

# Evolution of the amelogenin gene in toothed and toothless vertebrates

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Amelogenin proteins constitute the major organic contents in forming enamel, but some previously published data indicate that the amelogenin gene could be present in several species lacking teeth or functional enamel. Therefore, amelogenin could have another, still unknown, function other than contributing to enamel formation. In order to test this hypothesis, the presence of this gene has been searched for in various vertebrates. We first compared the mammalian amelogenin sequences available in the literature to obtain the best chance of a successful detection of this gene in phylogenetically distant species. Using this analysis, we have shown that the occurrence of the amelogenin gene in the Y chromosome of primates and of an artiodactyl species is probably due to two independent duplications from genes on the X chromosome. Primers for PCR have therefore been synthesized and tested in eight species, five possessing teeth (human, two phylogenetically distant lizards, and two phylogenetically distant actinopterygians) and three lacking teeth (chicken and two phylogenetically distant turtles). The amelogenin gene has been detected in all species except those lacking teeth. This result indicates that the unique role of amelogenin in amniotes is to contribute to enamel formation.

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Amelogenins are the most important class of proteins in mammalian enamel as they represent approximately 90% of the proteins secreted by the ameloblasts into the extracellular matrix of forming enamel (1–3). The other enamel proteins are proteases and anionic proteins (known as non-amelogenins), some of which have been recently characterized, such as tuftelin (4), enamelin, and ameloblastin (also named amelin or sheathlin) (5, 6). During the maturation stage, the organic matrix of enamel is progressively degraded and removed, and mineral crystals are deposited perpendicular to the cell surface, forming the highly mineralized tissue that is enamel (7). The amelogenins are thought to play a role in the organization of the mineral by preventing random proliferation of crystal nuclei and by regulating growth kinetics, orientation and size of the formed enamel crystals (8, 9). The prismatic organization of the mineral crystals differentiates enamel from other superficial mineralized tissues (10), and this distinction has allowed paleontologists to recognize the typical

enamel-like structure in the dermal ornamentation of the oldest vertebrates, 460 million years old (11).

The amelogenin gene or the cDNA has been cloned in several mammalian species: human (12), mouse (13, 14), rat (15), pig (16), bovine (17), and opossum (18). The sequences already known are very similar, indicating a low substitution rate. This result is quite surprising because it is generally assumed that a low substitution rate indicates that the protein interacts with other different molecules. The high percentage of amelogenin proteins in forming enamel does not favor such interactions.

In eutherians (placental mammals), the amelogenin gene is shared on the heterologous part of the sex chromosomes, some species having the gene on both the X and Y sex chromosomes, and others only on the X chromosome (3). This gene is autosomal in prototherians (monotremes) and metatherians (marsupials) (19). The recently published analysis of phylogenetic relationships of amelogenin genes (18) appears to be confusing as the tree does not conform to either the phylogenetic

relationships of species (20) or to the understanding of the evolution of sex chromosomes (21, 22). However, this analysis (18) is flawed because the tree-generating algorithm used requires a constant molecular rate of evolution, which is probably not true for amelogenin because the third introns of the Y chromosome in primates accumulate mutations five times more rapidly than those of the X chromosome (23). Moreover, the central part of the amelogenin is composed of 9-base repeats (18), which are particularly difficult to align. Such repetitions are known to produce instability in DNA during the replication process or in interphase (24).

