Validation of 17 microsatellite markers for parentage testing and individual identification in domestic yak (*Bos grunniens*)

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ABSTRACT

Background. Yak (*Bos grunniens*) is the most important domestic animal for people living at high altitudes, especially on the Qinghai-Tibetan Plateau. Yak ordinarily feed by grazing. Yak grazing has a strong impact on pedigree record accuracy because it is difficult to control mating in grazing yak. Polled yak are less dangerous to herdsman than horned yak. For this reason, polled yak breeding is preferred by herdsmen.

Methods. A standardized set of microsatellite loci was assessed and applied to parentage testing and individual identification in yak. Seventy-one microsatellite loci were selected from literature databases, ~~. M~~most of them ~~were~~ used for cattle. Thirty-five microsatellite loci generated excellent results from PCR amplification and were evaluated for parentage testing and individual identification in 236 unrelated polled yaks. Seventeen loci had polymorphic information content (PIC) > 0.5 and were in Hardy-Weinberg equilibrium without linkage disequilibrium. Of these 35 markers, the seventeen loci were used in a core set of multiplexed markers suitable for parentage testing and individual identification in polled yak.

Results. The multiplex PCRs and loading systems provided very high exclusion probabilities (EP) determined from combined core set genotypes. The followed combined exclusion probabilities were obtained: EP1 (exclusion probability for one candidate parent when one confirmed parental genotype is missing) = 0.99718116; EP2 (exclusion probability for one candidate parent when one confirmed parental genotype is available) = 0.99997381; EPP (exclusion probability for one alleged parental genotype pair is available) = 0.99999998. The combined EI (exclusion probability of finding two identical genotypes) and combined ESI (exclusion probability of finding the identity of two siblings) were > 0.99999999 and 0.99999899, respectively. The results of this analysis indicated that this combination of seventeen microsatellite markers could improve process efficiency, reliability, and utility in paternity testing and individual identification.

Discussion. Fluorescent detection primers can identify many microsatellite alleles that are applicable in polled yak population genetics analyses. Fluorescence labeling is useful for multiplex amplification and loading systems, decreases detection time, reduces testing costs,

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and generates copious amounts of microsatellite genotype data. Polymorphic microsatellite loci are suitable for parentage/relatedness analysis in other yak breeds with locus allele frequencies similar to those of the polled yak.

Keywords Polled yak, Microsatellites, Short tandem repeats, Simple sequence repeats, Forensic genetics, ~~Exclusion probabilities~~Identification, Parentage testing, - Conservation biology, Genetic diversity

INTRODUCTION

Yak (*Bos grunniens*), a member of the Bovidae, has successfully adapted to the severe cold and low oxygen levels characteristic of high altitude regions (~2,500-5,500 m) such as the Himalayas in south-central Asia, the Qinghai-Tibetan Plateau, Mongolia, and Russia (Wu 2016). These regions are known for their high elevations, pristine natural environments, and frequent seasonal variations (Haynes & Yang 2013; Ishizaki et al. 2005; Mizuno et al. 2015). The ability of yak to survive in such rugged natural environments is the result of numerous behavioral, physiological, and genetic adaptations (Barsila et al. 2014; Ding et al. 2015; Hu et al. 2012; Huang et al. 2012; Qiu et al. 2012; Wang et al. 2017b). For instance, yak have steady daily fasting heat production levels at constant ambient air temperatures even with increasing altitude (Ding et al. 2014; Han et al. 2003). Yak can thrive in an extreme environment such as the Tibetan Plateau where few other animals can survive. Yak have made significant contributions to human life in the Tibetan Plateau by providing meat, milk, fur, leather, transportation, and more (Hu et al. 2016; Lee et al. 2017; Medhammar et al. 2012; Wang et al. 2018).

