Genome-based development of 15 microsatellite markers in fluorescent multiplexes for parentage testing in captive tigers

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As one of the most endangered species, tiger (*Panthera tigris*) inbreeding has become an urgent issue to address. Using a microsatellite (short tandem repeat, STR) identification system, paternity testing may be helpful to avoid inbreeding in captive breeding programs. In this study, we developed a genome-based identification system named TPI-plex (tiger pedigree identification multiplex system). By analyzing the entire tiger genome, 139,967 STR loci were identified and 12.76% of these displayed three to six alleles among three re-sequenced individual tiger genomes. A total of 204 candidate STRs were identified and screened with a reference population containing 31 unrelated captive tigers. Of these, 15 loci were chosen for inclusion in the multiplex panel. The mean allele number and mean expected heterozygosity (He) were 7.3333 and 0.7789, respectively. The cumulative probability of exclusion (CPE) and total probability of discrimination power (TDP) reached 0.999999472 and 0.999999999999995, respectively. The results showed that the TPI-plex system can be applied in routine pedigree identification for captive tigers. We also added a sex identification marker named TAMEL into the TPI-plex for sex determination.

1   **Genome-based development of 15 microsatellite markers in**

2   **fluorescent multiplexes for parentage testing in captive tigers**

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Abstract

As one of the most endangered species, tiger (*Panthera tigris*) inbreeding has become an urgent

issue to address. Using a microsatellite (short tandem repeat, STR) identification system,

paternity testing may be helpful to avoid inbreeding in captive breeding programs. In this study,

we developed a genome-based identification system named TPI-plex (tiger pedigree

identification multiplex system). By analyzing the entire tiger genome, 139,967 STR loci were

identified and 12.76% of these displayed three to six alleles among three re-sequenced individual

tiger genomes. A total of 204 candidate STRs were identified and screened with a reference

population containing 31 unrelated captive tigers. Of these, 15 loci were chosen for inclusion in

the multiplex panel. The mean allele number and mean expected heterozygosity (H_e) were

7.3333 and 0.7789, respectively. The cumulative probability of exclusion (CPE) and total

probability of discrimination power (TDP) reached 0.999999472 and 0.999999999999995,

respectively. The results showed that the TPI-plex system can be applied in routine pedigree

identification for captive tigers. We also added a sex identification marker named TAMEL into

the TPI-plex for sex determination.

1.Introduction

The tiger (*Panthera tigris*) was listed as an endangered (EN) species by the International Union

for Conservation of Nature (IUCN) in 1986. As a recognized keystone species, tigers play a key

39   role in maintaining healthy ecosystems *(Cho et al. 2013)*. Unfortunately, the rapid loss of tigers

40   was remarkable. Fewer than 4000 wild tigers survived in areas that occupied only 7% of their

41   historical range and the tigers were divided into two currently recognized subspecies *(Kitchener*

42   *et al. 2017; Wilting et al. 2015)*. The World Wildlife Fund (WWF) worked in alliance with local

43   governments and agencies to protect wild tiger populations. Moreover, there has been an effort to

44   conserve tigers through captive breeding programs and the efforts appeared to have paid off. The

45   captive tiger population has greatly outnumbered the wild ones since 2007 *(Luo et al. 2008)*. In

46   China, the number of captive tiger population has grown rapidly since 2002 and has reached

47   between 5,000 and 6,000 in the last two years (https://eia-international.org/where-are-the-

48   tigers/).

49   For EN animals, the real purpose of protective captivity should not only concentrate on

50   population expansion but genetic diversity preservation *(Reed & Frankham 2003)*. Inbreeding

51   led to reduced genetic diversity and had a deleterious effect on the biological fitness of the

52   population *(Keller & Waller 2002; Reed & Frankham 2003; Ruiz‑López et al. 2012)*. Research

53   showed that tigers are one of the most inbred animals in captivity (*Begany & Criscuolo*

54   *2009*).The captive South China tiger population was found to be suffering from inbreeding

55   depression and a decline in genetic diversity *(Xu et al. 2007)*. High levels of inbreeding brought

56   stillbirths and high infant mortality and very low genetic diversity within the captive population

57   of Asian lions *(Atkinson et al. 2018)*. Having a better understanding of the mating system of a

58   species is the foundation to a successful breeding and captive management *(Ferrie et al. 2013)*.

59   Mastering the parent-child relationship of captive tigers is highly recommended in avoiding

60   captive tiger inbreeding. The breeding programs of many captive species managed by zoos or

61   other organizations depend on studbooks to record individual pedigrees *(Ferrie et al. 2013;*

62   *Jones et al. 2002)*. Accurate and complete pedigree information is essential for effective pedigree

63   analysis *(Ferrie et al. 2013)*. However, the recorded data in the studbook may be missing or

64   incorrect and the information in the studbook should be supported by genetic analysis *(Ferrie et*

65   *al. 2013; Xu et al. 2007)*.

66   Microsatellites (short tandem repeats, STRs) proved to be one of most powerful genetic

67   markers for kinship analysis of animals and have been generally applied for this purpose

68   *(Ichikawa et al. 2001; Luikart et al. 1999; Pei et al. 2018; Queller et al. 1993; Webster &*

69   *Reichart 2005)*. Isolating polymorphic microsatellites efficiently from the species genome is a

70   crucial precondition of paternity testing for the proven STR method *(Webster & Reichart 2005)*.

