



Sex identification based on AMEL gene PCR amplification from blue sheep (*Pseudois nayaur*) fecal DNA samples

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ABSTRACT. The use of noninvasive genetic sampling to identify the sex of wild animals is an extremely valuable and important tool in molecular ecology and wildlife conservation. Sex determination using the amelogenin gene has been conducted in many species because only a single pair of primers is required to amplify both X- and Y-linked alleles. However, this method has not been used in field research with the feces of wildlife. In this study, we applied this method to 222 fecal samples from wild blue sheep (*Pseudois nayaur*) using amelogenin primers (SE47/SE48) after testing the effectiveness of sex determination using tissue samples and fecal samples from blue sheep of known sex. We found this method to be highly reliable (80.2%) for blue sheep. Amelogenin can be used to identify the sex of wild animals using fecal samples.

Key words: Amelogenin; Fecal sample; Non-invasive genetic sample; Blue sheep; Polymerase chain reaction-based sex-typing

INTRODUCTION

Identifying the sex of free ranging wildlife is a critical component in molecular ecology and conservation genetics (Griffiths et al., 1998; Schliebe et al., 1999). This information is essential for understanding population dynamics, dispersal, habitat use, population structure, and behavior (Mossman and Waser, 1999; Woods et al., 1999; Lucchini et al., 2002; Pajares et al., 2007). Because of the elusive behavior and rarity or lack of sexual dimorphism in many wildlife species, sex determination is difficult (Taberlet et al., 1993; Rosel, 2003). Feces is a non-invasive sample that is easy to collect and contains a large amount of ecological information (Waits and Paetkau, 2005; Brinkman and Hundertmark, 2009). The development of techniques that utilize DNA collected through non-invasive sampling from wild animals, particularly feces, has created new opportunities for sex determination in wildlife (Reed et al., 1997; Ding et al., 1998; Oliveira and Duarte, 2013).

In mammals, the sex-determining *SRY* and *Zfx/y* genes (Griffiths and Tiwari, 1993; Taberlet et al., 1993) and the enamel protein gene for amelogenin (Chen et al., 1999; Yamauchi et al., 2000; Bradley et al., 2001; Ensminger and Hoffman, 2002; Di Fiore, 2005) have been used to identify sex. Of these, amelogenin (*AMEL*) requires a single pair of primers only to amplify both X- and Y-linked alleles and shows a relatively large difference in length between the X- and Y-chromosome fragments (Sullivan et al., 1993; Ennis and Gallagher, 1994; Yamauchi et al., 2000; Pfeiffer and Brenig, 2005).

Helan Mountain, China, forms a “continental island” for many wildlife species as it is separated from other pockets of suitable habitat by surrounding desert, cities, and rivers. Particularly, blue sheep (*Pseudois nayaur*) inhabiting this area comprise a special peripheral population with no immigration and emigration. The blue sheep population has increased in recent years, and how individuals will disperse to ensure that the population remains healthy and robust remains a significant issue in the management of this animal. Determining the sex of these wild animals is a central component of understanding their spatial ecology and population dynamics. In this study, we applied sex identification methods using the *AMEL* gene using fecal samples collected from blue sheep. This method is frequently used for bovid tissue samples, but rarely for fecal samples, and may present an efficient method of sex identification in this remote species.

MATERIAL AND METHODS

Samples

Tissue samples extracted from the fur of wild blue sheep that died of natural causes of known sex (N = 17) were used as positive controls and collected from Helan Mountain, Tibet, Qinghai, Gansu, and Xinjiang in 2011. Fecal samples of known sex (N = 3) were collected from the Shanghai Zoo in 2013 and used to test the effectiveness of the *AMEL* primers. Fecal samples (N = 222) from wild animals were also collected from Helan Mountain as part of an ongoing study of blue sheep. Sex identification was only performed for samples in which successful amplification of at least 1 microsatellite locus was achieved.

