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AMELX and AMELY Structure and Application for Sex Determination of Iranian Maral deer (*Cervus elaphus maral*)

Research Article

T. Farahvash[1], R. Vaez Torshizi[1*], A.A. Masoudi[1], H.R. Rezaei[2] and M. Tavallaei[3]

[1] Department of Animal Science, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran
[2] Department of Environmental Science, Faculty of Fisheries and Environmental Science, Gorgan University of Agricultural Science and Natural Recourses, Gorgan, Iran
[3] Human Genetic Research Center, Baqiyatallah University of Medical Science, Tehran, Iran

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*Correspondence E-mail: rasoult@modares.ac.ir

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ABSTRACT

In order to have a good perspective of wild animals, it is necessary to determine their population and genetic structure. It provides an opportunity to decide on better conservation managements. In the wilderness, due to the escapable nature and sometimes not having the distinguishable bisexual appearance, sex identification could be difficult by observing animals. The X- and Y- chromosome linked amelogenin (AMELX and AMELY) due to its independent and different evolution on both chromosomes could play an important role in sex determining of wild animals. To determine the sex ratio and also the genetic structure of AMELX and AMELY in Maral deer (*Cervus elaphus maral*), 37 samples were collected from populations were located in north parts of Iran. Results showed that in female deer, the amelogenin gene had one banding patterns (231 bp, for X chromosome) and the male deer had two banding pattern (231 bp and 180 bp for X and Y chromosomes, respectively). The AMELY of Maral had in/del mutation (54 bp). The genetic distance (D) of AMELX from Maral deer and Red deer was 0.12 ± 0.02, it was calculated zero for AMELY. The phylogenetic analysis of AMELX and AMELY of different deer species, showed no distance for AMELY and the D was 0.048 ± 0.009 for AMELX. It is recommended that sex determination of wild animals, especially mammalian populations using amelogenin gene would be a useful and simple method which could provide further information for genetic conservation strategies.

KEY WORDS amelogenin, *Cervus elaphus maral*, sex determination, wilderness.

INTRODUCTION

In order to genetic conservation of a population, it is necessary to have information on the structure and genetic diversity of the population (Yamazaki *et al.* 2011). Sex determination of the wild animal populations is an effective technique to evaluate the population structure and to decide on a useful conservation management and keep the population dynamic (Shaw *et al.* 2003). The main sample which is available from the wild animals is fecal that is collecting without knowing the sex of the animal. In this situation, genetic markers could be useful means for achieving genetic information of the populations. The result would help in gathering statistical and evolutional information to make the best conservation management decision (Carranza *et al.* 2009). Numerous molecular techniques have been improved in mammals for sex determination that some are based on polymerase chain reaction. For example, SRY locus on the Y chromosome had widely been applied in this manner (Matsubara *et al.* 2001). The main problem with

applying this marker is that a male individual would be distinguished only when the SRY locus was not amplified. However, this condition may also happen due to the experimental errors. So to solve the problem, another gene (mostly Cytb or an autosomal microsatellite marker) should be included in the experiment (Barbosa *et al*. 2009). The application of two pairs of primers would raise the cost and also make difficulty, since the annealing temperature and PCR protocol should be the same as the SRY gene (Takahashi *et al*. 1998). Considering this fact, a simple method that is able to recognize both X and Y chromosome at the same time is of great importance (Pilgrim *et al*. 2005). Amelogenin gene, in mammalians, is both X- and Y-chromosome linked (AMELX and AMELY, respectively) and this gene controls the development of the enamel. The conserved structure of this gene turns it into a useful marker for sex determination. This gene is conserved and has independent different evolution of X and Y chromosomes (Royo *et al*. 2007; Sullivan *et al*. 1993). Because of the in/del mutation in AMELY, two distinguishable bands with different sizes would be amplified on agarose gel (Pfeiffer and Brenig, 2005; Babo *et al*. 2002). Amelogenin was first used to sex identification of cow that was reported two different patterns of amplification: classI for X chromosome with 280 bp length in female cow and class I and class II for X and Y chromosome with 280 bp and 217 bp length in male animals, respectively (Ennis and Gallagher, 1994). The similar pattern of one and two amplified bands is reported for sheep (Pfeiffer and Brenig, 2005). The Cervidae has escapable nature of life style which results in difficulty in sex determination of deer from the appearance of animal, so the amelogenin gene could be an informative marker for this manner. Maral deer are a big game animal of Iran, which is suffering from decreased population size, abundance of natural habitats and illegal hunting that expose these animals to decline genetic diversity. Determining sex ratio of maral populations would provide additional information to decide on conservation management of these populations. This study has been conducted to evaluate the structure of amelogenin gene from maral deer, also to determine the sex ratio of some captive Maral deer populations of Iran using AMELX and AMELY.