71   Until now, there have been two alternative sources of microsatellite loci for the STR-based

72   method of tiger paternity identification. First, one may select a set of microsatellite primers

73   derived from the domestic cat *(Felis catus) (Menotti-Raymond et al. 1999)* to amplify the tiger's

74   microsatellite DNA *(Wu et al. 2011; Zhang et al. 2003)*. These investigations introduced an

75   initial single-locus amplification in the target DNA and failed to provide the accuracy of

76   paternity testing by calculating the cumulative probability of exclusion (CPE). Second, one may

77   isolate microsatellite loci from the tiger genome *(Sharma et al. 2008; Williamson et al. 2002; Wu*

78   *et al. 2009; Zhang et al. 2006a; ZHANG et al. 2006b)*. These investigations preselected specific

79   repeated motifs as probes without knowing their abundance in the tiger genome and therefore

80   inevitably introduced biases and limited the microsatellite types into a small fixed subset *(Castoe*

81   *et al. 2010; Malausa et al. 2011)*. These methods are both quite labor-intensive.

82   Therefore, establishing a more efficient method for screening polymorphic loci unbiasedly

83   from all types of microsatellite loci presenting in the tiger genome is essential for establishing a

84   paternity test. Here, we developed a multiplex system in a single reaction tube that can serve as a

85   convenient, effective, and accurate method for paternity testing in the captive tigers. In this

86   study, we screened highly polymorphic microsatellite loci on a genome-wide scale using

87   bioinformatics analysis. We also validated the tiger amelogenin locus based on homology

88   analysis for sex identification. We incorporated all the selected autosomal STR loci and the sex

89   determination locus into one polymerase chain reaction (PCR) to establish an STR five-color

90   fluorescent-multiplex system for simple and effective use. We used a reference population that

91   consists of a group of unrelated individuals to investigate alleles, allelic frequencies, genotypes,

92   genotype frequencies of each STR locus and assessed the validity and accuracy of the multiplex

93   system for paternity testing and individualization in captive tigers.

94 2. Materials and Methods

95   2.1 Sample collection

96   A total of 42 captive continental tigers were sampled (*Table S1*). 20 blood samples were

97   collected from individuals T01-T20 via the femoral vein after anesthetization and 22 hair

98   samples were collected from individuals RT01-RT22 (*Table S1*). Individuals T01-T20 are from

99 Changsha Ecological Zoo. We want to identify their parent-child relationships. Individuals T12-

100 T20 are parents and do not have blood relationship, but they may mate and produce offspring

101 T01-T11. There into, T01, T02, T06, T07, T08 and T09 six tigers were born from the same

102 womb, and T03, T04 and T05 three tigers were from the same womb. However, the biological

103 parents were in doubt. The available recorded information was provided by the zoo staff (*Table*

104 *S2*). Individuals RT01 to RT22 are other 22 unrelated tigers from different places. The study was

105 approved by the *Institutional Review Board on Bioethics and Biosafety of BGI* (FT 16084).

106 2.2 DNA isolation

107 Genomic DNAs from whole blood were extracted by TIANamp Blood DNA Kits® (TIANGEN

108 Biotech Beijing), following the manufacturer's instructions. The hair shaft of each hair sample

109 was cut off and the remaining part containing the hair follicle was placed into a 1.5 ml Eppendorf

110 tube and washed with double distilled water and absolute ethyl alcohol, respectively, and

111 digested by proteinase K. The genomic DNAs from the hair samples were isolated using Chelex

112 100.

113 2.3 Selection of tiger markers

114 We downloaded the tiger reference genome (GCA_000464555.1 PanTig1.0) from the NCBI

115 database and used Tandem Repeats Finder (v4.09) *(Benson 1999)* for annotating the STR loci in

116 the reference genome. We also downloaded the re-sequenced data from three tigers from the

117 NCBI database (SRR640236, SRR640237 and SRR640238) *(Xu et al. 2013)* and filtered the raw

118   data by SOAPnuke (v2.0) *(Chen et al. 2017)*. Based on the annotation results, we typed the STRs

119   of the three tigers using lobSTR *(Gymrek et al. 2012)*. We screened all of the valid STR loci with

120   high polymorphisms that satisfied the following criteria: (1) the locus is a tetra-nucleotide or

121   penta-nucleotide repeat, (2) the repeat units repeat 10 to 20 times in the reference genome, (3)

122   three to six alleles in three re-sequenced individuals exist at the site.

123   To identify a tiger's sex, we validated the amelogenin locus based on homology analysis.

124   We downloaded human amelogenin sequences (AMELX and AMELY) from the NCBI database,

125   aligned them to the tiger genome, and found two homologous sequences in the tiger. Clustal X

126   (v2.1) *(Jeanmougin et al. 1998)* was used to find the deletion polymorphism of amelogenin

127   sequences.

128   Primer pairs were designed in flanking regions using OLIGO 7 (v7.56) *(Rychlik 2007)* and

129   the size range of the amplification products was controlled between 100-500 bp. The specificity

130   of the primers was validated by PCR, and loci that could be easily amplified were reserved. The

131   forward primers of the normal primer pairs were then labeled with different fluorescent-dye.

132   Loci with inefficient amplification or split peaks were excluded.

133 ## 2.4 Multiplex amplification assay

134   The selected loci were incorporated into a multiplex amplification system. For the loci whose

135   primers were labeled with the same dye color, their allele size ranges did not overlap each other.

136   All of the fluorescence-labeled primer pairs were mixed in proportions that were determined

137   from the results of multiple experiments guaranteeing good amplification at each site and peak

138   height during capillary electrophoretic separation. The final primer concentration of each primer

139   pair was in the range of 0.2 to 1.3μM/μL. The primer mixture was used to amplify genomic

140   DNAs by PCR and amplification was performed in a 10 μL reaction volume. The reaction

141   mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3, 25°C), 2.0 mM $MgCl_2$, 0.1 mg/ml

142   BSA, 0.2 mM dNTPMix (dATP, dTTP, dCTP and dGTP mixed equally), 0.2 units DNA

143   polymerase (EzAmp® Fast Taq DNA Polymerase) and 0.1-2 ng genomic DNA. For each

144   reaction, the PCR conditions were as follows: 1 cycle of 95 °C for 5 min, 30 cycles of 95 °C for

145   10 s, 58 °C for 1 min, 70 °C for 20 s, and 1 cycle of 60 °C for 1h with Applied Biosystems®

146   Veriti® Thermal Cycler.

147 ## 2.5 Electrophoresis separation and data analysis

148   The PCR products (1 μl) were mixed with loading buffer composed of Hi-DiTM formamide and

149   internal size standard Salmon 500 Plus at a 9:0.3 (v/v) ratio. The electrophoretic separations were

150   performed on an ABI 3500 Genetic Analyzer (Thermo Fisher, USA). Then the collected data

151   were analyzed with GeneMapper® ID-X Software Version 1.5.

