA	H_0	H_E	F_{IS}	P_{HW}	Null$_{all}$
MF_loc06	CA(20)	F: [VIC]ACCCACGACATTCAGCCTTC R: AGAGCTTGGGACCCTGTACT	MN150461	1	172-188	9	0.910	0.870	-0.0465	0.574	0
MF_loc07	AC(20)	F: [NED]CGCAGCAATTCTCCCAGGA R: CCTTCTGTATAAGGCTGGTGT	MN150462	1	117-137	10	0.676	0.897	0.2427	0.042	0.067
MF_loc13	GT(18)	F: [VIC]CTTTCCTCCCTTTCCCGAGG R: GAACCCTCCTTGAGTGAGCC	MN150464	1	225-250	12	0.846	0.887	0.0464	0.193	0.001
MF_loc15	TG(17)	F: [6FAM]AGCTCATAATATACCATGCTGACA R: TCTCAGGATGTCTGGCTCCA	MN150466	1	165-188	11	0.756	0.928	0.1847	0.170	0.065
MF_loc18	CA(16)	F: [VIC]GCTTAGGGAGCCCTATGTTGT R: GCAAGTGGTTTCTGTTTCTGC	MN150467	1	122-142	10	0.897	0.848	-0.0589	0.870	0
MF_loc03	GT(21)	F: [6FAM]GGTGGTGTTCTGATACGAGTGT R: TGACAGTTACCCATCCACCC	MN150458	2	237-255	8	0.872	0.827	-0.0545	0.132	0
MF_loc04	TG(21)	F: [VIC]CCTTGTCTCCTGGCCTCATT R: ACTGTGCCAATTATAATCCTCCC	MN150459	2	191-229	15	0.962	0.880	-0.0932	0.996	0
MF_loc11	GT(18)	F: [PET]TCTCTGTGGCTGCATCAGTC R: AGAGTCGCATCCAGAAAGATGT	MN150463	2	280-336	13	0.897	0.846	-0.0614	0.415	0
MF_loc28	AC(12)	F: [NED]GGACTACAGACTTCCGTGCT R: GCTGCCTGGTGAATTGCTTT	MN150468	2	181-185	3	0.359	0.450	0.2011	0.578	0.041
MF_loc05	GT(20)	F: [NED]CCAGGAAAGCTGGGTGAAGA R: TGGTTTCCCAGCTCACTTCC	MN150460	3	260-276	8	0.756	0.747	-0.0123	0.557	0.005
MF_loc14	AC(17)	F: [NED]GACCGAGGCAGGAATAGAGT R: GGCAGGCGAGGCTAAGTTAT	MN150465	3	298-312	8	0.897	0.844	-0.0639	0.574	0
MF_loc36	TCAT(10)	F: [6FAM]CCAAAGGACTCGCTCGTCTT R: GGCCGTACCCACATTAAATTCA	MN150469	3	281-309	8	0.859	0.761	-0.1290	0.744	0

3 **Notes:** GB: GenBank accession number; MP: Multiplex locus was assigned to; NA: Number of alleles per locus; H_0: Observed

4 heterozygoty; H_E: Expected heterozygoty; P_{HW}: Hardy-Weinberg *p*-value; Null$_{all}$: null allele frequencies.