The ~13 million domestic yak in China constitute ~90% of the global yak population. There are fourteen yak breeds in China, of which one is artificial (Wu 2016). Polled yak strain have been bred for many years at the foot of the Ashidan Mountain. Polling is very useful in herd management because it reduces the risk of horn-inflicted injury or death among the

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71 herdsman. ~~Polled yak breeding is very valuable in pasturing areas.~~ Accurate genealogical
72 records help estimate genetic parameters and improve breeding programs, and~~. They~~ ensure
73 efficient and effective breeding progress ~~and~~ avoiding excessive inbreeding. When yak graze,
74 however, mating is very difficult to control. Semen samples could be mislabeled in error
75 during preparation, and mating records might be confused as a result of clerical errors made
76 during artificial insemination. Therefore, accurate yak pedigree records compiled by paternity
77 testing and individual identification are essential in polled yak breeding.

78 Molecular markers ~~like microsatellite DNA~~can indicate the degree of genetic relatedness
79 between animals and facilitate paternity verification and individual recognition (Estoup et al.
80 2002; Zhang et al. 2006). Microsatellites, namely short tandem repeats (STR) or simple
81 sequence repeats (SSR), are tracts of repetitive DNA in which certain motifs ranging in length
82 from 1-10 nucleotides are repeated 5-50 times~~x~~ (Carneiro Vieira et al. 2016). Microsatellite
83 DNA can be used to develop pedigreed animal populations and evaluate animal breeding to
84 support genetic improvement by selective breeding (Weising et al. 1998). The application of
85 microsatellites as molecular markers for animal identification and parentage verification is
86 highly accurate and effective in both breeding~~routine~~ applications and forensic casework
87 (Budowle et al. 2005; Iyengar & Hadi 2014; Linacre et al. 2011).

88 ~~Recently, DNA~~ Analysis of microsatellite markers has been used to verify parentage in
89 breed registries and identify individual animals linked to a particular database or owner.
90 Microsatellite panels have been well characterized for cattle (Zhao et al. 2017), horses (Kang
91 et al. 2016), sheep (Rosa et al. 2013), dogs (Jeong et al. 2015) and parrots (Coetzer et al.
92 2017). Many highly polymorphic microsatellites have been identified, characterized, and
93 mapped for bovine breeds. Marker panels have also been developed (Carolino et al. 2009).
94 Microsatellite marker panels have been used to identify and assign individuals to families and
95 populations (Stevanovic et al. 2010). The application of microsatellites in the identification of
96 yak relationships has seldom been reported. Moreover, there are no reports on paternity
97 testing for yak breeds. Consequently, there is a strong requirement for yak identity control and
98 parentage verification.

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Anim Genet. 2005 Dec;36(6):484-9.
Application of bovine microsatellite markers for genetic diversity analysis of Swiss yak (Poephagus grunniens).
Nguyen TT1, Genini S, Ménétrey F, Malek M, Vögeli P, Goe MR, Stranzinger G.
Yi Chuan. 2013 Feb;35(2):175-84.
[Genetic diversity of DNA microsatellite for Tibetan Yak].
Li D1, Chai ZX, Ji QM, Zhang CF, Xin JW, Zhong JC.
Dorji, T. et al. (2002). Genetic diversity in Bhutanese yak (Bos grunniens) populations using microsatellite markers. Proceedings of the third international congress on yak, in Lhasa, China, 4-9 September 2000. International Livestock Research Institute (ILRI), Nairobi, pp. 197-201.
Han Jianlin et al. (2002). Low level of cattle introgression in yak populations from Bhutan and China: Evidences from Y-specific microsatellites and mitochondrial DNA markers. Proceedings of the third international congress on yak, in Lhasa, China, 4-9 September 2000. International Livestock Research Institute (ILRI), Nairobi, pp. 190-196.
Hanotte, O. et al. (2000). Cattle microsatellite markers for amplification of polymorphic loci in Asian Bovidae. In: Shrestha, J.N.B. (ed), Proceedings of the 4th global conference on conservation of domestic animal genetic resources held in Kathmandu, Nepal, 17-21 August 1998, pp. 47-49.
Hishida, O. et al. (1996). Cross-species amplification and polymorphism of microsatellite loci in Asian bovidae. Proceedings of the eighth AAAP Animal Science Congress, ...

