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 managements 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 evolotional information to make the best conservation management decisions (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: class I 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, Gorgan (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'-CCCGCTTGCTCTGGTCG-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 30 sec, 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 were determined using the one and two banding patterns.

**The Sequencing**
20 µL of PCR products were sequenced (Macrogen 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 (Tamura et al. 2013) software. The polymorphic and parsimony informative sites were determined using DNAsp.51001. In order to phylogenetic analyzing 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 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.
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>
<thead>
<tr>
<th>Location</th>
<th>Captive populations</th>
<th>No.</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>East Azerbaijan</td>
<td>Aynali</td>
<td>7</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Qazvin</td>
<td>Ziyaran</td>
<td>5</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>Qazvin</td>
<td>Barajin</td>
<td>10</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Guilan</td>
<td></td>
<td>5</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Gorgan</td>
<td>Ghorogh</td>
<td>4</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Semnan</td>
<td>Parvar</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Mazandaran</td>
<td></td>
<td>3</td>
<td>-</td>
<td>3</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>No</th>
<th>Species</th>
<th>Name</th>
<th>Acc. No.</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cervus elaphus</td>
<td>Red deer</td>
<td>AY453391</td>
<td>Pfeiffer and Brenig (2005)</td>
</tr>
<tr>
<td>2</td>
<td>Cervus nippon</td>
<td>Sika deer</td>
<td>AB028027</td>
<td>Yamauchi et al. (2000)</td>
</tr>
<tr>
<td>3</td>
<td>Dama dama</td>
<td>Follow deer</td>
<td>KJ42361</td>
<td>Nichols and Spong (2014)</td>
</tr>
<tr>
<td>4</td>
<td>Capreolus capreolus</td>
<td>Roe deer</td>
<td>KJ42360</td>
<td>Nichols and Spong (2014)</td>
</tr>
<tr>
<td>5</td>
<td>Bos taurus</td>
<td>Cow</td>
<td>EU569299</td>
<td>Pursak and Grzybowski (2008)</td>
</tr>
</tbody>
</table>

**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 2 bands (231 bp for AMELX and 180 bp for AMELY). A: female animal; B: male animal and C: negative control.

**The sequence results**

The sequences of Maral AMELX and AMELY were as followed:

*Cervus elaphus maral AMELX*

CAGCCCTTCCAGGCACGCCCATCCAGCCACAGCC
TCACCAACCCCCTACAGGCCACGGCACCTGTCGACCC
CCATCAGGCCCTTCGCCACCTGTCA
CCCTGACCTTTCTGCCACCTTGCACCC
CTCTGGTGCCTTCTCCAGTATCCCATGCCAGCTTTTGC
CCCTGACCTTTCTGCACCTTGCACCC
CCCTGACCTTTCTGCCACCTTGCACCC
CCCTGACCTTTCTGCCACCTTGCACCC

*Cervus elaphus maral AMELY*

CAGCCCTTCCAGGCACGCCCATCCAGCCACAGCC
TCACCAACCCCCTACAGGCCACGGCACCTGTCGACCC
CCATCAGGCCCTTCGCCACCTGTCA
CCCTGACCTTTCTGCCACCTTGCACCC
CTCTGGTGCCTTCTCCAGTATCCCATGCCAGCTTTTGC
CCCTGACCTTTCTGCCACCTTGCACCC
CCCTGACCTTTCTGCCACCTTGCACCC
CCCTGACCTTTCTGCCACCTTGCACCC

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 homology and 92%, 82%, 83%, 70% and 83% Y homology with Red deer, Sika deer, Follow deer, Roe deer, sheep and cow, respectively. However, Pfeiffer and Brenig (2005) reported 97% X homology and 96% Y homology for sheep and Red deer with the original sequence of the cow, respectively.
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 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

<table>
<thead>
<tr>
<th>Species</th>
<th>AMELX (bp)</th>
<th>AMELY (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cervus elaphus maral</td>
<td>231</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>Cervus elaphus</td>
<td>231</td>
<td>180</td>
<td>Pfeiffer and Brenig (2005)</td>
</tr>
<tr>
<td>Cervus nippon</td>
<td>255</td>
<td>205</td>
<td>Pfeiffer and Brenig (2005)</td>
</tr>
<tr>
<td>Dama dama</td>
<td>221</td>
<td>167</td>
<td>Yamauchi et al. (2000)</td>
</tr>
<tr>
<td>Capreolus capreolus</td>
<td>219</td>
<td>165</td>
<td>Yamauchi et al. (2000)</td>
</tr>
<tr>
<td>Bos taurus</td>
<td>607</td>
<td>607</td>
<td>Nichols and Spong (2014)</td>
</tr>
</tbody>
</table>

**Figure 4** The in/del position (54 bp) of AMELY in comparison with AMELX sequence
The alignment of AMELY sequence from Maral deer, Red deer, Sika deer, Follow deer and Roe deer showed there were not 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.

**ACKNOWLEDGEMENT**

This study has been conducted with the financial support of Tarbiat Modares University. The authors want to thank Department of Environment Islamic Republic of Iran, for the support and samples they were provided. We also very much welcome the facilities and technical support from Noor Human Genetic Research Center, Baqiyatallah University of Medical Science.

**REFERENCES**