MATERIALS AND METHODS

Sample collection and DNA extraction

A total of 37 samples, included tissue, fecal and blood samples were collected from East Azerbaijan (Aynali), Qazvin (Ziyaran and Barajin), Guilan, Gorghan (Ghorogh), Semnan (Parvar) and Mazandaran naturally reserved Maral populations. DNA was extracted by using Bioneer Dynabio Blood/Tissue DNA Extraction Mini Kit (Bioneer, South Corea) and AccuPrep Stool DNA Extraction Kit (Bioneer, South Corea).

Primers

The primers were the same as described by Ennis and Gallagher (1994):
SE47:5'-CAGCCAAACCTCCCTCTGC-3' and
SE48:5'-CCCGCTTGGTCTTGTCTGTTGC-3'.

Polymerase chain reaction

PCR reaction was carried out in a 25 µL mixture containing 12.5 µL Taq DNA polymerase 2X mix red Amplicon master mix, 1 µl of each external primers (5 pmol/µL) and 0.5 µL DNE template (5 ng/µL). Cycling was carried out under the following conditions, 95 ˚C for 15 min followed by 35 cycles of 95 ˚C for 30sec, 57 ˚C for 40 s, 72 ˚C for 30 s and the final extension of 5 min at 72 ˚C.

Sex determination of amplified samples

PCR products were run on 2% agarose gel and the sex of the animals was determined using the one and two banding patterns.

The Sequencing

20 µL of PCR products were sequenced (Macrogene Company, South Korea). The results were blast using blastn procedure of NCBI (http://www.ncbi.nlm.nih.gov/BLAST). The AMELX and AMELY sequences were trimmed with SEQSCAPE2.6. The genetic distance (D) was calculated by MEGA.6 software (Tamura *et al*. 2013) software. The polymorphic and parsimony informative sites were determined using DNAsp.51001 software. In order to phylogenetic analyzing of the data, the AMELX and AMELY sequences of Red deer, Sika deer, Follow deer, Roe deer and cow were obtained from NCBI (Table 1). The model parameters were calculated by the model test 2.1.10 software and phylogenetic analysis was carried out for AMELX and AMELY sequences using maximum likelihood method for MEGA.6.

RESULTS AND DISCUSSION

Sex determination using amelogenin amplification

The amplification of all the samples was successful. The sex of animals was determined by using 2% agarose gel by the following pattern: female animals: 1 band, 231 bp length and male animals: 2 bands: first, 231 bp and the second, 180 bp length (Figure 1).

Figure 1 shows that AMELY has two bands pattern. It is the consequence of an in/del mutation in this gene so it has two bands with different sizes, one with the very same of the X chromosome and the other with a shorter length.

Table 1 The AMELX and AMELY sequences of Red deer, Sika deer, follow deer, Roe deer and cow

No	Species	Name	Acc. No.	References
1	*Cervus elaphus*	Red deer	AY453391	Pfiffer and Brenig (2005)
2	*Cervus nippon*	Sika deer	AB028027	Yamauchi *et al.* (2000)
3	*Dama dama*	Follow deer	KJ542361	Nichols and Spong (2014)
4	*Capreolus capreolus*	Roe deer	KJ542360	Nichols and Spong (2014)
5	*Bos taurus*	Cow	EU569299	Pursak and Grzybowski (2008)