152 ## 2.6 Allele sequencing and genotyping

153   A homozygote was selected at each locus and the amplification products were sent to Sangon

154   Biotech (Sangon, Shanghai, China) for Sanger sequencing in both forward and reverse directions

155   after performing agarose gel electrophoresis for validation. The sequenced alleles were named by

156   the number of repetitions of the motif according to the nomenclature of STR recommended by

157   the International Society for forensic genetics (ISFG) *(Mayr et al. 1995; Olaisen et al. 1998)*.

158   The repetition numbers of the motifs of unsequenced alleles were deduced in reference to the

159   repeat sequence structures of both the sequenced alleles and the reference genome, and the

160   observed sizes of both sequenced and unsequenced alleles. The panel and bin files were

161   programmed for the GeneMapper® ID-X Software Version 1.5 to genotyping analysis. The

162   allelic ladders of each locus were listed in Table S3.

2.7 Population genetic analysis

163

164   From the 42 captive continental tigers, a total of 31 unrelated tigers (T12-T20 and RT01-RT22)

165   were selected as the reference population for population genetic analyses of the 15 autosomal

166   STR loci. The Hardy-Weinberg equilibrium (HWE) for each locus was tested by using Genepop

167   v4.7 *(Raymond & Rousset 1995; Rousset 2008)*. Expected heterozygosity (H_e: Equation 1),

168   probability of exclusion (PE: Equation 2), and discrimination power (DP: Equation 3) of each

169   locus, cumulative probability of exclusion (CPE: Equation 4), and cumulative discrimination

170   power (TDP: Equation 5) were calculated according to the formulas below *(Frankham et al.*

171   *2010; Kloosterman et al. 1993; Li et al. 2018; Wu et al. 2009; YiPing 2016)*.

172    $H_e = 1 - \sum_{i=1}^{k} p_i^2$ (Equation 1, k is the number of alleles and p_i the allele frequency of the

173   i^{th} allele at the target locus)

174    $PE = \sum_{i=1}^{k} p_i (1 - p_i)^2 - 1/2 \sum_{i=1}^{k-1} \sum_{\substack{j=i+1 \\ i \neq j}}^{k} p_i^2 p_j^2 (4 - 3p_i - 3p_j)$ (Equation 2, p_i and p_j are

175   respectively the allele frequency of the ith and j^{th} allele at the target locus, with i not equal to j)

176    $CPE = 1 - \prod_{i=1}^{k} (1 - PE_k)$ (Equation 3, PE_k is PE for each of k loci)

177      $DP = 1 - \sum_{i\ =\ 1}^{k} g_i^2$ (Equation 4, g_i is the frequency of each genotype)

178      $TDP = 1 - \prod_{i=1}^{k}(1 - DP_k)$ (Equation 5, DP_k is DP for locus k)

179   2.8 Sensitivity testing

180   To evaluate the sensitivity of the TPI-plex amplification system, the DNA from the individual of

181   T08 was chosen as the control DNA and used to perform the sensitivity testing experiment. A

182   series of template DNA quantities were diluted in a 10 µL PCR reaction system and the DNA

183   concentrations, from high to low, were as follows: 2 ng 10 µL^{-1}, 1 ng 10 µL^{-1}, 0.5 ng 10 µL^{-1},

184   0.25 ng 10 µL^{-1}, 0.125 ng 10 µL^{-1}, 0.0625 ng 10 µL^{-1}, and 0.03125ng 10 µL^{-1}. Each quantity of

185   DNA was analyzed in triplicate and a negative control group was set up.

186   2.9 Specificity testing

187   As DNA from human or other non-human species may be mix in detected material, the TPI-plex

188   amplification system was tested with DNA from a range of species including human, sheep,

189   chick, duck, dog and rat, under the same PCR amplification condition to estimate potential

190   interference. Genomic DNAs from human and dog samples were extracted from whole blood

191   with TIANamp Blood DNA Kits® (TIANGEN Biotech Beijing), while others were extracted

192   from fresh tissue (purchased from markets) using TIANamp Genomics DNA Kits® (TIANGEN

193   Biotech Beijing). Each DNA was analyzed in duplicate and a negative control group was set up.

194 **3. Results and discussion**

195 3.1 Establishment of TPI-plex identification system

196 **3.11 Selection of tiger markers**

197 Microsatellites have a high mutation rate as compared to other types of known genetic

198 polymorphisms *(Ballantyne et al. 2010; Webster & Reichart 2005)*. Microsatellites analyses are

199 based on simple PCR, which allows for typing in samples of low DNA quality or concentration

200 *(Dawson et al. 2013; Webster & Reichart 2005)*. Therefore, microsatellites are the most

201 commonly used genetic marker in parentage identification of animals *(Vignal et al. 2002;*

202 *Webster & Reichart 2005)*, the paternity testing in tigers is no exception *(Wu et al. 2011; Zhang*

203 *et al. 2003)*. A total of 139,967 valid STR loci were identified across the whole tiger genome and

204 31.48% and 12.76% of these STR loci display two and three to six alleles (*Fig. S1A*),

205 respectively. As the tiger genome sequence shows a 95.6% similarity to that of the domestic cat

206 *(Cho et al. 2013)*, we mapped all the detected STR loci from the tiger genome onto a cat

