



## Rodent Amelogenin in *Akodon azarae* and *Lagostomus maximus*

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### ABSTRACT

The amelogenin gene, *AMEL*, encodes an important protein for tooth enamel formation during development. It is highly conserved among mammals. The *AMEL* gene, which is located in sex chromosomes in humans, exhibits a length polymorphism for the X and Y copies of the gene. This length polymorphism has been reported in several mammal species, and the differences between the X and Y chromosomes are used for sex determination by amplification of the polymorphic regions. In this work, we analyzed *AMEL* in *Akodon azarae* and *Lagostomus maximus*. In order to assess the identity of the individuals and verify the presence or absence of chromosomal rearrangements, a cytogenetic study was performed. To amplify the *AMEL* region, specific primers were designed using the genomic information of *Rattus norvegicus*, *Mus musculus* and humans. The sequenced PCR product confirmed the presence of the *AMEL* region in both species obtaining, for the first time, a partial sequence for the gene in the subject species. This sequence would be homologous to the amelogenin intron 3 in *R. norvegicus* and *M. musculus*. The future sequencing of the full length gene and the possibility to differentiate between X and Y in *A. azarae* and *L. maximus* is our next objective.

**Key Words:** Amelogenin, *AMELX*, *AMELY*, *Lagostomus*, *Akodon*

### RESUMEN

El gen amelogenina, *AMEL*, codifica para la formación de una proteína importante en la formación del esmalte dental durante el desarrollo y está altamente conservado entre los mamíferos. El gen *AMEL*, localizado en los cromosomas sexuales en humanos y otras especies, presenta un polimorfismo de longitud entre las copias del gen en el cromosoma X y en el cromosoma Y. Estas diferencias del gen *AMEL* entre el X y el Y se utilizan para la determinación del sexo mediante la amplificación de las regiones polimórficas. En este trabajo se analizó el gen *AMEL* en *Akodon azarae* y *Lagostomus maximus*. Mediante el estudio citogenético, se confirmó la identidad específica y se verificó la ausencia de arreglos cromosómicos en los individuos utilizados. Se diseñaron "primers" específicos destinados a amplificar la región del gen *AMEL* en base a la información del genoma de *Rattus norvegicus*, *Mus musculus* y humanos. Se obtuvo, por primera vez, una secuencia parcial para el gen *AMEL* en *A. azarae* y *L. maximus*. Esta secuencia sería homóloga al intrón 3 de *AMEL* en *R. norvegicus* o *M. musculus*. El estudio de la secuencia completa del gen en estas especies es un objetivo futuro para determinar la presencia de polimorfismos entre *AMELX* y *AMELY* y su posible utilización en la determinación del sexo.

**Palabras Claves:** Amelogenina, *AMELX*, *AMELY*, *Lagostomus*, *Akodon*

## INTRODUCTION

### *Amelogenin gene use on gender determination*

Karyotyping, Y antigen presence, X-linked enzymes and polymerase chain reaction (PCR) are used as methods of gender determination for mammals. The PCR is an accurate, reliable and sensitive technique that can be used successfully for sex determination and is based on *AMEL* length differences between the X and Y copies (Yamamoto et al., 2002; Phua et al., 2003; Pfeiffer and Brenig, 2005). The information of the *AMEL* sequence in mammals is important for its possible application in embryos sex determination by PCR, as well as for DNA analysis from animal remains (Ikawa et al., 2005).

Sex determination using *AMEL* has been performed in mammals such as *Sus scrofa* (pigs), Cetacea (whales) and Artiodactyla (ruminants). In pigs, two amelogenin genes located on the sex chromosomes have been described. In cetaceans and ruminants, a large insertion in the Y amelogenin locus has been discovered (specifically in exon 4), allowing its use as sexing method by PCR and providing a relevant use for phylogenetic inference (Ikawa et al., 2005; Macé and Crouau-Roy, 2008). *AMEL* is not useful for sex determination in *M. musculus*, since there is only one copy of the gene located on the X chromosome.