DNA extraction and polymerase chain reaction (PCR) amplification

We extracted DNA from feces using the E.Z.N.A.TM Stool DNA Kit (Omega Bio-

Tek, Norcross, GA, USA) according to manufacturer protocols. DNA was amplified via PCR using the primers SE47 (5'-CAGCCAAACCTCCCTCTGC-3') and SE48 (5'-CCCGCTTGGTCTTGTCTGTTGC-3') as described previously (Ennis and Gallagher, 1994). We amplified the amelogenin segment from tissue and feces. According to this method, a male sample contains 2 bands in the agar gel, while a female sample contains 1 band. If 2 bands were found twice out of 6 runs, the fecal sample was regarded as being produced by a male; otherwise the sample was regarded as belonging to a female. PCR amplification was carried out in a total volume of 15 μ L consisting of 1.5 μ L DNA extract, 1.25 U Premix Ex Taq, 0.5 μ L 20 mg/mL bovine serum albumin (Takara, Otsu, Japan), and 10 μ M of each primer (SE47, SE48). The PCR protocol consisted of a denaturing step at 94°C for 5 min, followed by 35 cycles at 95°C for 30 s, 62°C for 30 s, and 72°C for 45 s. Final extension was performed at 72°C for 7 min. PCR products were separated by 2% agarose gel electrophoresis for 33 min and then photographed.

RESULTS

Sex identification using tissue samples

Every sample was scored as female or male based on the presence of amplification products of appropriate size by electrophoresis and all 17 samples of known sex were correctly typed (Table 1). PCR amplification in male and female animals consistently displayed a sex-specific band pattern showing length variations that were characteristic of *AMELX* and *AMELY* in all blue sheep analyzed (Figure 1).

Table 1. Sex identification using tissue samples from blue sheep of known sex.

Sample ID	Sample	Origin of sample	Known sex	Determined sex
HLS2	Tissue	Helan Mountain	M	M
HLS3	Tissue	Helan Mountain	F	F
HLS8	Tissue	Helan Mountain	F	F
HLS9	Tissue	Helan Mountain	M	M
HLS35	Tissue	Helan Mountain	M	M
HLS47	Tissue	Helan Mountain	M	M
G1	Tissue	Gansu	M	M
G3	Tissue	Gansu	M	M
LZ1	Tissue	Tibet Linzhi	M	M
NM1	Tissue	Tibet Namucuo	M	M
RT1	Tissue	Tibet Rkaze	F	F
QH1	Tissue	Qinghai	M	M
QH2	Tissue	Qinghai	M	M
XJ1	Tissue	Xinjiang	M	M
XJ2	Tissue	Xinjiang	M	M
SC18	Tissue	Sichuan	F	F
SC19	Tissue	Sichuan	M	M

We cloned the PCR products from the collected tissue samples and observed PCR fragments specific to males (263 and 218 base pairs, bp) and females (263 bp). There was no difference in this length across different blue sheep populations (e.g., *P. nayaur nayaur* in Tibet and *Pseudois nayaur szechuanensis* in Qinghai, Gansu, Xinxiang, Sichuan, and Ningxia). Male and female blue sheep differed by 10 bp; the 2 amplicons differed in size by 45 bp and were easily separated and visualized using bench-top procedures (Figure 2).

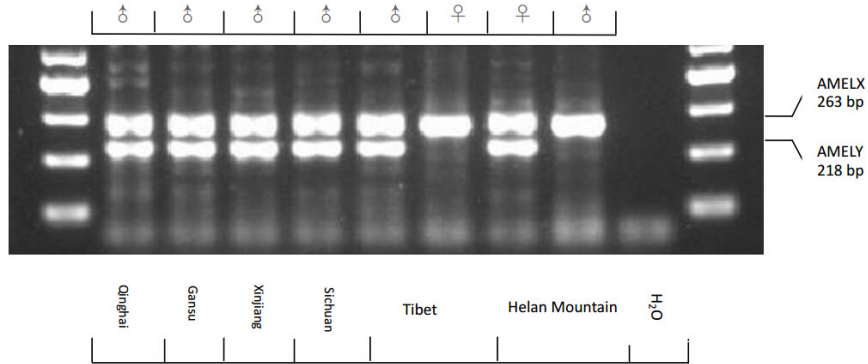


Figure 1. Sex identification based on amelogenin amplification (*AMELX* and *AMELY*) in blue sheep.