207 karyotype, based on their genomic homology, to show their distribution on chromosomes *(Fig.*

208 *S1B)*. We generated 204 candidate STR loci (*Table S4*) of which 84.11% displayed three alleles

209 and covered nineteen chromosomes of the tiger (*Fig. 1*). Then, primers were designed for 49

210 STR loci of 204 candidate STR loci based on the tiger reference and 27 pairs of primers on 27

211 STR loci were effective. Three loci were excluded because of weak or split peaks after the

212 detection of fluorescent-labeled PCR products. The remaining 24 loci were distributed on 14

213   chromosomes and were named as DA1S1290, DA1S1470, DA2S1059, DA2S1575, DA3S1145,

214   DA3S461, DA3S1123, DB1S1259, DB1S1096, DB1S542, DB2S734, DB2S23, DB3S187,

215   DB4S1505, DB4S2706, DB4S2753, DC1S1364, DD2S793, DD3S899, DD3S86, DD4S705,

216   DE1S613, DF1S579 and DF2S497, respectively (*Fig. 1*).

217   In humans, sex identification in forensic multiplexes is based upon the amelogenin gene on

218   both the X and Y chromosomes, which is commonly used in sex genotyping *(Akane et al. 1991;*

219   *Nakahori et al. 1991)*. Similarly, we discovered two sequences of the tiger amelogenin gene on

220   scaffolds of ATCQ01070658.1 and ATCQ01738.1 by homology searching (*Fig. S2*).

221   Interestingly, on the tiger amelogenin locus, we found a deletion polymorphism (20 bp) which

222   may be used to identify the tiger's sex. We named the tiger amelogenin locus *TAMEL*,

223   amelogenin X-linked as *TAMELX* on ATCQ01070658.1 and amelogenin Y-linked as *TAMELY*

224   on ATCQ01738.1, respectively.

225   ## 3.12 TPI-plex identification system development

226   We established an STR five-color fluorescent-multiplex system, with 15 autosomal STRs loci

227   and a sex identification locus (*Table 1*). The 16 loci (DA1S1290, DA1S1470, DA2S1059,

228   DA2S1575, DA3S1123, DA3S1145, DB1S542, DB1S1259, DD2S793, DD3S86, DD4S705,

229   DE1S613, DF2S497, DF1S579, and TAMEL; *Fig. 1*) are distributed on 12 chromosomes and the

230   16-plex identification system used fluorescent forward primers labeled at the 5' end with blue

231   (6'-FAM), green (HEX), yellow (TAMRA), or red (ROX) dyes (*Table 1*).

232   To characterize each locus, we sequenced PCR amplification products from homozygotes

233   *(Xu et al. 2005)* and sequencing results *(Table S5)* provided the repeat numbers of motif units

234   *(Table S6)*. Sequenced alleles were defined on the basis of the nomenclature of STRs *(Mayr et*

235   *al. 1995)*. Most of these loci are simple repeats of tetra-nucleotide or penta-nucleotide motifs

236   *(Table S6)*. DE1S613, DF1S579, DA3S1145, and DD2S793 are compound repeats due to their

237   two different types of motifs. DB1S1259 and DD2S705 are complex repeats composed of tetra-

238   and a few penta-nucleotide motifs. Their alleles were designated using the method which

239   assumed the region was a general tetrameric repeat structure *(Hellmann et al. 2006)*. The

240   amplified fragments of both DB1S1259 and DD4S705 loci consist of a complex hypervariable

241   region mainly based on a tetrameric motif of (AAAG) repeat and a pentameric motif of

242   (AAAAG) repeat. The nomenclature was based on assuming a general tetrameric repeat structure

243   (Table S6). Eleven out of the fifteen loci had the same repeat sequences as the reference genome,

244   while four loci (DB1S1259, DE1S613, DF1S579, and DA3S1145) displayed polymorphisms in

245   repeat sequences.

246   3.2 Evaluation of the TPI-plex identification system

247   3.21 The CPE and TDP of TPI-plex system

248   To assess the TPI-plex, we calculated the allele frequencies *(Table 2)* and genotype frequencies

249   *(Table S7)* in the reference population (n=31). The repetition numbers of the motifs of

250   unsequenced alleles were deduced (see the method section 2.6). We provided the peak ratio of

251   each locus for correct allele calling *(Table S6)*. We ran this TPI-plex for each individual in the

252   reference population (n=31), and found that all of the STRs are polymorphic (*Tables 2 and S7*)

253   and allele number ranged from five to eleven. The observed and expected genotype distribution

254   of 15 autosomal STR loci was listed in Table S8. DA3S1145, DA3S1123, DD3S86, DA1S1470,

255   DA2S1059, DB1S1259, and DF1S579 displayed a departure from HWE (*P < 0.05, Table 3*). The

256   deviation from HWE in allele frequencies at these microsatellite loci can be caused by natural

257   selection, inbreeding, human disturbance, population degradation and small population size

258   *(Antoro et al. 2006; León et al. 1997; Rousset & Raymond 1995; Schaid et al. 2006; Yu et al.*

259   *2002).*

260   We evaluated the TPI-plex power in individualization and paternity testing. The expected

261   heterozygosity ranged from 0.6852 to 0.8902, and the average H_e was 0.7789. The PE of each

262   locus ranged from 0.4183 to 0.8183, average PE being 0.6028 and the CPE reached 0.999999472

263   (*Table 3*). The DP ranged from 0.8262 to 0.9417 with an average of 0.8808. The TDP of all the

264   15 loci reached 0.999999999999995 (*Table 3*). In a previous study using 21 microsatellite loci

265   and more than one multiplex panel for the Amur tiger, the CPE reached 0.9999 *(Wu et al. 2009)*.