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A highly informative set of DNA markers whose alleles can be easily scored is critical if it is to be used effectively in paternity testing and individual identification. The objective of the present study was to develop and evaluate multiplex microsatellite systems designed for the purposes of polled yak parentage control and kinship analyses. Many of these microsatellite loci are already commonly used to characterize genetic parameters for cattle. This study aimed to validate a set of highly polymorphic microsatellites for use in parentage testing and individual identification in polled yak. The results of this study can be used to develop powerful, efficient genetic tools for breeders to verify parentage verification and ~~match~~ individual identification.

~~yaks when their identities are ambiguous or missing.~~

MATERIALS AND METHODS

Marker selection and primer design

Microsatellites were selected from cattle breeding literature. The markers had to meet the following criteria: (a) high PIC and heterozygosity; (b) a large number of alleles; (c) no known null alleles; (d) homogeneous repeat motifs (Schnabel et al. 2000). A total of 71 bovine microsatellite markers were selected, of which 65 were derived from cattle references and 6 exclusively for yak (Li 2004). All 71 microsatellites and their flanking sequences were found on the cattle genome. Their corresponding sequences on the yak genome were sought. Primers for most of the loci used in earlier studies were adapted to the yak genome because of ~~mutations or~~ low scores. The following 14 loci did not need adjustment: BM1824, BM2113, BMS2533, ETH121, ETH225, ILSTS008, INRA124, RM099, INRA126, UMN0103, UMN0307, UMN0920, UMN2303, UMN3007, and UMN3008. The primers used in this study are listed in Table S1.

Sample collection

The polled yaks were selected from the herds on Ashidan Mountain in Qinghai province. To

Commented [WU14]: These outdated requirements are impossible to accommodate in practice (null alleles do always exist; they just must be infrequent; most STRs have heterogenous/complex structures) or redundant (PIC and H are correlated) ; moreover this reference still demands: «Loci non-syntenic or separated by more than 40 cM» (also preposterous) and «Allele size range.» (whatever it means).

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125 avoid consanguinity, only samples without genetic relationship were selected in pedigree.

126 Blood was drawn from the jugular veins of 236 unrelated individuals, including 38 sires and
127 198 dams. All yaks were handled in strict accordance with good animal practice according to
128 *the Animal Ethics Procedures and Guidelines of the People's Republic of China*, and the study
129 was approved by *the Animal Administration and Ethics Committee of Lanzhou Institute of*
130 *Husbandry and Pharmaceutical Sciences of Chinese Academy of Agricultural Sciences*
131 (Permit No. SYXK-2016-0039). The blood was mixed with preservation buffer (1.5 mg mL^{-1}
132 EDTA and 137 mmol L^{-1} NaCl) at a 5:1 ratio. The blood was stored at -80 °C in an ultra-cold
133 freezer prior to DNA extraction.

134

135 **DNA extraction and quantification**

136 Genomic DNA was extracted from white blood cells digested with proteinase-K. The samples
137 were centrifuged at 5,000 g for 2 min. The clear aqueous layer was then transferred to a new
138 test tube and 0.5 mL of 10 mg mL^{-1} RNase A was added to it. The sample was then extracted
139 with phenol:chloroform:isoamyl alcohol (25:24:1) followed by chloroform. The DNA was
140 precipitated with ethanol then resuspended in 50 mL TE buffer (10 mM Tris-HCl and 1 mM
141 EDTA, pH 8.0). The extracted DNA was quantified using a NanoDrop 2000 fluorometer
142 (Thermo Fisher Scientific, Waltham. MA, USA). $OD_{260:280}$ = 1.7-1.9 and $OD_{260:230}$ = 2.0-2.2
143 (data not shown).

144

145 **Preliminary primer screening**

146 Unlabeled primer pairs (Table S1) were ordered for each microsatellite marker and sample
147 fragments were amplified. The PCRs were conducted in 20-µL aliquots consisting of 20-50
148 ng genomic DNA, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 2.0 mM of each
149 dNTP, 5 ng BSA, and 1.0 U *Taq*™ Hot Start Version polymerase (TaKaRa Bio Inc., Kusatsu,
150 Shiga, Japan). Primer concentrations ranged from 1.0-5.5 µM. The thermal cycle parameters
151 were 5 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 30 s at 55-58 °C (Table S1), and