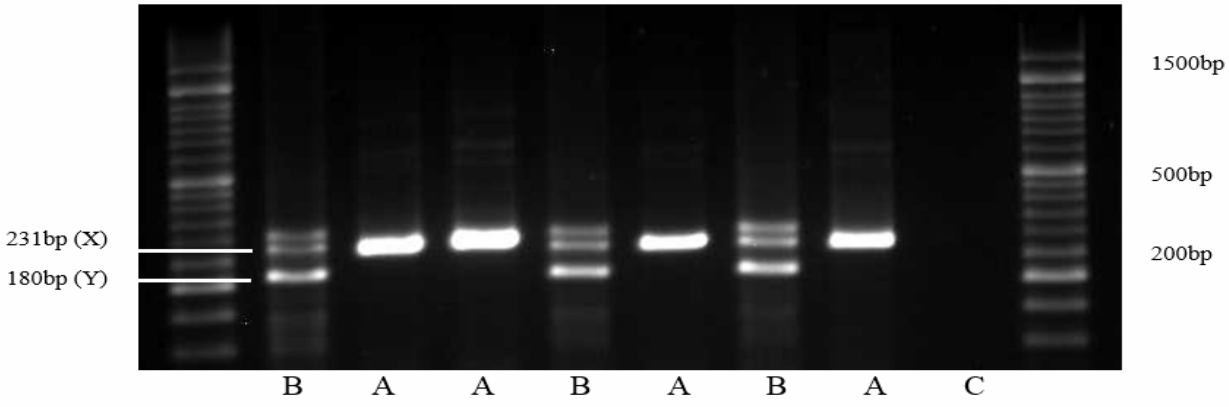

Figure 1 The results of amplification of amelogenin gene were used to determine the sex of maral deer
Female animals had 1 band (231 bp) and male animals had 2bands (231 bp for AMELX and 180 bp for AMELY) A: female animal; B: male animal and C: negative control

This pattern has been reported by other researchers in cow (Ennis and Gallagher, 1994), sheep (Pfeiffer and Brenig, 2005), Red deer (Gurgul *et al.* 2010; Pajares *et al.* 2007; Pfeiffer and Brenig, 2005) and Sika deer (Yamazaki *et al.* 2011; Yamauchi *et al.* 2000). This is the most important advantage of amelogenin gene for sex determination of wilderness. Because of this fact, there is a possibility to amplify two primers at the same tube and get reliable results with no need to test more primers. This method could be done in all no toothless mammalian species (Royo *et al.* 2007).

It should be noted that there is a third band in the male animals but it does not have influence on the sex determination.

Most researchers have been reported this third band and some suggested that it is likely due to poor amplification of poor samples especially fecal samples (Pfeiffer *et al.* 2005; Yamauchi *et al.* 2000). The results of sex determination of Maral deer naturally reserved populations are shown in Table 2.

Table 2 The results of sex determination of Iranian Maral deer populations

Location	Captive populations	No.	Male	Female
East Azerbaijan	Aynali	7	3	4
Qazvin	Ziyaran	5	-	5
Qazvin	Barajin	10	3	7
Guilan	-	5	2	3
Gorghan	Ghorogh	4	3	1
Semnan	Parvar	3	2	1
Mazandaran	-	3	-	3

The sequence results

The sequences of Maral AMELX and AMELY were as followed:

***Cervus elaphus maral* AMELX**

CAGCCCTTCCAGGCCCAGCCCATCCAGCCACAGCC
TCACCAACCCCTACAGCCCCAGTCACCTGTGCACC
CCATCCAGCCCTTGCCACCCCTGCAGCCCCTGTCA
CCTGTGCACCCCATCCAGCCCTTGCCCCCACAGCC
ACCTCTGCCTCCGATATTCCCCATGCAGCCTTTGCC
CCCTGTGCTTCCTGACCTGCCTCTGGAAGCTTGG-
CCAGCAACAGACAAGACCAAG

***Cervus elaphus maral* AMELY**

CAGCCCTTCCAGGCCCAGCCCATCCAGCCACAGCC
TCACCAACCCCTACAGCCCCAGTCACCTGTGCACC
CCATCCAGCCCTTGCCACCTCTGCCTCCGATATTCC
CCATGCAGCCTTTGCCCCCTGTGCTTCCTGACCTGC
CTCTGGAAGCTTGGCCAGCAACAGACAAGAC-
CAAGCGG

The nucleotide composition and protein sequences of AMELX and AMELY were calculated (Table 3). Sequences were blasted and they had 96%, 89%, 86%, 84% and 82% X homogeny and 92%, 82%, 83%, 70% and 83% Y homogeny with Red deer, Sika deer, Follow deer, Roe deer, sheep and cow, respectively. However, Pfeiffer and Brenig (2005) reported 97 and 96% X homogeny and 90 and 86% Y homogeny for sheep and Red deer with the original sequence of the cow, respectively.

Table 3 Nucleotide composition and protein sequences of Maral AMELX and AMELY

	Nucleotide composition (bp)				Protein sequence
AMELX	T(U)	C	A	G	QPFQAQPIQPQPHQPLQPQSPVHPIQPLPPLQPLSPVHPIQPLPPQPPLPPI FPMQPLPPVLPDLPLEAWPATDKTK
	16.9	48.1	18.6	16.5	
AMELY	T(U)	C	A	G	QPFQAQPIQPQPHQPLQPQSPVHPIQPLPPLPPIFPMQPLPPVLP-DLPLEAWPATDKTKR
	17.2	45.6	19.4	17.8	

Other study mentioned 91% and 87% AMELX and AMELY similarity between red deer and cow, respectively (Gurgul *et al*. 2010).