266   Compared with this report, our method greatly simplifies this process and is more efficient.

267   **3.22 Sensitivity testing**

268   Sensitivity testing can be used to find the DNA template usage limitation of the multiplex typing

269   assay *(Zhang et al. 2015)*. Here, we used DNA from the individual of T08 as control DNA. We

270   performed the typing assay in triplicate in the range of total input DNA (0.03125~2 ng 10 μL^{-1})

271   under the same PCR conditions. We calculated the mean percentage of detected sites in the

272 sensitivity testing and found that all 16 loci can be detected with DNA from 2 ng down to 0.5 ng.

273 When the DNA amount were 0.25ng, 0.125 ng, 0.0625 ng, and 0.03125 ng, the mean

274 percentages of detected loci were 93.75%, 93.75%, 37.5%, and 18.75%, respectively. When the

275 DNA template amount decreased to 0.125ng, only one loci could not be detected. Furthermore,

276 we calculated the mean peak height in the sensitivity testing. When the DNA template amount

277 varied from 2 ng down to 0.5 ng, the mean peak height was from 10,950.37 relative fluorescence

278 units (RFU) down to 5,752.40 RFU. In summary, the minimal DNA template was 0.5 ng for the

279 TPI-plex.

280 **3.23 Species specificity**

281 We performed the TPI-plex on the common species of human, sheep, chick, duck, dog, and rat

282 and showed that there was an off-range peak 180 to 210 bases long at DA2S1059 in human

283 (193.0 bases), sheep (193.2 bases), dog (207.9 bases), and rat (182.9 bases). We detected a

284 second OL peak at DC1S1364 in sheep (357.0 bases), and dog (372.6 bases).

285 ## 3.3 Applications of the TPI-plex identification system

286 **3.31 Sex determination**

287 Tigers have a chromosomal XY sex-determination system. The sex of tigers can be identified by

288 analyzing the amplification products of the sex identification marker, TAMEL. We added the sex

289 identification marker to the multiplex system for the first time and designed paired primers at

290 both ends of the 20 bp deletion sequences. At the TAMEL locus, a male and female tiger display

291 heterozygous (87 bp and 109 bp, namely, Y and X, *Fig. 2A,* boxed in carmine) and homozygous

292 bands (109 bp, namely, X, *Fig. 2B,* boxed in carmine), respectively. Furthermore, we validated

293 this sex marker by identifying the sexes of a group of tigers with known sex information (*Table*

294 *S1*: RT01, RT04, RT05, RT06, RT07, RT08, RT10, RT11, RT14 and RT15) using the TPI-plex

295 system and compared our detected sex with information from these tiger's breeders. We found

296 that our detection on all individuals was correct (*Fig. S3*), suggesting that the TAMEL locus can

297 identify the sex correctly.

298 3.32 Tiger pedigree identification

299 Using STR genotyping results, we determined the parent-child relationship between the 20 tigers

300 from Changsha Ecological Zoo and validated the pedigree reconstruction using information

301 provided by the zoo. As shown in *Fig. 3*, there were three families in all (Family 1: T13, T19,

302 T01, T02, T06, T07, T08 and T09, *Fig. 3A*; Family 2: T13, T20, T03, T04 and T05, *Fig. 3A*; and

303 Family 3: T15, T18 and T10, *Fig. 3B*) and T13 connects two families as having a common

304 father. Another 4 individuals, T12, T14, T16 and T17, have no blood relationships with the

305 others. Table S9 lists genotyping information of the three families.

306   We checked information from the zoo and found that T01, T02, T06, T07, T08, and T09

307 tigers were born from the same womb (*Table S2*). These six individuals and T11 shared the same

308 dam, T19. Our genotyping results show that T19 was indeed biological mother of the seven

309 tigers, since one of two alleles at each locus in the seven offspring individuals inherited from

310   T19, which was in accordance with Mendelian inheritance. In addition, T03, T04 and T05 were

311   three tigers born in a single birth.

312   Interestingly, zoo staffs cannot confirm whether white tiger, T10, is from orange tiger

313   family (T16 and T14) or from white tiger family (T18 and T15). Our identification results

314   showed that T15 and T18 were biological parents of T10.

315   Conclusions

316   We used the bioinformatics analysis method to identify tiger microsatellite loci on a genome-

317   wide scale for the first time and screened 15 highly polymorphic microsatellite loci distributed

318   on 11 chromosomes from the tiger genome. We obtained a sex determination locus by validating

319   the tiger amelogenin locus based on homology analysis. The 15 loci together with the sex

320   determination locus were incorporated into one PCR reaction and a STR five-color fluorescent-

321   multiplex system named TPI-plex was established. The TPI-plex system's CPE and TDP reached

322   0.999999472 and 0.999999999999995, respectively, suggesting that this TPI-plex can be applied

323   for routine pedigree identification and individualization for tigers. The sex identification locus

324   provided sex information of individuals.

325   Compared with other methods, our identification process is time- and cost-saving, as the

326   TPI-plex system is a single reaction multiplex system. Our research could contribute to the

327   supplementation and correction of studbook records by identifying and verifying the pedigree

328   relationships among captive individuals and could also play a positive role in promoting pedigree

329   management and breeding control of tigers in captive institutions. It is of great significance to

330   effectively avoid inbreeding in order to protect the genetic diversity of captive tigers.

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466

Figure 1

Distribution of selected STRs on chromosomes.

The 204 candidate STRs are labeled on chromosome using different colors based on the allele polymorphism. Yellow, orange and red represent the allele number is 3, 4 and 5, respectively. The 24 validated loci were denoted, among which fifteen retained loci are denoted with black fonts, nine excluded loci are denoted with gray fonts. The position of sex identification locus TAMEL is marked with white line on chromosome X and denoted with black fonts.