<i>Pseudola nayaur</i> (Gansu)_X	CCCCCTGGCTTGTCTGTCTGCTGGCCAAAGCTCCAGAGG	40
<i>Pseudola nayaur</i> (Qinghai)_X	CCCCCTGGCTTGTCTGTCTGCTGGCCAAAGCTCCAGAGG	40
<i>Pseudola nayaur</i> (Xinjiang)_X	CCCCCTGGCTTGTCTGTCTGCTGGCCAAAGCTCCAGAGG	40
<i>Pseudola nayaur</i> (Sichuan)_X	CCCCCTGGCTTGTCTGTCTGCTGGCCAAAGCTCCAGAGG	40
<i>Pseudola nayaur</i> (Tibet)_X	CCCCCTGGCTTGTCTGTCTGCTGGCCAAAGCTCCAGAGG	40
<i>Pseudola nayaur</i> (Hingxia)_X	CCCCCTGGCTTGTCTGTCTGCTGGCCAAAGCTCCAGAGG	40
<i>Pseudola nayaur</i> (Gansu)_Y	CCCCCTGGCTTGTCTGTCTGCTGGCCAAAGCTCCAGAGG	40
<i>Pseudola nayaur</i> (Qinghai)_Y	CCCCCTGGCTTGTCTGTCTGCTGGCCAAAGCTCCAGAGG	40
<i>Pseudola nayaur</i> (Xinjiang)_Y	CCCCCTGGCTTGTCTGTCTGCTGGCCAAAGCTCCAGAGG	40
<i>Pseudola nayaur</i> (Sichuan)_Y	CCCCCTGGCTTGTCTGTCTGCTGGCCAAAGCTCCAGAGG	40
<i>Pseudola nayaur</i> (Tibet)_Y	CCCCCTGGCTTGTCTGTCTGCTGGCCAAAGCTCCAGAGG	40
<i>Pseudola nayaur</i> (Hingxia)_Y	CCCCCTGGCTTGTCTGTCTGCTGGCCAAAGCTCCAGAGG	40
Consensus		
<i>Pseudola nayaur</i> (Gansu)_X	CAGTCAAGAACGATGAGGAGGAGGAGGTCATGGGAAAT	80
<i>Pseudola nayaur</i> (Qinghai)_X	CAGTCAAGAACGATGAGGAGGAGGAGGTCATGGGAAAT	80
<i>Pseudola nayaur</i> (Xinjiang)_X	CAGTCAAGAACGATGAGGAGGAGGAGGTCATGGGAAAT	80
<i>Pseudola nayaur</i> (Sichuan)_X	CAGTCAAGAACGATGAGGAGGAGGAGGTCATGGGAAAT	80
<i>Pseudola nayaur</i> (Tibet)_X	CAGTCAAGAACGATGAGGAGGAGGAGGTCATGGGAAAT	80
<i>Pseudola nayaur</i> (Hingxia)_X	CAGTCAAGAACGATGAGGAGGAGGAGGTCATGGGAAAT	80
<i>Pseudola nayaur</i> (Gansu)_Y	CAGTCAAGAACGATGAGGAGGAGGAGGTCATGGGAAAT	80
<i>Pseudola nayaur</i> (Qinghai)_Y	CAGTCAAGAACGATGAGGAGGAGGAGGTCATGGGAAAT	80
<i>Pseudola nayaur</i> (Xinjiang)_Y	CAGTCAAGAACGATGAGGAGGAGGAGGTCATGGGAAAT	80
<i>Pseudola nayaur</i> (Sichuan)_Y	CAGTCAAGAACGATGAGGAGGAGGAGGTCATGGGAAAT	80
<i>Pseudola nayaur</i> (Tibet)_Y	CAGTCAAGAACGATGAGGAGGAGGAGGTCATGGGAAAT	80
<i>Pseudola nayaur</i> (Hingxia)_Y	CAGTCAAGAACGATGAGGAGGAGGAGGTCATGGGAAAT	80
Consensus	cagtcaggaaagca t agggagagagg tcatgggaaat	
<i>Pseudola nayaur</i> (Gansu)_X	ATGAGGACAGAGTGGCTGTGGGACCAAGGCTGGATGG	120
<i>Pseudola nayaur</i> (Qinghai)_X	ATGAGGACAGAGTGGCTGTGGGACCAAGGCTGGATGG	120