Other contradictory results have been obtained concerning the amelogenin gene. Since the study of HEROLD *et al.* (25), polyclonal and, later, monoclonal antibodies against amelogenin and non-amelogenin (enamelin) proteins of various mammals have been used to detect enamel-like proteins in the teeth of non-mammalian vertebrates, such as reptiles, lissamphibians, actinopterygians (ray-finned fish), chondrichthyans (sharks) (26–28), and even in the horny “teeth” of hagfish (27). The somewhat confusing analysis of the results has been clarified by two recent works. SLAVKIN & DIEKWISCH (29) have confirmed that the amelogenin gene is present in hagfish, and ZYLBERBERG *et al.* (30) have immunolocalized amelogenin-like proteins in the ganoine, which is an enamel (31) covering the scales in a polypterid (an actinopterygian). These results indicate that the amelogenin gene was probably present 460 Mya in the common ancestor of both lineages, among the jawless “craniates” *sensu* JANVIER (11). The possible presence of amelogenins in extant hagfish which lack true teeth and a dermal skeleton has led the authors to postulate that amelogenins could have (or could have had) another function than that known in mammalian tooth enamel (29). Moreover, WATSON *et al.* (19) have detected the amelogenin gene by Southern blot in the monotreme *Platypus*, a species which lacks teeth after birth and therefore has no selective constraint to maintain enamel. This could indicate either another function for this gene, or that teeth have been lost recently in this lineage. Unfortunately, the few known monotreme fossils cannot give more precise information about the possible moment of tooth loss in the monotreme lineage (between 110 and 5 Mya) (32). The presence of the amelogenin gene in birds (they lost their teeth 60 Mya) was suggested after intraocular graft experiments (33). Chick epithelium recombined with mouse molar mesenchyme was shown to be able to deposit an enamel-like matrix after ameloblasts had differentiated. However, the presence of the amelogenin gene in chicken has not been detected by Southern blot of genomic DNA (34).

Taken together, these results could indicate that amelogenins have had, or still have, another role than producing the organic matrix of enamel.

We have undertaken the present work to elucidate the relationship between the presence of the amelogenin gene and the presence or absence of enamel in various vertebrate groups. If the amelogenin gene is detected within a group which lost its teeth over 50 Mya, this detection could suggest another function for this gene. We have concentrated our efforts on sauropsids (e.g. turtles, lepidosaurs, crocodiles and birds), the sister group of synapsids (mammals), but we also have included two actinopterygian taxa as outgroups. Squamates have teeth covered with enamel similar to mammals in terms of mineral content (35), whereas turtles and birds lost their teeth respectively 200 and 60 Mya. We have first undertaken a phylogenetic analysis of the previously published mammalian sequences to design primers in the most conserved region of the gene. The quality of the alignment has been inferred from the coherence between the tree resulting from this alignment and the known mammalian phylogeny (20). Then, Southern blots of the PCR products hybridized with the mammalian probe have been used to detect the amelogenin gene in the studied species.

## Material and methods

### Re-analysis of previously published sequences

Previously published sequences of amelogenin were found in Genbank and EMBL databases or in previously published papers. Alignments of DNA sequences have been performed using Clustal X 1.4 (36) with further check by hand. The total length of alignment without introns and including gaps from ATG to Stop codon is 675 nucleotides. For further analysis, only one sequence per species was used, except for bovine and human, for which both the X and Y chromosome sequences were retained. Rat sequence was excluded because it is similar to the mouse one. The phylogenetic relationships of these genes were established using the Kimura two-parameter substitution rate method (37). The neighbor-joining method for tree construction (38) or maximum parsimony analysis (39) was used with 1000 bootstrap replicates for each method to ensure the significance of the trees. The trees were rooted at the divergence of metatherians and eutherians (20). The central part with repeats in exon 6 (18) was removed from the analysis because the molecular mechanisms generating them (slipped-strand mispairing, gene conversion or unequal crossing-over) (24, 40) do not permit differentiation between homology and homoplasy in alignment and will confound all the tree-

generating algorithms (41). Phylogeny of the third intron in primates (23) was also produced to elucidate the relationships between the amelogenin genes on the X and Y chromosomes, obtained in the amelogenin mammalian phylogeny.

The tree calculated by the neighbor-joining method represents the best tree obtained from the distance matrix computed according to the number of substitutions between all couples of species. With the maximum parsimony analysis it is possible to find among all the possible trees which one would require the lowest number of substitutions to explain the current sequences. For this analysis, only common derived state of characters between several taxa (i.e. apomorphic characters) are retained. Therefore, the common ancestral state of characters (i.e. plesiomorphic characters) is not taken into account to produce the regrouping of taxa. Both methods have limits, and the percentage obtained for each node by the bootstrap replicates is a measure of the statistical significance of the node.