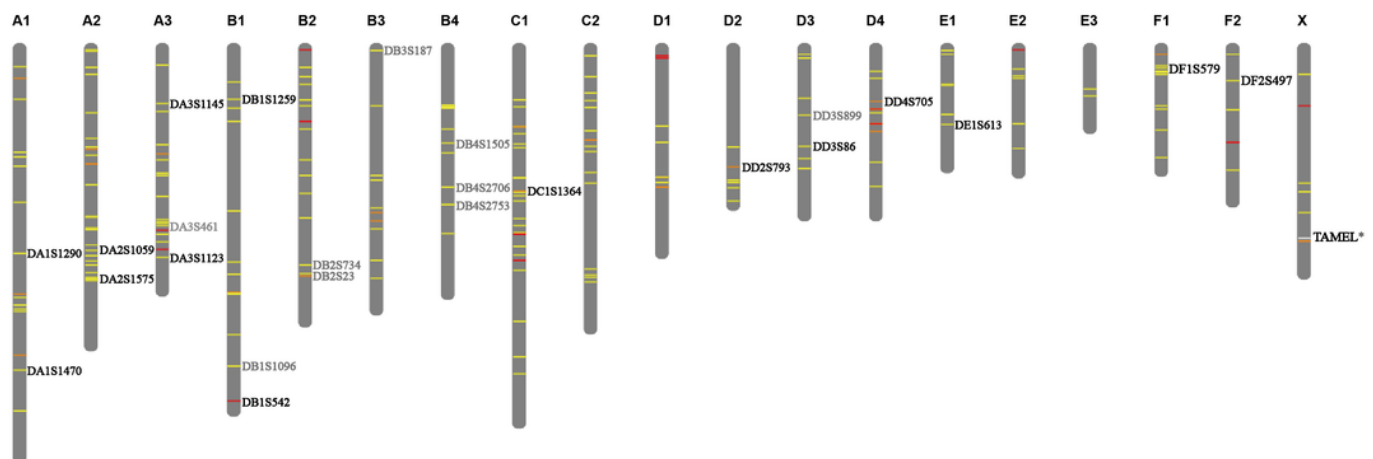



Figure 2

Amplification products of a male (A) and a female (B), detected by ABI 3500 Genetic Analyzer.

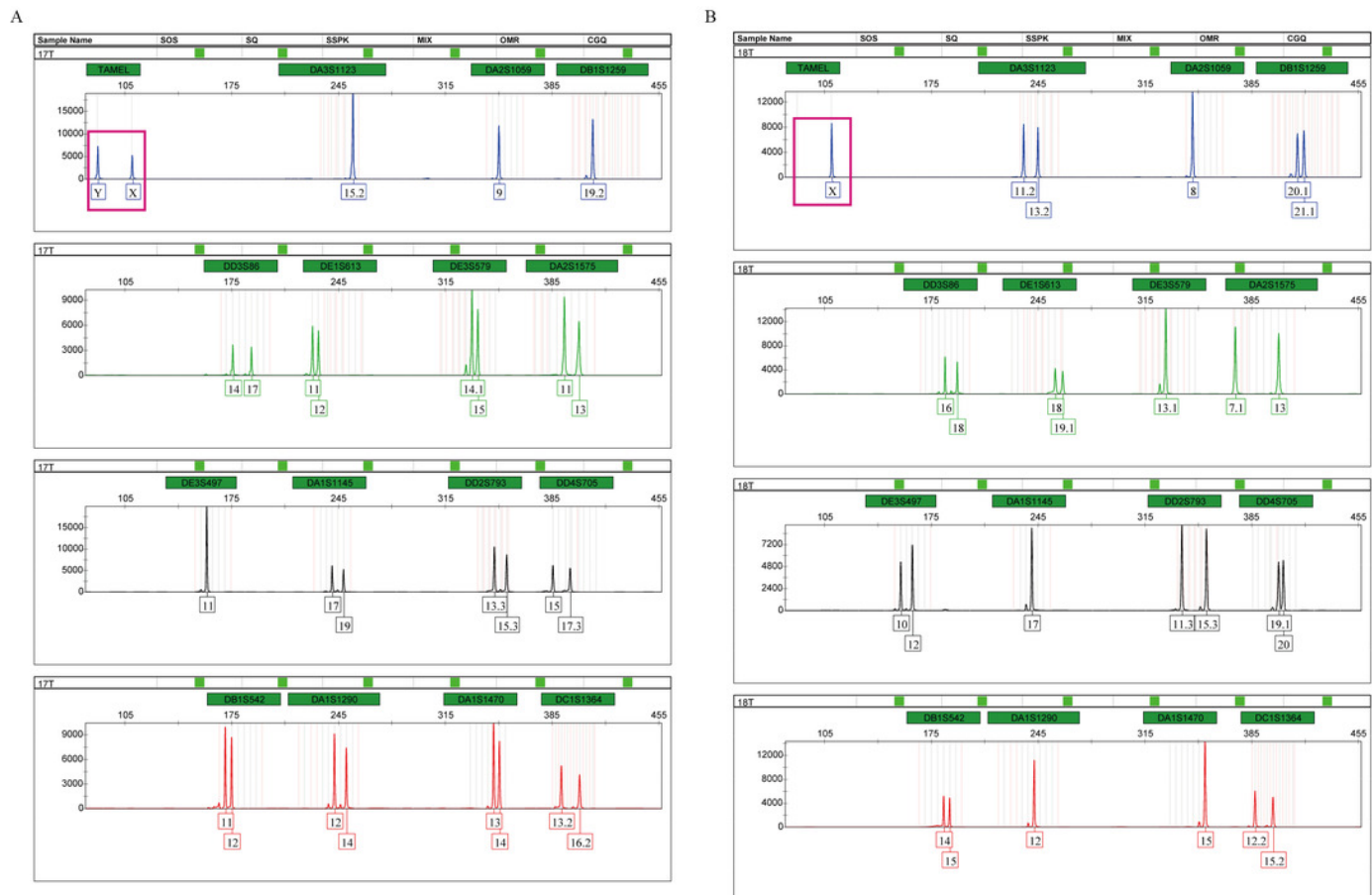
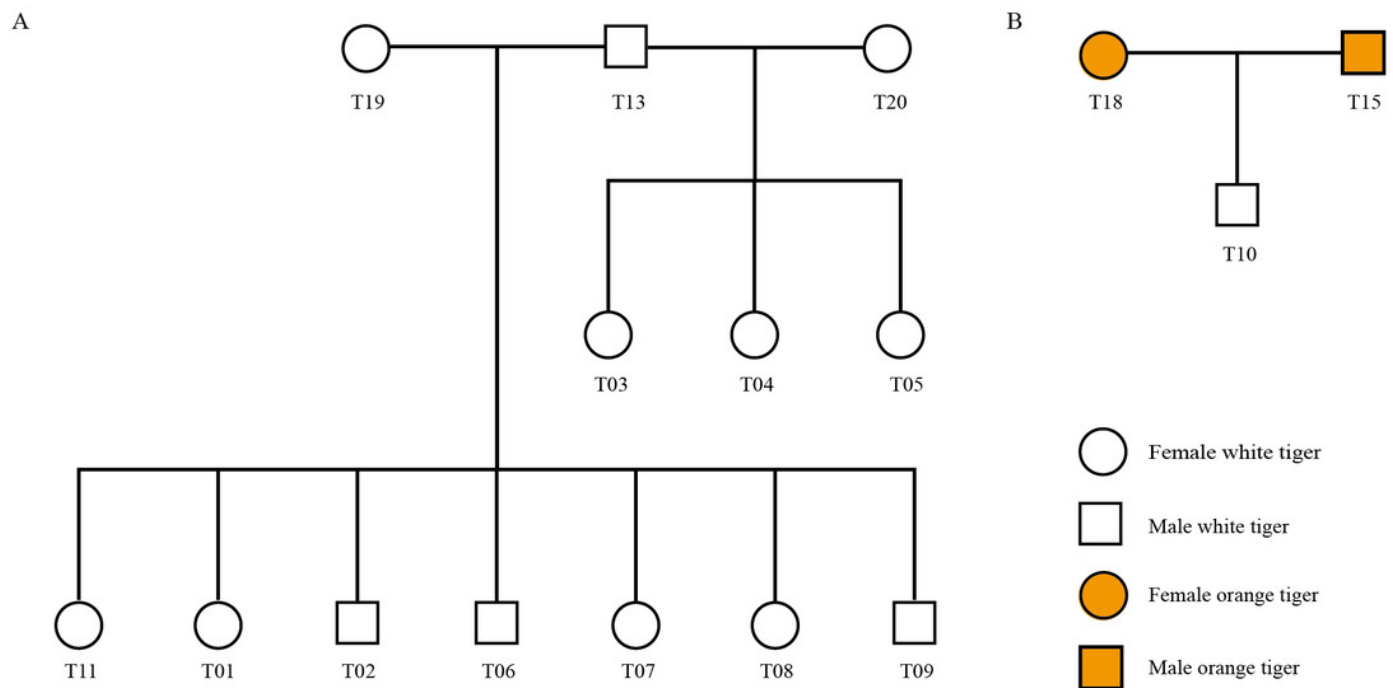