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152 20 s at 72 ºC, then a final extension step of 5 min at 72 ºC in a Verity PCR Veriti 96-Well
153 Thermal Cycler (Applied Biosystems Corp., Foster City, CA, USA). After amplification, the
154 PCR products were visualized on 2% agarose gel (Gene Tech Co. Ltd., Chai Wan, Hong
155 Kong). A 5-µL sample was mixed with 1 µL loading dye (TaKaRa Bio Inc., Kusatsu, Shiga,
156 Japan). Electrophoresis was run for 35 min at 85 V and the samples were visualized with an
157 GelDoc™ XR+ gel imaging system (Bio-Rad Corp., Hercules, CA, USA).
158 The amplification fragments with high specificity, high amplification efficiency, and loci
159 that were either non-syntenic or separated by >10 cM (to avoid strong genetic linkage) (Table
160 S2) were manually cut out of the agarose gel and send to the biological companies for
161 sequencing. The samples were forwarded to Invitrogen (Carlsbad, CA, USA) and Thermo
162 Fisher Scientific (Waltham, MA, USA) without prior purification. Fragment analysis was
163 conducted on an ABI 3730xl automated sequencer (Applied Biosystems Corp., Foster City,
164 CA, USA). Only forward primers were used to sequence the targets. Repeat marker sequences
165 were revealed with Sanger sequencing (Invitrogen, Carlsbad, CA, USA, and Thermo Fisher
166 Scientific, Waltham, MA, USA).

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167

168 **Genetic information acquisition**

169 Confirmed primer sequences flanking microsatellite loci were synthesized with a fluorescent
170 label attached to the 5' end of each forward primer. The forward primers set was fluorescently
171 labeled with FAM™, HEX™, or TAMRA™ dyes (Thermo Fisher Scientific, Waltham, MA,
172 USA). Microsatellites were separately amplified by PCR to identify loci with high levels of
173 ~~allelic~~ polymorphism. The amplification systems and conditions were similar to those used
174 with the unlabeled primers described above.
175 After amplification, 1 µL amplified fragment mix was added to 0.5 µL loading buffer (blue
176 dextran, 50 mg mL^{-1}; EDTA, 25 mM) and 4 µL deionized formamide then denatured by
177 incubation for 5 min at 95 ºC. Then, 0.5 µL internal size standard (Thermo ABI 4322682;
178 Thermo Fisher Scientific, Waltham, MA, USA) was added to each sample. Fluorescently
179 labeled PCR products were identified by capillary electrophoresis (ABI3730xl Genetic

180 Analyzer, Applied Biosystems Corp., Foster City, CA, USA). The fluorescently labeled DNA
181 fragments were analyzed with GENESCAN v. 3.7 (Applied Biosystems Corp., Foster City,
182 CA, USA) then with GENOTYPER v. 3.7 NT (Applied Biosystems Corp., Foster City, CA,
183 USA) and automatically sorted according to the internal size standard. The numbers of the
184 microsatellite repeat motifs were calculated on the basis of the fragment lengths of the PCR
185 product (Table S3).

186

187 **Genetic information analysis**

188 Genotypic data were initially processed in Microsoft Office Excel 2007, manually checked
189 for errors, and transformed into other input files for various analyses. Performance
190 characteristics [observed heterozygosity (HO); expected heterozygosity (HE); polymorphism
191 information content (PIC); estimated null allele frequency (F(null)); Hardy-Weinberg
192 equilibrium (HWE); and genotypic disequilibrium] were measured with GENEPOP v. 4.6
193 (Raymond & Rousset 1995; Rousset 2008) and CERVUS v. 3.0.7 (Kalinowski et al. 2007;
194 Kalinowski et al. 2010; Slate et al. 2000).