<i>Pseudola nayaur</i> (Xinjiang)_X	ATGAGGACAGAGTGGCTGTGGGACCAAGGCTGGATGG	120
<i>Pseudola nayaur</i> (Sichuan)_X	ATGAGGACAGAGTGGCTGTGGGACCAAGGCTGGATGG	120
<i>Pseudola nayaur</i> (Tibet)_X	ATGAGGACAGAGTGGCTGTGGGACCAAGGCTGGATGG	120
<i>Pseudola nayaur</i> (Hingxia)_X	ATGAGGACAGAGTGGCTGTGGGACCAAGGCTGGATGG	120
<i>Pseudola nayaur</i> (Gansu)_Y	ATGAGGACAGAGTGGCTGTGGGACCAAGGCTGGATGG	120
<i>Pseudola nayaur</i> (Qinghai)_Y	ATGAGGACAGAGTGGCTGTGGGACCAAGGCTGGATGG	120
<i>Pseudola nayaur</i> (Xinjiang)_Y	ATGAGGACAGAGTGGCTGTGGGACCAAGGCTGGATGG	120
<i>Pseudola nayaur</i> (Sichuan)_Y	ATGAGGACAGAGTGGCTGTGGGACCAAGGCTGGATGG	120
<i>Pseudola nayaur</i> (Tibet)_Y	ATGAGGACAGAGTGGCTGTGGGACCAAGGCTGGATGG	120
<i>Pseudola nayaur</i> (Hingxia)_Y	ATGAGGACAGAGTGGCTGTGGGACCAAGGCTGGATGG	120
Consensus	at g aggcagagtgctgtgg gacag gctggatgg	
<i>Pseudola nayaur</i> (Gansu)_X	GTTCACAGGCTGCTGGGCTCAGGGGTCAGAGGCTG	160
<i>Pseudola nayaur</i> (Qinghai)_X	GTTCACAGGCTGCTGGGCTCAGGGGTCAGAGGCTG	160
<i>Pseudola nayaur</i> (Xinjiang)_X	GTTCACAGGCTGCTGGGCTCAGGGGTCAGAGGCTG	160
<i>Pseudola nayaur</i> (Sichuan)_X	GTTCACAGGCTGCTGGGCTCAGGGGTCAGAGGCTG	160
<i>Pseudola nayaur</i> (Tibet)_X	GTTCACAGGCTGCTGGGCTCAGGGGTCAGAGGCTG	160
<i>Pseudola nayaur</i> (Hingxia)_X	GTTCACAGGCTGCTGGGCTCAGGGGTCAGAGGCTG	160
<i>Pseudola nayaur</i> (Gansu)_Y	GTTCACAGGCTGCTGGGCTCAGGGGTCAGAGGCTG	160
<i>Pseudola nayaur</i> (Qinghai)_Y	GTTCACAGGCTGCTGGGCTCAGGGGTCAGAGGCTG	160
<i>Pseudola nayaur</i> (Xinjiang)_Y	GTTCACAGGCTGCTGGGCTCAGGGGTCAGAGGCTG	160
<i>Pseudola nayaur</i> (Sichuan)_Y	GTTCACAGGCTGCTGGGCTCAGGGGTCAGAGGCTG	160
<i>Pseudola nayaur</i> (Tibet)_Y	GTTCACAGGCTGCTGGGCTCAGGGGTCAGAGGCTG	160
<i>Pseudola nayaur</i> (Hingxia)_Y	GTTCACAGGCTGCTGGGCTCAGGGGTCAGAGGCTG	160
Consensus	gttcacaggctgctgggctcagggtcagaggctg	
<i>Pseudola nayaur</i> (Gansu)_X	CAGAGGCTGCTAGGCTCAGAGGCTCAGAGGCTGTTGA	200
<i>Pseudola nayaur</i> (Qinghai)_X	CAGAGGCTGCTAGGCTCAGAGGCTCAGAGGCTGTTGA	200
<i>Pseudola nayaur</i> (Xinjiang)_X	CAGAGGCTGCTAGGCTCAGAGGCTCAGAGGCTGTTGA	200
<i>Pseudola nayaur</i> (Sichuan)_X	CAGAGGCTGCTAGGCTCAGAGGCTCAGAGGCTGTTGA	200