### *Amelogenin gene function*

Amelogenin is important for enamel matrix formation, hence it is important in human health (Snead et al., 1989). In humans, amelogenin is encoded by a single copy gene located in the short arm of the X and Y chromosomes. The product of this gene is a protein secreted by ameloblast cells and necessary for teeth enamel formation. Mutations in the *AMEL* gene are responsible for the “amelogenesis imperfecta” in humans, an inherited disease that severely affects the formation of teeth (Crawford et al., 2007).

### *Other findings on the amelogenin gene*

The *AMEL* locus in humans and other mammals is located in the Y and X non-recombining region (pseudautosomal boundary of sex chromosomes). Human male carries two copies of the *AMEL* (Xp22.1-p22.3 and near Y centromere). The Y copy (*AMELY*) is larger than the *AMELX* (the X copy contains a 6 bp deletion in the exon 1, Sullivan et al., 1993). The polymorphism has allowed the application of the amelogenin gene on human sex determination tests in forensic science and anthropology. A comprehensive study on *AMEL* and PAB (pseudautosomal boundary) in mammals can be found in studies conducted by Iwase and colleagues (2003).

The *AMEL* gene has been highly conserved during evolution and exhibits a high degree of homology among mammals (Sasaki and Shimokawa, 1995). Furthermore, homologous sequences to mammalian *AMEL* are present in amphibians, reptiles and monotremes (Toyosawa et al. 1998). The *AMEL* locus is an autosomal region that has been added to the sex chromosomes during the mammalian evolution after eutherians diverged from monotremes and marsupials. The amelogenin locus has been found in an “ancient pseudautosomal boundary” in humans, chimpanzees, *Saimiri*, *Otolemurs* and *Lemur catta*, as well as in primates and three other mammals: *Bos taurus*, *Sus scrofa* (pigs) and *Equus caballus* (Iwase et al., 2003). Recent findings on molecular evolution of amelogenin have determined that the dental proteins (including amelogenin) were dental-specific in at least the last toothed ancestor of modern birds (Delgado et al., 2005; Sire et al., 2005 and Sire et al., 2008). Besides amelogenin, RNA transcripts have been under the intensive effect of alternative splicing in rodents such as *Mus sp.* and *Rattus sp.*, in which the primary sequences are highly conserved (Bartlett et al., 2006; Morril and Rickords, 2008). According to the NCBI database, several research laboratories are currently studying the *AMELX* in 78 species belonging to 68 genera of 18 orders of mammals (including marsupials and Prototheria).

## MATERIALS AND METHODS

### *Specimens*

The animals studied were obtained from wild populations (Buenos Aires province, Argentina). The *Akodon azarae* specimens were trapped with sherman live traps at a field located at Diego Gaynor (34°16'60''S; 59°13'60''W) and *Lagostomus maximus* was obtained from the population of the Estación de Cría de Animales Silvestres, ECAS, Ministry of Agriculture, Villa Elisa (34°50'25''S; 58°05'51''W). Six *L. maximus* (five females and one male) and six *A. azarae* (four females and two males) were used in this work. The specimens were cytogenetically diagnosed and the chromosomal study was carried out by standard procedures. Genomic DNA was obtained from liver samples of each specimen. The animals were treated in accordance with international welfare standards (using resources available from the Canadian Council on Animal Care, <http://www.ccac.ca>).

### *Genomic DNA*

Genomic DNA was prepared from liver samples from *A. azarae* and *L. maximus* following standard procedures as follow. A piece of liver tissue was transferred to a cold 15 ml blue top tube and resuspended in 5 ml of salting out/lysis buffer (10 mM Tris-Cl pH 7.5, 400 mM NaCl, 2 mM EDTA pH 8). Then, 200 µl of 20% SDS and 300 µl of 5 mg/ml proteinase K were added. The samples were incubated at 50°C until tissue had been completely digested. The extraction and precipitation of the genomic DNA was performed with neutralized phenol/chloroform/isoamyl alcohol (25:24:1). After mix and spin, the upper phase was transferred to a new tube and precipitated after adding 3M NaCl with 2.5 vol. ethanol at room temperature. The total yield obtained was approximately 20-50 µg of DNA, 0.1-0.25 µg/µl (by adding 0.5-1 ml of 70% ethanol (-20°C)). The ethanol was removed and the DNA was let dry at room temperature, resuspen-

ded in TE buffer and incubated at 65°C for 15 minutes. The DNA was resuspended at a final concentration of 500 ng/µl.