195

196 **Multiplex PCR conditions**

197 Genotyping 236 yak for 35 loci (Table 1) produced a core set of 17 loci with high PICs (Table
198 2). Four multiplex PCRs were assembled, each of which contained four or five microsatellite
199 markers. A list of the primer sequences used in the multiplex appears in Table 2. The
200 multiplex PCR amplification was performed in 15-µL volumes per sample. Each of these
201 consisted of ~25 ng genomic DNA, 10 mM Tris-HCl (pH 8.3), 35 mM KCl, 1.8 mM $MgCl_2$,
202 5.0 mM of each dNTPs, and 2.5 U *Taq*™ Hot Start Version polymerase (TaKaRa Bio Inc.,
203 Kusatsu, Shiga, Japan). The primer concentrations are shown in Table 2. For PCR
204 amplification, a thermal cycler (Veriti 96-Well, Applied Biosystems Corp., Foster City, CA,
205 USA) was run at 95 ºC for 5 min followed by 25 cycles of 95 ºC for 30 s, 55-58 ºC for 30 s
206 and 72 ºC for 30 s, then 10 cycles of 95 ºC for 30 s, 53 ºC for 30 s, and 72 ºC for 30 s,

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finishing with a final extension at 72 °C for 5 min. Genetic typing of these microsatellite loci was performed in a manner similar to that described above.

Paternity test and individual identification

Different types of exclusion probability were determined with CERVUS v. 3.0.7. These included (a) EP1, the exclusion probability for one candidate parent when one confirmed parental genotype was missing; (b) EP2, the exclusion probability for one candidate parent given the genotype of a known parent of the opposite sex; (c) EPP, the exclusion probability for one candidate parent pair; (d) EI, the exclusion probability for the identity of two unrelated individuals; and (e) ESI, the exclusion probability of finding the identity of two siblings (Kalinowski et al. 2007; Kalinowski et al. 2010; Slate et al. 2000).

Three parental exclusion probabilities were calculated corresponding to different scenarios. EP1 assumes that genotypes are known for the offspring and one putative parent but not the other. EP2 assumes that genotypes are known for the offspring, one confirmed parent, and one putative parent (both parents genotyped). EPP assumes that genotypes are known for the offspring and one parent pair; it is the probability of a misattributed progeny (mismatched with both parents) from two genotyped parents (Jamieson & Taylor 1997).

RESULTS

Microsatellite loci genetic characteristics

The numbers of alleles, allele size ranges, HO, HE, PIC, F(null), and HWE for the 35 microsatellite loci for the polled yak are presented in Table 1. ~~A total of 214 alleles were identified in the polled yak population.~~ Significant ($P < 0.001$) heterozygote deficits were detected at loci BM2943, INRA035, and RM099 because they were monomorphic. For the 32 polymorphic loci left, the number of alleles per locus ranged from 3 (CSSM013 and CSSM033) to 12 (SPS115) and the PIC varied from 0.084 (MM12) to 0.815 (ILSTS028).

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Commented [WU22]: Monomorphic loci cannot show «heterozygote deficits»; HO=HE by definition.

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233 Four of the 32 polymorphic loci had PIC < 0.25 (low). Eleven of them had moderate PIC
234 values ranging from 0.25 to 0.50. The remaining 17 loci had high PIC (>0.50) (Table 1).
235 The 17 genetic markers with high PIC were reproducible, informative, and robust for
236 parentage testing and identification program. The numbers of alleles ranged from 5 to 12
237 within this subset of 17 polled yak loci. Heterozygosities ranged from 0.573 (POTCHA) to
238 0.806 (ILSTS028) with an overall average of 0.662. Deviations between observed and
239 estimated heterozygosity ranged from 0.003 (INRA005) to 0.103 (TGLA126). The 17
240 markers had an average PIC of 0.636 ranging from 0.529 (INRA005) to 0.815 (ILSTS028).
241 F(null) alleles were estimated to be in the range of -0.0218 for YAK08 to +0.0819 for
242 TGLA126 (Table 1).

243

244 **Hardy-Weinberg and genotypic disequilibrium tests**

245 Exclusion probabilities were calculated from the allele frequencies based on the underlying
246 assumptions of HWE. The HWE tests for the 17 microsatellite loci were not significant (P >
247 0.05). The 17 loci with high PIC (>0.5) were selected for yak paternity testing. Non-random
248 gamete association to form genotypes also influences the allele frequencies used to calculate
249 genotype frequencies. In natural populations, this effect probably occurs because of
250 population substructuring. Genotypic disequilibrium within the polled yak resulted in 136
251 comparisons. There were no interlocus disequilibria.