<i>Pseudola nayaur</i> (Tibet)_X	CAGAGGCTGCTAGGCTCAGAGGCTCAGAGGCTGTTGA	200
<i>Pseudola nayaur</i> (Hingxia)_X	CAGAGGCTGCTAGGCTCAGAGGCTCAGAGGCTGTTGA	200
<i>Pseudola nayaur</i> (Gansu)_YGTGA	155
<i>Pseudola nayaur</i> (Qinghai)_YGTGA	155
<i>Pseudola nayaur</i> (Xinjiang)_YGTGA	155
<i>Pseudola nayaur</i> (Sichuan)_YGTGA	155
<i>Pseudola nayaur</i> (Tibet)_YGTGA	155
<i>Pseudola nayaur</i> (Hingxia)_YGTGA	155
Consensus	gtga	
<i>Pseudola nayaur</i> (Gansu)_X	GGCTGGCTGGATGGCTGGGCTGGAAGGCTCTGGG	240
<i>Pseudola nayaur</i> (Qinghai)_X	GGCTGGCTGGATGGCTGGGCTGGAAGGCTCTGGG	240
<i>Pseudola nayaur</i> (Xinjiang)_X	GGCTGGCTGGATGGCTGGGCTGGAAGGCTCTGGG	240
<i>Pseudola nayaur</i> (Sichuan)_X	GGCTGGCTGGATGGCTGGGCTGGAAGGCTCTGGG	240
<i>Pseudola nayaur</i> (Tibet)_X	GGCTGGCTGGATGGCTGGGCTGGAAGGCTCTGGG	240
<i>Pseudola nayaur</i> (Hingxia)_X	GGCTGGCTGGATGGCTGGGCTGGAAGGCTCTGGG	240
<i>Pseudola nayaur</i> (Gansu)_Y	GGCTGGCTGGATGGCTGGGCTGGAAGGCTCTGGG	195
<i>Pseudola nayaur</i> (Qinghai)_Y	GGCTGGCTGGATGGCTGGGCTGGAAGGCTCTGGG	195
<i>Pseudola nayaur</i> (Xinjiang)_Y	GGCTGGCTGGATGGCTGGGCTGGAAGGCTCTGGG	195
<i>Pseudola nayaur</i> (Sichuan)_Y	GGCTGGCTGGATGGCTGGGCTGGAAGGCTCTGGG	195
<i>Pseudola nayaur</i> (Tibet)_Y	GGCTGGCTGGATGGCTGGGCTGGAAGGCTCTGGG	195
<i>Pseudola nayaur</i> (Hingxia)_Y	GGCTGGCTGGATGGCTGGGCTGGAAGGCTCTGGG	195
Consensus	ggctggctggatggctgggctggaaggctctggg	
<i>Pseudola nayaur</i> (Gansu)_X	CGGSCAAGGAGGATTTGGCTG	262
<i>Pseudola nayaur</i> (Qinghai)_X	CGGSCAAGGAGGATTTGGCTG	262
<i>Pseudola nayaur</i> (Xinjiang)_X	CGGSCAAGGAGGATTTGGCTG	262
<i>Pseudola nayaur</i> (Sichuan)_X	CGGSCAAGGAGGATTTGGCTG	262
<i>Pseudola nayaur</i> (Tibet)_X	CGGSCAAGGAGGATTTGGCTG	262
<i>Pseudola nayaur</i> (Hingxia)_X	CGGSCAAGGAGGATTTGGCTG	262
<i>Pseudola nayaur</i> (Gansu)_Y	CGGSCAAGGAGGATTTGGCTG	217
<i>Pseudola nayaur</i> (Qinghai)_Y	CGGSCAAGGAGGATTTGGCTG	217
<i>Pseudola nayaur</i> (Xinjiang)_Y	CGGSCAAGGAGGATTTGGCTG	217
<i>Pseudola nayaur</i> (Sichuan)_Y	CGGSCAAGGAGGATTTGGCTG	217
<i>Pseudola nayaur</i> (Tibet)_Y	CGGSCAAGGAGGATTTGGCTG	217
<i>Pseudola nayaur</i> (Hingxia)_Y	CGGSCAAGGAGGATTTGGCTG	217
Consensus	cggscaaggagagattggctg	