### *PCR amplification and sequencing*

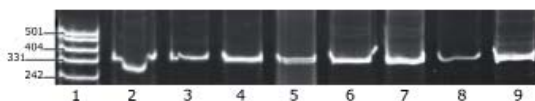
Sense and antisense primers were designed for amplifying the *AMEL* region. The nucleotide sequence of primers used for amelogenin region amplification in *A. azarae* and *L. maximus* DNA were 5'AGCTACCACCTCATCCT 3' (forward) and 5'TGCCCTATCATGGAGCCT 3' (reverse). The PCR reaction was performed in a 20 µl final volume following the basic protocol provided by the Taq Polymerase manufacturer (Invitrogen, USA; Cat N° 11615-010). The amplification was performed in a Thermal Cycler TC9600-G from Multigene (Labnet, Edison, NJ, USA). The initial denaturation at 94°C for 3 minutes was followed by 33 cycles: denaturation at 94°C for 45 seconds, primer annealing at 55°C for 30 seconds, an extension at 72°C for 1 minute and a final extension at 72°C for 10 minutes. The PCR products were identified by 12% acrylamide gel electrophoresis. Once the results from the amplification were confirmed by electrophoresis, the DNA was carefully purified and sequenced by Macrogen, Korea (<http://dna.macrogen.com>).

## RESULTS

The chromosomal study (Giemsa-stained chromosomes) showed a karyotype  $2n = 56$  ( $FNa = 106$ ) with a constriction in an autosomal pair for *Lagostomus maximus* and  $2n = 38$  ( $FNa = 38$ ) for *Akodon azarae*. These results confirm that our specimens belong to *L. maximus* and *A. azarae* and have no mutations or chromosomal rearrangements (translocations, inversions or numerical abnormalities such as polyploidy or aneuploidy). The *A. azarae* females studied in this work were XY\* (Bianchi and Contreras, 1967).

The products from the PCR amplification were analyzed by electrophoresis in 12% polyacrylamide gels from which fragments of 331

base pairs were excised and purified for sequencing purposes in each species (Figure 1). The sequences obtained are shown in Figure 2. These sequences were submitted to the GeneBank (NCBI). The accession numbers for the sequences of *A. azarae* are FJ001812 and FJ001810, while FJ001811 is the accession number for *L. maximus*. After searching the database using the Blast algorithm, we found that the amplified sequence might be homologous to the amelogenin gene intron 3 in *Rattus norvegicus* and *Mus musculus*, and the nucleotides were aligned with the human AMELX using the BLAST tool to perform sequence comparisons on GeneBank (NCBI, public domain; Altschul et al., 1997). The BLAST analysis showed that the two sequences of *A. azarae* presented a 93% of identity. There was a 92% of identity between the sequences of *A. azarae* and *L. maximus* and 2 gaps were found in a fragment (of 204 base pairs). Finally, there was a 75% and a 73% of identity between the human sequence and *A. azarae* and *L. maximus*, respectively (fragments ranging from 200 to 265 base pairs) (Figure 2). The high degree of similarity, shown by this analysis, was expected for several reasons, as it is discussed below.