252

253 **Multiplex amplification and loading**

254 Multiplexes were organized such that four groups of four or five loci with high PIC were
255 co-amplified by PCR. Non-overlapping allele lengths were chosen in such a way that they
256 would have the same fluorescent color label. Typical fluorescence signals of detections for the
257 core 17 microsatellite loci are shown in Figure 1. Using the three available fluorescent colors,
258 we multiplex-loaded and scored two groups of eight or nine loci in each single gel lane.

259

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Commented [WU25]: A frequency estimate cannot be a negative value

Commented [WU26]: Misplaced; does not fit the heading. Moreover EP is a biased statistic for measuring information content; please use the standard matching probability or conversely discriminating power.

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Commented [WU29]: I do not understand - the number of pairwise combinations of 17 loci is 153.

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Parentage inference

The EP1 of the 17 markers ranged from 0.189 (BM720) to 0.503 (ILSTS028) and their average value was 0.285. The average EP2 was higher than that for the EP1. The average for all markers was 0.451 and the range was from 0.328 (INRA005) to 0.673 (ILSTS028). For all 17 markers, the combined EP1 and EP2 were 0.99718116 and 0.99997381, respectively. For the alleged parents, the combined EPP was 0.99999998. The combined EI and ESI were $>$ 0.99999999 and 0.99999899, respectively (Table 2). Therefore, the identity and parentage exclusion probabilities based on the 17 microsatellite loci were highly discriminant.

Commented [WU33]: That is a truism; if I understand correctly, your EPs, EP2 is always greater than EP1 by definition

Commented [WU34]: Same comment as above on statistics; the recommended statistical reporting for paternity/kinship is a likelihood ratio (never mentioned).

DISCUSSION

Previous studies implied that 3-4% of the losses in genetic progress were caused by 10% increases in pedigree errors per year in dairy breeding (Israel & Weller 2000). Nevertheless, other studies indicated that these dairy breeding pedigree errors have increased to 22% since the 1980s (Ron et al. 1996; Visscher et al. 2002). In fact, the yak pedigree error rate may be $>$ 22% since yak feed primarily by grazing. On the other hand, wrong insemination records caused by clerical- and insemination errors and by stain disappearance may also influence pedigree error rates. Therefore, it is necessary to identify and correct the pedigree through paternity testing and individual identification. These practices are essential for reliable breeding programs aimed at the genetic improvement of yak. Several reports have been published on the use of microsatellite markers for cattle identification (Sharma et al. 2015; Zhao et al. 2017), but the performance characteristics of a yak identification panel have not yet been characterized. Paternity testing in yak breeding would increase profitability by improving selective breeding program efficiency.

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Commented [WU36]: Same as above; please rephrase the two sentences into a single one.

Commented [WU37]: Rephrase omitting 22% and just state that grazing increases the difficulties in kinship attribution.

Commented [WU38]: Rephrase, avoiding repetition of 'character'- rooted words.

The method of choice for livestock identification and parentage verification has been based on microsatellites (Jan & Fumagalli 2016; Jeong et al. 2015; Wang et al. 2017a). For swine (*Sus scrofa*) and cattle (*Bos taurus*), single nucleotide polymorphisms (SNPs) have been applied in identification and parentage verification (Eggen 2012; Rohrer et al. 2007). A recent