Figure 3

Pedigree tree of the identified tigers.

The information of selected loci in the multiplex system and primer sequences of each locus.

1   **Table 1:**

2   **The information of selected loci in the multiplex system and primer sequences of each**

3   **locus.**

Locus	Scaffold	Position on scaffold	Repeat motif	Size range (bp)	Dye	Primer sequences (5'-3')
TAMEL	-	-	-	80-115	6'-FAM	GAGAGGCCAARTAGGAGTGTGC TTCAAGATGTTTCTCAGTCC
DA3S1123	scaffold 94140	28202	AAAAC	206-276	6'-FAM	ATGTATGTCTCCTGCACATGCTTCCAC CTGTCATGGATAATGTGCTTGAGTCCC T
DA2S1059	scaffold 67413	66413	AGAT	332-380	6'-FAM	CCTTGTACGAAAACAGGCAGTAAGCC A AGGCAAGACATTCACTTCTTAGGCA
DB1S1259	scaffold 87533	4877	AAAG	388-448	6'-FAM	TCCTCTTCTGGTGGGAACTTCTC ATTTTGCAATTTCTTAGCATAGTGGC
DD3S86	scaffold 62023	14317	AAAG	157-205	HEX	TCCTTTTGGGATTTAGTACACCAG ATTTTAAAGTCAACACCTGTA
DE1S613	scaffold 91793	6047	AAGG	222-270	HEX	GCCCACCACCTTCAAAATAGCCAT CCTCTTGACAGCCCTCCTTATACCTC
DF1S579	scaffold 79	17437	AAAG	307-355	HEX	CCCTTGCTTTTAATGAGGCATAACCTT GCTTCCCACTCCCTATGTATGGC
DA2S1575	scaffold 88063	10431	AAAAG	368-428	HEX	TATGCTTAGGTCTGCTCATCAAGGG GATGATCATAAGCGGAAATGCACGAG
DF2S497	scaffold 72035	19788	AAAG	132-178	TAMRA	CACTGGTATGTTTAAGGGAATGTCA CACATAGCACATCATCTTAACACA
DA3S1145	scaffold 74228	13337	AAAG	215-263	TAMRA	TGGGGTAGTCGTTATACAACCGAA CTTAACATCAGTGAGCCCAATGTGGA
DD2S793	scaffold 140970	2179	AGAT	317-365	TAMRA	AACATAAGTGGGAAGCTGGTATCTGA TCTCCCCTTAGCTGTTATGTGCAGT
DD4S705	scaffold 96585	34146	AAAG	377-425	TAMRA	GTTGCTAAGGCTCACCCATACAAA CAATAAAGAGCCCTTTGATCAGTCC
DB1S542	scaffold 96253	30364	AAGG	159-207	ROX	CGGTTCCTGTTACACTTCTTAGCCTT AGGTACAATAATATTACACGGAAAGC A
DA1S1290	scaffold 89346	24116	AAAG	212-272	ROX	CTGAGTCTTCAAGCTGGGTTATCACA ATACACAGCTTCTCAAATGCCATCC
DA1S1470	scaffold 74155	15379	AAAG	314-362	ROX	CACACACCACAGAGCACTAGGCA ATCTGGTCTGGGTCTTTTAACTCCT
DC1S1364	scaffold 80644	19735	AAAG	378-426	ROX	CTGCCATAATCCAGATGTGTAAACCA AACAAATCTCAAACATTTCGGCTCT

Alleles and allelic frequencies of the 15 microsatellite loci in the reference population (n=31).