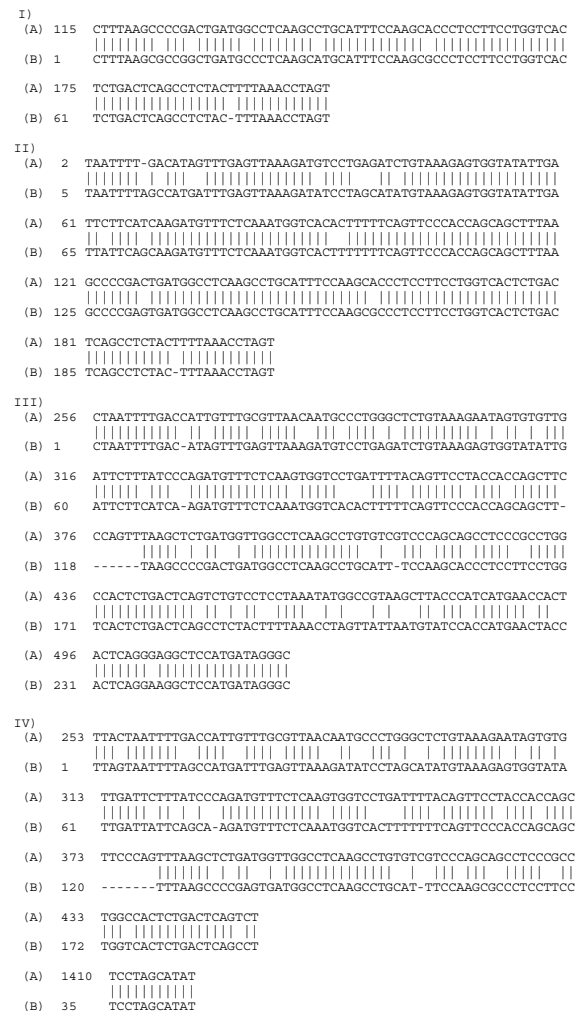


**FIGURE 1**

Electrophoresis on 12% acrilamida gel of PCR products showing the 331 base pairs used for sequencing from genomic DNA. Lanes 1-DNA ladder (AB pUC 19/MspI Marker DNA). 2- *Mus musculus* male; 3-*M. Musculus* female; 4- *Lagostomus maximus* male; 5-*L. maximus* female; 6- Human, male 7- Human, female; 8- *Akodon azarae*, male; 9- *A. azarae*, female.

## DISCUSSION

The chromosomal analysis allowed us to establish the absence of chromosomal mutations in the specimens analyzed. The published cytogenetics data of *Lagostomus* sp. and *Akodon* sp. support that our results, at chromosomal le-



**FIGURE 2**

Alignment and comparison by BLAST (NCBI) of sequences, AMELX, intron 3. I - *A. azarae* FJ001810 (A) and FJ001812 (B). Identities (i) 84/90 (93.3%); gaps 1/90 (1%). II - *A. azarae* FJ001810 (A) and *L. maximus* FJ001811 (B), (i) = 188/204 (92.16%); gaps 2/204 (1%). III- human, M55418 (A) and *A. azarae*, FJ001810 (B), (i) = 201/265 (75.85%); gaps 10/265 (3.8%). IV- human M55418 (A) and *L. maximus* FJ001811(B), (i) = 146/200 (73%), Gaps = 9/200 (4,5%). Notice a short segment with identities 100% and gaps 0%.

vel, are unambiguous (Bianchi and Contreras, 1967; Vidal et al., 1973). The works of Sire et al. (2005) and Delgado et al. (2005) have shown some aspects of the amelogenin gene in vertebrates. Our search on the NCBI data bases showed that amelogenin gene is the subject of analysis

in many mammals. The fact that the amelogenin gene is actually under intensive studies shows the importance of this gene for dentistry and for studies referred to sex determination in mammal species besides man. Furthermore, during the past few years, the *AMEL* research has allowed to determine the partial amelogenin sequence in 78 mammalian species. The species studied belong to 62 genera and 18 orders of mammals, of which only 9 species are rodents. The complete sequence is known in seven species, so far. This gene is being intensively studied probably due to the possible application to the determination of gender.

The high similarities found between the sequences compared (*Lagostomus*, *Akodon* and human) are consistent with the fact that a high degree of conservation was reported for the entire *AMEL* region. The amelogenin gene is located outside the pseudoautosomal region in sex chromosomes. The pseudoautosomal region is the only recombining region during male meiosis due to crossing-over (see Marais and Galtier, 2003). The *AMELY* is located in the non-recombining region of the Y chromosome and the *AMEX* is in a region with a low rate of recombination. This fact may explain, in part, the minimal variation found between the intron 3 of the species studied.

It has been established that X-linked genes in *Mus sp* fall into the same strata as orthologous genes in humans, which supports the evolutionary origin of X and Y chromosomes from an autosomal pair (Sandstedt and Tucker, 2004).

The issue whether the amelogenin gene is reliable for gender identification in *L. maximus* and *A. azarae* will be probably answered after a complete sequencing of the *AMEL* locus be obtained.

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