287 study debated whether SNPs rather than microsatellites should be used for parentage
288 verification (Kaiser et al. 2017). Nevertheless, the interpretation of complex parentage cases
289 may require >700 SNP markers. The simplified international exchange of microsatellite
290 profiles involves only 32 genotypes as opposed to the 1,400 genotypes for SNPs (Strucken et
291 al. 2016). For these reasons, microsatellites are preferred over SNPs for parentage testing.
292 The cattle identification and parentage verification panel recommended by the ISAG
293 (International Society for Animal Genetics) consists mainly of dimeric microsatellite repeat
294 motifs. Similarly, all of the microsatellite markers applied in the present study were dimeric.
295 The various repeat patterns have both advantages and disadvantages. Dinucleotide
296 microsatellites may have mutations or stutter bands which can introduce allele interpretation
297 errors (Walsh et al. 1996). For trimeric-, tetrameric-, and pentameric repeat motifs, stutter
298 slippage efficiency during PCR is ~~even~~ lower (Gill et al. 2005), and ensures clear peak
299 discrimination. On the other hand, an appropriate mutation rate is beneficial for
300 multigenerational parentage verification. For trimeric-, tetrameric-, pentameric-, and
301 hexameric microsatellites, gaps during sequence variant visualization within repeat units
302 result in larger bin sizes (Gill et al. 2000). When sequence variants span too widely, however,
303 it becomes increasingly difficult to confine the microsatellite marker within the same
304 fluorochrome to a single multiplex PCR system. Multimeric repeats can also be compound.
305 The microsatellite markers used in the present study were all dimeric with homogeneous
306 repeat motifs, so the genotyping was unambiguous.
307 Microsatellite loci must have few null alleles, to be in Hardy-Weinberg equilibrium
308 ~~balance~~, and absence of gametic association (~~no~~ linkage disequilibrium)~~a~~ ~~if they are~~ to be
309 ~~effectively~~ used in ~~exclusion probability determinations for~~ paternity testing and individual
310 identification~~verification~~. Null alleles ~~are loci that~~ fail to amplify to detectable levels by PCR
311 because of primer binding site mutations and other errors (Dakin & Avise 2004; Kline et al.
312 2011). Null alleles frequencies are estimated by comparing the observed and expected
313 numbers of homozygotes for a locus (Dąbrowski et al. 2015; Dakin & Avise 2004). ~~There~~
314 ~~may be an increased risk of null alleles for certain markers.~~ This fact must be considered

Commented [WU39]: Reference? See AMORIM A, PEREIRA L (2005) Pros and cons in the use of SNPs in forensic kinship investigation: a comparative analysis with STRs. Forensic Sci Int. 150:17-21.

Commented [WU40]: In contradiction with ISFG recommendations (Linacre et al 2011)

Commented [WU41]: I see no advantage on di- over tetra. See below

Commented [WU42]: How?
In contradiction with the use of Exclusion based rationale.

Commented [WU43]: I have difficulties understanding and I do not see the impact on genotyping quality.

Commented [WU44]: rare

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315 when genotyping individual yak for paternity testing, . For example, when there is an apparent opposite homozygosity

316 between [illegible] parent and offspring [illegible] The estimated null allele frequencies were highest for HEL10 (F(Null)

317 = 0.703) and MGTG4B (F(Null)) = 0.163) in this study. Therefore, these loci were excluded

318 from the core set used in parental identification.

319 The formulae for estimating exclusion probabilities assume random mating and non-

320 random association among alleles and loci. The allele frequencies used in the calculation of

321 genotype frequencies and exclusion probabilities must be consistent with Hardy-Weinberg

322 assumptions. The 17 loci selected as core microsatellite markers were all in Hardy-Weinberg

323 equilibrium. We tested for linkage disequilibrium because linked loci are relatively ineffective

324 at determining paternity or individual exclusion and the yak locus locations on chromosomes

325 are unknown. Genotypic disequilibrium tests showed no consistent deviations in populations

326 not expected *a priori* to have them. None of the 17 loci was in disequilibrium, which means

327 that they could be used in polled yak exclusion probability (PE) determincalculations.

328 The microsatellites used in cattle parentage testing could not be applied to yak

329 identification directly because it was uncertain as to whether the primers used for cattle would

330 produce the desired results for yak. In addition, allelic frequencies differ between cattle and

331 yak. In the present study, the primers were designed according to yak gene sequences then

332 tested by PCR amplification and electrophoresis. There is a strong correlation between PIC

333 and exclusion probability. In addition, allelic frequencies differ between cattle and

334 yak; . Eeven among various cattle breeds, the PIC of the same microsatellites have different

335 values (Mao et al. 2008; Vohra et al. 2017), and the same applied to yak breeds (Zhang et al.

336 2008). For this reason, when determining the exclusion probabilities of parentage testing and

337 individual identification for a new breed, the allele frequencies and PIC should be calculated

338 *de novo*. In the current study, the polled yak population was repeatedly tested on the proposed

339 microsatellite markers. Since some markers have similar allele frequencies and PIC among

340 yak breeds, they are expected to generate accurate results for other yak populations.