#### Design of primers for PCR

Several primers have been constructed to cover various well-conserved gene regions defined by the alignment of the genes described above. The nucleotides of the five 3' end codons of the primers were degenerated to amplify all sequences exhibiting the same amino acid sequence as the mammalian consensus one. Each couple of primers was tested for primer dimer and specificity using the X sequence of human cosmid for this region with the Amplify 1.2 program (42). Only primers giving one clear potential amplification were retained. Four degenerate primers (20–23 nucleotides long; named hereafter A and B for direct primers and C and D for reverse ones) were finally retained and synthesized (Eurogentec, Seraing, Belgium). The sizes of putative amplifications for human gene X are A-C: 816 bp, A-D: 823 bp, B-C: 488 bp and B-D: 495 bp. Sequences of primers are (Y = C, T; R = A, G; N = A, T, G, C):

A: 5'-GTGCTTACCCCNVTNAARTG-3'

B: 5'-GTACGAACCNATGGGNGGNTGG-3'

C: 5'-CTTCCTCCCGYTTNGTYTTRT-3'

D: 5'-TTAATCCACYTCYTCNCGYTTNG-3'

#### Genomic DNA and PCR

Genomic DNA from a female human (*Homo sapiens*), two phylogenetically distant squamates (a lacertid lizard: *Gallotia stehlini* and the monitor lizard: *Varanus niloticus*), two phylogenetically dis-

tant turtles (the leatherback turtle: *Dermochelys coriacea* and the European freshwater turtle: *Emys orbicularis*), a polypterid (*Polypterus senegalus*), and the zebrafish (*Danio rerio*) were prepared from blood cells (43) followed by purification using phenol/chloroform (Bioprobe Systems Laboratories, Montreuil-sous-Bois, France) and ethanol precipitation (Merck, Darmstadt, Germany) (44). DNA from chicken (*Gallus domesticus*) was provided by Dr. Danièle Eusèbe (ENS, Montrouge, France). PCR was performed using LongExpand PCR kit (Boehringer Mannheim, Germany) with the preconized procedure (buffer 1), and products were separated on agarose (Bioprobe) gel (0.8%) using TBE buffer (Bioprobe) (44).

#### Southern blots of PCR products

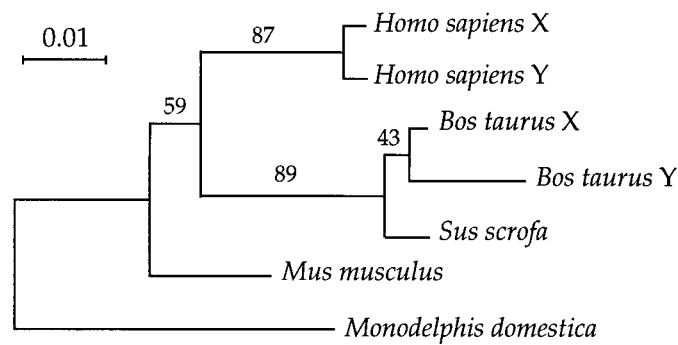
Fragments of human DNA PCR product obtained with primers A and C were labeled using digoxigenin by random priming (Boehringer). Ten  $\mu$ l of the products of each PCR were electrophoresed in 0.8% agarose gels, 20 cm long, 1 cm thick at 50 V for 14 h in TBE buffer. Gel-fractionated DNA samples (0.25 M HCl (Merck), 40 and 10 min; [1.5 M NaCl (Sigma-Aldrich, Saint-Quentin Fallavier, France), 0.5 M NaOH (Sigma-Aldrich)]; [H<sub>2</sub>O]; [10 mM TRIS-base (Sigma-Aldrich), 1.5 M NaCl (Sigma-Aldrich) pH 7.5]; each of them twice for 15 min) were transferred onto Nytran N membrane (Schleicher & Schuell Céra Labo, Ecquevilly, France) using Posiblot (Stratagene Ozyme, Montigny-le-Bretonneux, France) at 70 mm Hg for 30 min. The membrane was exposed at 365 nm, 120 mJ/cm<sup>2</sup> (Stratalinker 1800, Stratagene Ozyme) to crosslink DNA. Hybridization of 4 pmol of probe in 20 ml of hybridization solution and revelation were performed as described by the manufacturer and revealed using lumigen PPD (Boehringer). Molecular weight markers were revealed with methylene blue (Sigma-Aldrich) on membrane after transfer (45).