The length of AMELX and AMELY of Maral deer was determined and compared with the same sequences of other deer species and the original sequence of the cow. Results are summarized in Table 4. Maral deer had the same length of AMELX and AMELY with Red deer. The Sika deer had the shortest sequence of AMELX and AMELY. The comparison of AMELX and AMELY sequences from Maral deer showed that the Y had shorter sequence (54 bp) (Figure 4). This is the consequence of in/del mutation in Y-chromosome amelogenin (from site 90 to site 143) and it is the reason why there are two different banding patterns. Pfeiffer and Brenig (2005) mentioned 51 bp in/del in AMELY from Red deer, whereas Gurgul *et al*. (2010) reported 49 bp. It is reported 54 bp in Sika deer (Yamauchi *et al*. 2000) and the in/del mutation of the AMELY of sheep and cow was reported to be 68 bp and 72 bp, respectively (Pfeiffer and Brenig, 2005; Ennis and Gallagher, 1994).

The phylogenetic analyses

In order to compare amelogenin sequences of Maral deer and Red deer, the AMELX and AMELY sequences from Red deer were downloaded from NCBI and aligned with the same sequences of Maral deer. Calculated genetic distance (D) was 0.12 0.02 and 0.00 ± 0.00 for AMELX and AMELY, respectively. These amounts indicated that the diversity of amelogenin sequence of Maral deer and Red deer was low.

It confirmed that the amelogenin gene is has a conserved sequence. AMELX sequence was aligned between Maral deer, Red deer, Sika deer, Follow deer and Roe deer. There were 25 polymorphic sites with no parsimony informative sites. The protein sequences of AMELX from these groups were aligned and no specific differences were seen. The calculated D was 0.048 ± 0.009.

Sika deer had the shortest sequence (214 bp) in comparison with other deer species. The phylogenetic tree of AMELX was illustrated using maximum likelihood method (Figure 5).

Table 4 The length of AMELX and AMELY of Maral deer, Red deer, Sika deer, Follow deer, Roe deer and the original sequence of cow

Species	Name	AMELX (bp)	AMELY (bp)	References
Cervus elaphus maral	Maral deer	231	180	-
Cervus elaphus	Red deer	231	180	Pfiffer and Brenig (2005)
		255	205	Pfiffer and Brenig (2005)
Cervus nippon	Sika deer	221	167	Yamauchi *et al*. (2000)
		219	165	Yamazaki *et al*. (2011)
Dama dama	Follow deer	607	607	Nichols and Spong (2014)
Capreolus capreolus	Roe deer	608	608	Nichols and Spong (2014)
Bos taurus	Cow	280	217	Pursak and Grzybowski (2008)

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Cervus_elaphus_maralX CAG CCC TTC CAG GCC CAG CCC ATC CAG CCA CAG CCT CAC CAA CCC CTA CAG CCC CAG TCA CCT GTG CAC CCC ATC CAG  [ 78]
Cervus_elaphus_maralY CAG CCC TTC CAG GCC CAG CCC ATC CAG CCA CAG CCT CAC CAA CCC CTA CAG CCC CAG TCA CCT GTG CAC CCC ATC CAG  [ 78]

Cervus_elaphus_maralX CCC TTG CCA CCC CTG CAG CCC CTG TCA CCT GTG CAC CCC ATC CAG CCC TTG CCC CCA CAG CCA CCT CTG CCT CCG ATA  [156]
Cervus_elaphus_maralY CCC TTG CCA CC- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --T CTG CCT CCG ATA  [156]

Cervus_elaphus_maralX TTC CCC ATG CAG CCT TTG CCC CCT GTG CTT CCT GAC CTG CCT CTG GAA GCT TGG CCA GCA ACA GAC AAG ACC AAG ---  [234]
Cervus_elaphus_maralY TTC CCC ATG CAG CCT TTG CCC CCT GTG CTT CCT GAC CTG CCT CTG GAA GCT TGG CCA GCA ACA GAC AAG ACC AAG CGG  [234]
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Figure 4 The in/del position (54 bp) of AMELY in comparison with AMELX sequence