341 In the present study, Microsatellite loci with high PIC (> 0.5) were selected for the

342 identification panels. Nevertheless, the PICs of these loci were still slightly lower than those

Commented [WU46]: The forensic standard would be 1%; for many other loci, the silent allele frequency estimate is over 10% and at least those should also be excluded.

Commented [WU47]: This is wrong: random association between alleles of different loci is assumed

Commented [WU48]: The first sentence is repeated here; rephrase globally.

Commented [WU49]: Can you provide the results of the tests for the 17 loci; maybe adding to table S5. Results of tests on LD also missing.

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343 used for cattle research (Stevanovic et al. 2010; Vohra et al. 2017). Therefore, a greater
344 number of microsatellite markers were selected for the present study, ~~. The~~a set of 17 STRs
345 proved~~microsatellites~~ sufficient~~ed~~ for PE determinations. The combined probabilities of
346 excluding a wrongly assigned sire were 99.718116%, 99.997381%, and 99.999998% for EP1,
347 EP2, and EPP, respectively. These results were similar to those reported for ~~multiplexing~~
348 ~~systems in~~ Angeln dairy cattle, ~~. They had~~with 16 microsatellites ~~markers~~ and nearly 99.9%
349 PE1 (Sanders et al. 2006).

350 We used several mapped cattle microsatellites to develop sets of yak loci apt for multiplex
351 PCR amplification and multiplex loading into a single gel lane~~. PCR-based methods, highly~~
352 ~~variable microsatellite loci, and fluorescent-based genotyping establish a new standard for~~
353 ~~parentage testing. Furthermore, multiplexing~~ reducinges human errors, ~~microsatellite~~ typing
354 costs and time. Nevertheless, selecting the markers comprising a universal panel depends on a
355 balance among the required panel accuracy, amplicon length, and ability to undergo a
356 successful multiplex reaction. Multiplex PCR amplifications are technically more difficult
357 than their single-locus counterparts. Multiplex amplifications are also less likely to transfer
358 across species than single-locus amplifications. There is also a relatively higher risk of
359 introducing genotyping errors with multiplex amplifications due to allelic drop-out or false
360 allele production (Taberlet & Luikart 1999). Many multiplex PCR and loading optimization
361 methods have been investigated for cattle paternity testing. Nevertheless, these methods
362 cannot be directly applied to yak identification. Therefore, we developed four multiplex
363 amplifications, each of which contained four or five loci and two multiplex loads each
364 containing eight or nine loci. They were run in two gel lanes. The main advantage of this
365 system is that the allele length ranges do not overlap within the same fluorochrome. Multiplex
366 primer sets to amplify 17 microsatellite markers and fluorescent dye technology combined
367 form a rapid and powerful yak parentage testing method.

368 We propose that a combination of 17 genetic markers could yield a polled yak panel with
369 enhanced processing efficiency, reliability, and utility. This system exploits the advancements
370 made in DNA fragment analysis technology. These markers combined with likelihood-based

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parentage testing will help improve breeding programs and accurately determine polled yak pedigrees. If some of the polled yaks identified by this system were not descendants of the expected breeding male yak, they would be eliminated from a breeding group by breeders to ensure genetic purity and improvement. On the other hand, when semen samples of high-grade male yak are mixed with those of other males, the detection system in this study could identify the semen using the blood sample of the high-grade male yak. Multiplex systems can also be used for the rapid assessment of breed population history, structure, and diversity, and for reconstructing relationships among breeds. These multiplexes may also work in other yak breeds with gene frequencies similar to that of the population tested in the present study.

CONCLUSIONS

The set of 17 microsatellite markers can be amplified in four multiplex PCR reaction systems and genotyped in two multiplex loading systems. The high variability displayed by these microsatellite loci demonstrates that highly precise genotyping panels could potentially be used in individual genotyping, parentage verification, and individual identification. The microsatellites ~~authenticated~~ reported in this study could also serve in studies of yak population structure, history, and diversity, providing ~~. They are important~~a resource~~s~~ for the genetic improvement of domestic yak.

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