## Results and Discussion

#### Phylogenetic relationships of the mammalian amelogenin genes

The phylogenetic relationships obtained with the non-repetitive part of the amelogenin gene are different from what was expected according to the evolution of the sex chromosomes (for both the neighbor-joining and the maximum parsimony methods) (Fig. 1). The groups obtained using these methods are highly significant. All the eutherian X and Y chromosomes are homologous and have diverged in their actual form between the metatherian divergence and the first separation of the euther-

## A Neighbor-joining method



## B Parsimony analysis

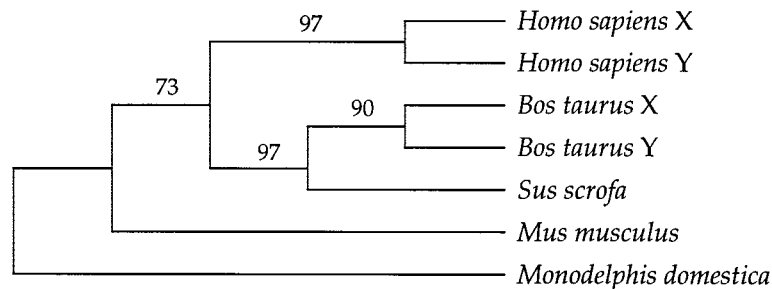


Fig. 1. Phylogeny of the mammalian amelogenin genes using (A) the neighbor-joining method (Kimura's two-parameter correction for multiple substitution and gap exclusion) and (B) the maximum parsimony method. The values at each node indicate the bootstrap values for 1000 replicates. The common names of species are: *H.s.*- human, *B.t.*- cow, *S.s.*- pig, *M.m.*- mouse and *M.d.*- opossum. The tree has been rooted at the divergence between marsupial and eutherian sequences.

ian lineages (21, 22, 46–48). Thus, we could expect to obtain a regrouping of both Y sequences on one side and all the X sequences on the other. Here, we obtain a regrouping coherent with mammalian phylogeny (20), but irrespective of the chromosomal origin of the gene. This tree is slightly different from that obtained when including a repeated part of the amelogenin gene (18) because here both human sequences are grouped together, whereas in the previously published analysis, the human X sequence was grouped with those of the Artiodactyla group. This new regrouping leads us to two hypotheses: either a gene conversion has homogenized X and Y sequences from the same organism (49), or independent duplications of this gene from X to Y chromosomes occurred both in primates and artiodactyls (18).

To discriminate between these two hypotheses, the phylogeny has been established taking into account the third intron of four species of primates (Fig. 2). The neighbor-joining method does not give two clear lineages for X and Y genes because the *Saimiri boliviensis* X intron is more closely related to *S. boliviensis* Y intron than to other X sequences. However, this regrouping is not well supported (bootstrap 44.3%) (Fig. 2A). Exhaustive

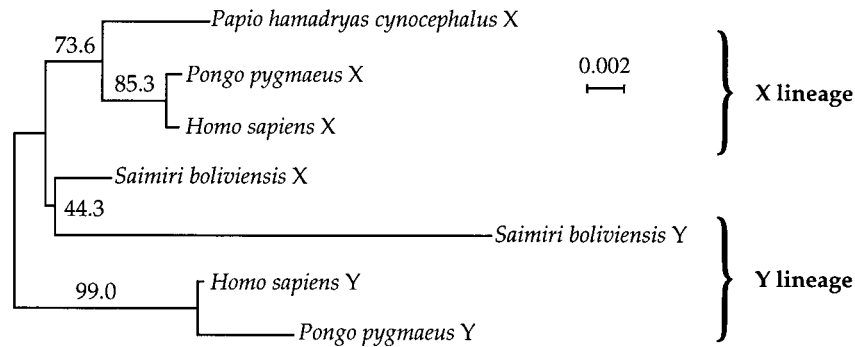
search for maximum parsimony gives only one tree with all nodes supported by 100% of bootstraps. In this tree, X and Y genes belong to two different lineages (Fig. 2B), implying that no exchange by gene conversion has occurred between them since the primate radiation. The regrouping of *S. boliviensis* sequences with the neighbor-joining method was in fact based on plesiomorphic characters retained in the *S. boliviensis* Y sequence compared to other two Y sequences. Gene conversion could also be detected if the same bases were present in X and Y sequences from one species and absent in all other sequences. No evidence for such an event is observed.