Figure 2. Alignment of female and male amelogenin sequences (*AMELX* and *AMELY*) from blue sheep inhabiting six areas. Sequence variations are indicated by the circles.

Sex identification using fecal samples

The results of sex identification using blue sheep fecal samples are shown in Table 2. We identified 98 samples as male and 124 samples as female. Based on these results, we determined the overall blue sheep sex ratio as 1.27:1 (♀:♂). The sex ratio of blue sheep inhabiting Lülugou as 1:1.82 (♀:♂), and in the remaining valley as 1.78:1 (♀:♂). Three fecal samples collected from the Shanghai Zoo (2 males and 1 female) were also successfully amplified from samples collected from the field via PCR using the *AMELX* and *AMELY* gene primers 6 times (Figure 3A and B).

Table 2. Sex identification using 222 fecal samples from blue sheep inhabiting Helan Mountain, Nigxia, China.

Location	Samples (N = 222)	Males (N = 98)	Females (N = 124)
Valley of Zihuagou	11	4	7
Valley of Gangou	12	3	9
Ridge between Zihuagou and Gangou	23	3	20
Valley of Gangoushui	61	24	37
Valley of Dananchi in Zizhuagou	53	24	29
Valley of branch road in Lvlugou	62	40	22

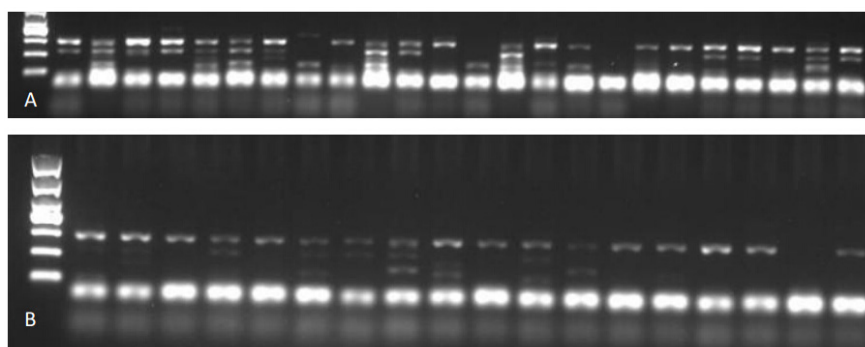


Figure 3. Blue sheep sex identification with *AMELX/AMELY* gene primers (SE-47, SE-48) fecal samples from Helan Mountain, Nigxia, (A) and fecal samples from Shanghai Zoo (B).

Although there were some non-specific bands or no bands present, these bands did not affect the results and thus the method used in this study was reliable.

Further, we analyzed the correct sex and the steady sex of tissue samples (from wild animals of known sex) and fecal samples (Shanghai Zoo animals of known sex); this data is presented in Table 3 and Figure 4.

Table 3. Correct sex and steady sex of all blue sheep samples.

Samples	Total number of samples	Correctly sexed (tested 6 times)	Steady sexed (tested 2 times)	Steady sexed (tested 4 times)	Steady sexed (tested 6 times)
Tissue	17	17	17	17	17
Fecal samples from Shanghai Zoo	3	3	3	3	2 (66.6%)
Fecal samples from Helan Mountain	222	NA	178 (80.2%)	80 (36%)	60 (27%)

Correct sex: the sample amounts of the sex identification correctly determined (known sex tissue samples and feces samples). Steady sex: the number of samples with the same result repeated many times.