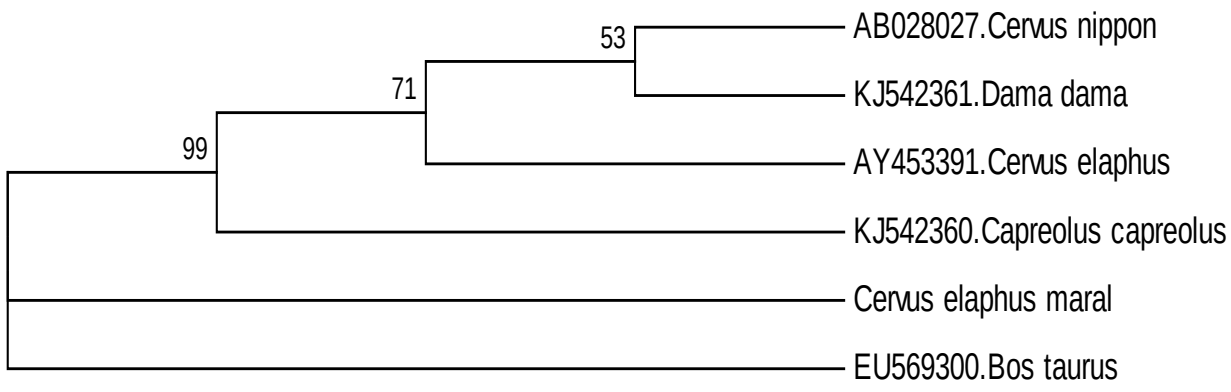

Figure 5 The phylogenetic tree of the AMELX. The phylogeny has been analyzed using Maral deer (*Cervus elaphus maral*), Red deer (*Cervus elaphus*), Sika deer (*Cervus nippon*), Follow deer (*Dama dama*) and Roe deer (*Capreolus capreolus*) AMELX sequences. *Bos taurus* was an out group and the tree has been analyzed using maximum likelihood method with HKY model and 1500 bootstrap

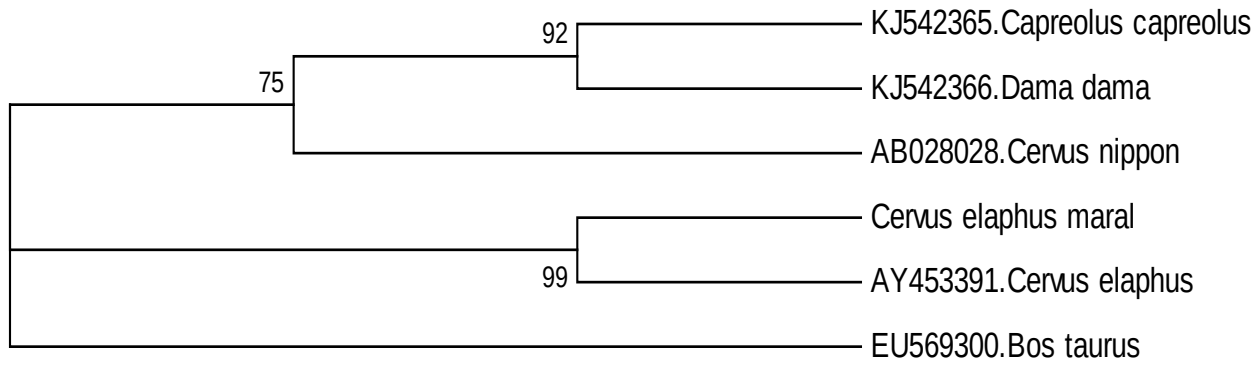

Figure 6 The phylogenetic tree for the AMELY. The phylogeny has been analyzed using Maral deer (*Cervus elaphus maral*), Red deer (*Cervus elaphus*), Sika deer (*Cervus nippon*), Follow deer (*Dama dama*) and Roe deer (*Capreolus capreolus*) sequences. *Bos taurus* was an out group and the tree has been analyzed using maximum likelihood method with Tajima-Nei model and 1500 bootstrap

The alignment of AMELY sequence from Maral deer, Red deer, Sika deer, Follow deer and Roe deer showed there were no any polymorphic sites. The protein coded by these sequences had no significant difference with the original sequence of the cow. Estimated D was 0.00. Figure 6 shows the phylogenetic relationship of AMELY sequences of deer populations. The results of phylogenetic analysis confirmed this fact that X- and Y- chromosome linked amelogenin have independent and different evolution.

CONCLUSION

Sex determination of wild animals is a useful method that would help to have a better conservation management of wilderness. Amelogenin gene due to its structure and different evolution of X- and Y- chromosomes linked amelogenin, could be a reliable molecular technique in sex identification and phylogenetic study of mammalian populations. The results of this study confirmed that AMELX and AMELY could be easily applied to determine the sex ratio of Iranian deer, especially Maral deer.

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