Thus, this result supports the hypothesis of two independent duplications from X to Y chromosomes in primate and artiodactyl groups as gene conversion within amelogenin appears to be a rare event if it exists at all. The sequence alignments appear to be congruent with the mammalian phylogeny and were used for primer design.

## Amelogenin gene detection

Four sets of PCR amplifications and Southern blots were produced. As the annealing temperature was

**A Neighbor-joining method**



**B Parsimony analysis**

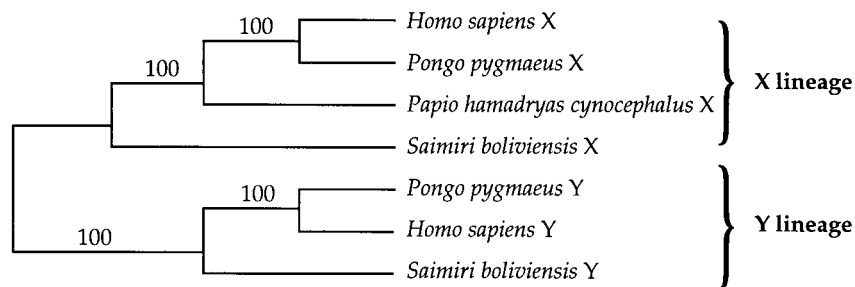


Fig. 2. Phylogeny of the third intron of the primate amelogenin genes using (A) the neighbor-joining method (Kimura’s two-parameter correction for multiple substitution and gap exclusion) and (B) the maximum parsimony method. The values at each node indicate the bootstrap values for 1000 replicates. The common names of species are: *H.s.*- human, *P.p.*- orangutan, *P.h.c.*- baboon, *S.b.*- squirrel monkey. Unrooted tree.