1 **Table 2:**

2 **Alleles and allelic frequencies of the 15 microsatellite loci in the reference population (n=31).**

Locus		Alleles and allelic frequency									
DA3S1123	Allele	11.2	12.2	13.2	14.2	15.2					
	Frequency	0.2097	0.2581	0.4194	0.0161	0.0968					
DA2S1059	Allele	8	9	10	11	12					
	Frequency	0.4355	0.3065	0.1452	0.0323	0.0806					
DB1S1259	Allele	18.1	19.2	20.1	20.2	21.1	22.1	22.2	23.2	25.1	25.3	26.3
	Frequency	0.0484	0.1290	0.1452	0.0806	0.1129	0.1613	0.0484	0.0323	0.0484	0.0968	0.0968
DD3S86	Allele	13	14	15	16	17	18	19				
	Frequency	0.0323	0.4516	0.0484	0.0806	0.2097	0.0968	0.0806				
DE1S613	Allele	11	12	13.2	14.2	14.3	15.2	15.3	16.2	17	18	19.1
	Frequency	0.0968	0.0161	0.1613	0.0161	0.1129	0.0161	0.0806	0.0323	0.0161	0.1774	0.2741
DF1S579	Allele	9.1	11.1	12.1	13.1	14.1	15	16	17	18		
	Frequency	0.1935	0.1129	0.1774	0.1129	0.0968	0.1613	0.0323	0.0645	0.0484		
DA2S1575	Allele	7.1	8.1	9.1	10	11	12	13	14			
	Frequency	0.0161	0.1935	0.0806	0.1613	0.0968	0.3065	0.1129	0.0323			
DF2S497	Allele	10	11	12	13	14						
	Frequency	0.4516	0.2742	0.1613	0.0968	0.0161						
DA3S1145	Allele	15	16	17	18	19						
	Frequency	0.2258	0.3387	0.2097	0.0806	0.1452						
DD2S793	Allele	9	10	11	12	13						
	Frequency	0.1613	0.3226	0.2258	0.0645	0.2258						
DD4S705	Allele	17.3	18	18.2	19.2	19.3	20.2	20.3	22	22.2	22.3	24.3
	Frequency	0.1290	0.1452	0.0645	0.0645	0.1935	0.0645	0.1452	0.0968	0.0161	0.0323	0.0484
DB1S542	Allele	11	12	13	14	15	16					
	Frequency	0.0161	0.4355	0.0161	0.2742	0.1613	0.0968					

3 **Continued Table 2:**

Locus	Alleles and allelic frequency

DA1S1290	Allele	7	10	11	12	13	14	15	
	Frequency	0.0806	0.1774	0.0645	0.1935	0.2419	0.1129	0.1290	
DA1S1470	Allele	9	10	11	12	13	14	15	
	Frequency	0.3387	0.2258	0.0161	0.0484	0.1290	0.1774	0.0645	
DC1S1364	Allele	12.2	13.2	14	14.2	15.2	16.2	17.2	18.2
	Frequency	0.1613	0.0645	0.0161	0.0161	0.2097	0.3871	0.1129	0.0323

Characterization of 15 STR loci in the reference population (n=31).

a D:departure from HWE, P<0.05. b NS: not significant, P>0.05.

1 **Table 3:**

2 **Characterization of 15 STR loci in the reference population (n=31).**

Locus	No. of alleles	H_e	HWE	PE	CPE	DP	TDP
DA3S1123	5	0.7040	D[a]	0.4818	0.4818	0.8304	0.8304
DA2S1059	5	0.6878	D	0.4646	0.7226	0.8387	0.9726
DB1S1259	11	0.8902	D	0.7856	0.9405	0.9282	0.99804
DD3S86	7	0.7263	D	0.5385	0.9726	0.8637	0.999732
DE1S613	11	0.8366	NS[b]	0.6929	0.9916	0.9303	0.9999813
DF1S579	9	0.8626	D	0.7346	0.99776	0.9157	0.99999843
DA2S1575	8	0.8127	NS	0.6521	0.999222	0.9136	0.999999864
DF2S497	5	0.6852	NS	0.4622	0.999581	0.8262	0.9999999764
DA3S1145	5	0.7627	D	0.5648	0.999818	0.8533	0.99999999653
DD2S793	5	0.7638	NS	0.5640	0.9999206	0.8783	0.999999999578
DD4S705	11	0.8783	NS	0.7643	0.9999813	0.9417	0.9999999999754
DB1S542	6	0.6993	NS	0.4817	0.99999029	0.8345	0.99999999999593
DA1S1290	7	0.8325	NS	0.6804	0.99999690	0.9282	0.999999999999707
DA1S1470	7	0.7794	D	0.5971	0.99999875	0.8553	0.999999999999958
DC1S1364	8	0.7617	NS	0.5774	0.999999472	0.8741	0.999999999999995
	Mean: 7.3333	Mean: 0.7789		Mean: 0.6028		Mean: 0.8808	

3 [a] D:departure from HWE, $P<0.05$. [b] NS: not significant, $P>0.05$.