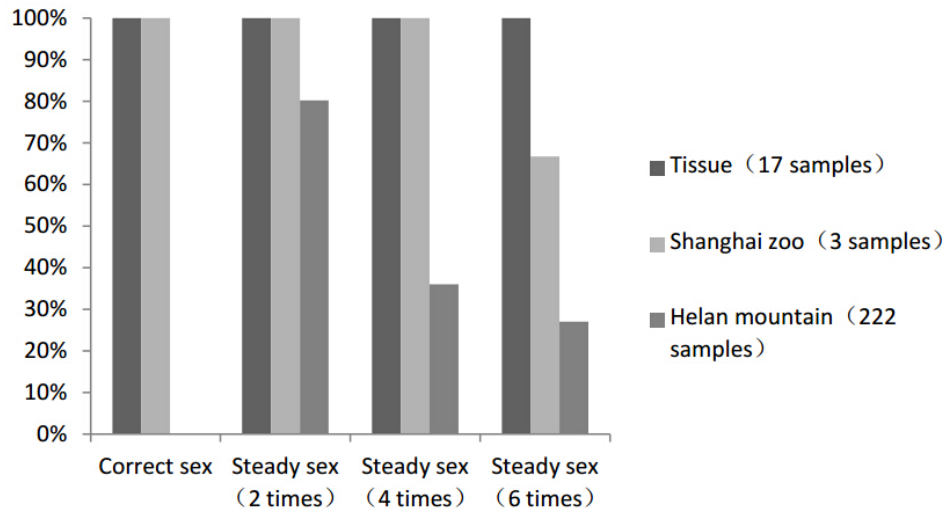


Figure 4. Correct sex and steady sex of all blue sheep samples in this study.

DISCUSSION

Under specific conditions, a lack of complementarity and allelic drop-out in low quality samples may lead to less efficient amplification of the Y chromosome fragment (Sipos et al., 2007; Bru et al., 2008; Gurgul et al., 2010) or generate non-specific products, resulting in misinterpretation of data. Other studies have shown that amplification of longer (>300 bp) fragments is problematic because of high amplification failure and allelic dropout (Buchan et al., 2005) when a small amount of DNA or a poor-quality template is used (Findlay and Quirke, 1996). This problem is more serious when using feces, hair, and other non-invasive samples. Methods based on fecal samples and *AMEL* circumvent this problem by using fresh samples and targeting much smaller regions for amplification. Importantly, the target fragments are short and are reliably amplified, even when using degraded DNA templates recovered from non-invasive samples.

The target fragments of the primers used in this study were short, and fecal samples collected from wildlife inhabiting Helan Mountain were as fresh as possible, improving the reliability of the results. The method tested here correctly attributed the sex of 100% of animals using tissue samples and therefore can be used for sex identification in blue sheep. We compared tissue samples across the 6 populations, and found no difference in the *AMEL* sequence across regions or between subspecies (*P. nayaur nayaur* and *P. nayaur szechuanensis*).

While fecal amplification using *AMEL* primers resulted in numerous extra bands, the target bands could be easily distinguished. Our accuracy rate of sex determination was 80.2% of samples over 2 trials, which is comparable to the success rate observed for the *SRY* primer (85%) (Yamazaki et al., 2011). Further, the sex ratio detected here (1.27 female:1 male) corresponded to the ratio found in other field studies of blue sheep inhabiting Helan Mountain (Wang et al., 1998; Cao, 2005; Liu et al., 2007). The sex ratio measured for animals inhabiting Lülugou ($\text{♀}:\text{♂} = 1:1.82$) and other valleys ($\text{♀}:\text{♂} = 1.78:1$) was consistent with the fact that males and females separate during the breeding season.

In conclusion, our assessment of the *AMEL* primer in blue sheep indicated that this method can be used to identify the sex of wild animals using fecal samples.

Conflicts of interest

The authors declare no conflict of interest.

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