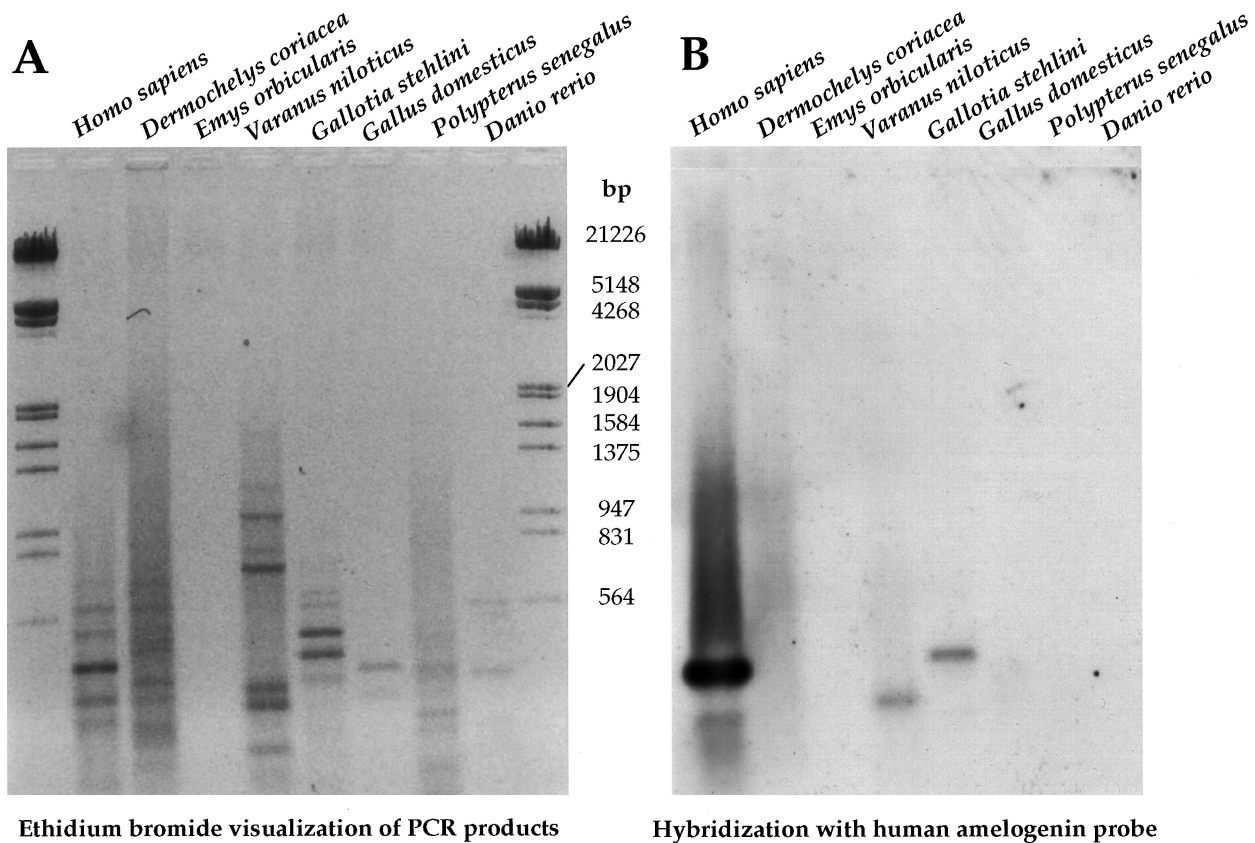
Table 1

Summary of the size of bands ( $\pm 20$  bp) detected as being amelogenin gene using PCR with four couples of primers. “NONE” indicates that a positive result was expected if the gene was present because the amelogenin gene is detected in more phylogenetically distant species using the same conditions

	Species	A-C	A-D	B-C	B-D
Human	<i>Homo sapiens</i>	816 bp	823 bp	488 pb	495 bp
Turtles	<i>Dermochelys coriacea</i>			NONE	NONE
	<i>Emys orbicularis</i>			NONE	NONE
Lizards	<i>Varanus niloticus</i>			417 pb	430 pb
	<i>Gallotia stehlini</i>			495 pb	514 pb
Chicken	<i>Gallus domesticus</i>			NONE	NONE
Actinopterygians	<i>Polypterus senegalus</i>				1151 pb
	<i>Danio rerio</i>				585 pb

relatively low, several bands were observed for each species (Fig. 3A for couple B-C). After hybridization with a human amelogenin probe, only one band (if any) is revealed per species (Fig. 3B for couple B-C), even after over-exposure (not shown). Each band corresponds to the amplification product of the amelogenin gene in the corresponding species. We can reject contamination during PCR since the size of each band is unique. The size variability probably results from changes in the number of repeats as already shown for mammals

(17). The results obtained with the various combinations of primers are summarized in Table 1. For each couple, positive control (human DNA) gives the required band. No specific amplification of the amelogenin gene, except for the human specimen, is detected with couples A-C and A-D. The primer couple B-C appears to amplify specifically the amniote sequences, except in turtle and chicken (both toothless amniotes). However, primer couple B-D permits the amplification (not shown) of all the sequences (including *Polypterus*



**Fig. 3.** PCR products obtained with B-C primer couple visualized using ethidium bromide after electrophoresis (A) and after Southern blot and hybridization with a human probe (B). See Table 1 for the estimates of the band sizes. The gray levels for image A have been inverted to obtain a better contrast. Positive bands after hybridization (B) are indicated by arrowheads in ethidium bromide gel (A).

and *Danio*), except again in turtle and chicken (Table 1).

In conclusion, the present study shows that we have obtained two couples of primers which theoretically could permit amplification and further detection of the amniote amelogenin gene. One of these couples has also permitted the amplification of the amelogenin gene in two actinopterygian species, proving the high conservation of the sites chosen for our primers. However, we have obtained no amplification/detection of the amelogenin gene in the three toothless taxa: the leatherback turtle, the European freshwater turtle and the chicken. The turtle species separated 150 Mya, and they lost their teeth approximately 200 Mya. For the chicken, our results confirm the previous finding obtained using Southern blot on genomic DNA (34). It is unlikely that we have failed to detect the amelogenin gene in the turtle and bird genomes because we were able to detect it in two actinopterygian species, a lineage which separated from the sauropsids 450 Mya. Thus, the amelogenin gene is probably absent in toothless sauropsids, a result which reinforces the idea that the unique role of amelogenin in amniotes is to contribute to enamel formation.

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