RESEARCH ARTICLE

Conservation of Amelogenin Gene Expression During Tetrapod Evolution

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ABSTRACT

Well studied in mammals, amelogenesis is less known at the molecular level in reptiles and amphibians. In the course of extensive studies of enamel matrix protein (EMP) evolution in tetrapods, we look for correlation between changes in protein sequences and temporospatial protein gene expression during amelogenesis, using an evo-devo approach. Our target is the major EMP, amelogenin (AMEL) that plays a crucial role in enamel structure. We focused here our attention to an amphibian, the salamander *Pleurodeles waltl*. RNAs were extracted from the lower jaws of a juvenile *P. waltl* and the complete *AMEL* sequence was obtained using PCR and RACE PCR. The alignment of *P. waltl* AMEL with other tetrapodan (frogs, reptiles and mammals) sequences revealed residue conservation in the N- and C-terminal regions, and a highly variable central region. Using sense and anti-sense probes synthetized from the *P. waltl AMEL* sequence, we performed in situ hybridization on sections during amelogenesis in larvae, juveniles, and adults. We demonstrated that (i) *AMEL* expression was always found to be restricted to ameloblasts, (ii) the expression pattern was conserved through ontogeny, even in larvae where enameloid is present in addition to enamel, and (iii) the processes are similar to those described in lizards and mammals. These findings indicate that high variations in the central region of *AMEL* have not modified its temporospatial expression during amelogenesis for 360 million years of tetrapod evolution. *J. Exp. Zool. (Mol. Dev. Evol.) 320B:200–209, 2013.* © 2013 Wiley Periodicals, Inc.

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Enamel formation consists in the deposition of an extracellular organic matrix, mostly composed of enamel matrix proteins (EMPs) that are progressively degraded by proteases while mineralization proceeds. This process results in a nearly protein-free, highly mineralized, and unique tissue. In living and extinct tetrapods (mammals, reptiles, and amphibians) the structural organization of enamel is typical and easily recognizable on ground sections observed in a light or scanning electron microscope, which means that enamel structure was already acquired in their last common ancestor, more than 360 million years ago (Ma) (Hedges, 2009). This leads us to postulate that the function of EMPs was also already acquired in early tetrapods, a hypothesis that is supported by the probable recruitment of EMPs in early vertebrates, long before tetrapod diversification (Sire

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et al., 2007). However, were the processes leading to enamel formation, that is, amelogenesis, kept temporospatially unchanged in the three tetrapod lineages during such long, separate evolutionary periods?

To answer this question we undertook evo-devo studies aiming to check whether or not EMP gene sequences have changed during tetrapod evolution and whether these changes correlate to variations in the temporospatial expression of EMPs during amelogenesis. Our main target is amelogenin (AMEL), the most abundant EMP (90% in forming bovine enamel: Fincham et al., '99) that plays an essential role as structural protein (Paine and Snead, 2005). In mammals, AMEL controls crystal deposition and hydroxyapatite crystallite organization into rods, and is essential for the development of normal crystallite size (Moradian-Oldak et al., 2003; Paine et al., 2003; Snead, 2003). In humans, mutations of *AMEL* result in a genetic disease, the X-linked *amelogenesis imperfecta*, which demonstrates the importance of AMEL for correct amelogenesis (Wright, 2006; Wright et al., 2011).

Evolutionary analyses of mammalian (Delgado et al., 2005a; Sire et al., 2005) and reptilian (Toyosawa et al., '98; Delgado et al., 2006) AMELs have revealed sequences of similar length, functionally important residues in the N-terminal (N-ter) and C-terminal (C-ter) regions, and a variable, central region characterized by numerous triplet repeats. The few amphibian AMELs known to date are shorter than the amniote sequences and possess also a variable central region (Diekwisch et al., 2009).

In rodents and humans, amelogenesis has been described in detail from morphogenesis to late differentiation stages, and at various levels of integration, including molecular biology (reviewed in Simmer et al., 2010). In contrast, in non-mammalian tetrapods, data on amelogenesis were mainly obtained at the cell and tissue level, and molecular data are restricted to a few studies in reptiles (Delgado et al., 2006; Diekwisch et al., 2009; Handrigan and Richman, 2011).

In a previous study we showed that the temporospatial expression of *AMEL* during lizard amelogenesis was similar to that described in mammals (Delgado et al., 2006). Here, we extended our evo-devo approach more than 100 Ma back in tetrapod evolution by studying, for the first time, *AMEL* expression during amelogenesis in an amphibian. We chose the salamander *Pleurodeles waltl*, a model species used in our laboratory for studying odontogenesis (Davit-Béal et al., 2006, 2007a, 2007b). In *P. waltl*, as in most non-mammalian vertebrates, teeth are continuously replaced during life. Each tooth that forms at a given position is called a tooth generation, and all generations constitute a tooth family. The first tooth generation develops early in ontogeny and teeth are functional in larvae, 3 days after hatching (Davit-Béal et al., 2006). These larval teeth are conical and composed of dentin and enameloid, covered with a hardly visible, thin enamel layer. The two other tooth generations that develop prior metamorphosis are larger but similarly shaped (a single cusp) and structured. Enameloid is still present but enamel is thicker. The fourth tooth generation, which develops during metamorphosis, and the following tooth generations in juveniles and adults display a different shape (two cusps) and structure. Enameloid is no longer present, and dentin is covered with enamel only. Enameloid is more related to dentin than to enamel but it could contain EMPs. Moreover, the organic matrix could be degraded by proteases as for enamel matrix (Davit-Béal et al., 2007b).

Odontogenesis in salamanders is similar to that described in other vertebrates, at least in juveniles and adults (Smith and Miles, '71; Wistuba et al., 2002). However, it is known that non-prismatic and prismatic enamel occurs in mammals, while enamel without prisms is the condition in non-mammalian vertebrates. The difference in enamel macrostructures is related to the presence (mammals) or the absence (non-mammals) of ameloblast Tomes' processes. However, some questions remain to be answered: taking into account the large evolutionary distance among the amphibian lineages, does the AMEL sequence of *P. waltl* differ largely from the other amphibian AMEL sequences reported to date? Is *AMEL* expression in *P. waltl* restricted to ameloblasts during tooth development? Given the enameloid–enamel transition through ontogeny, is there a similar temporospatial expression of *AMEL* in larvae, pre- and post-metamorphic juveniles and adults? Is this expression also similar to that described in lizards and mammals? Finally, could *P. waltl* be considered a good model species allowing a better understanding of amelogenesis processes and ameloblast function during tetrapod evolution?

In order to answer these questions we sequenced and compared *P. waltl* AMEL to mammalian, reptilian, and amphibian sequences, then we studied its temporospatial expression during amelogenesis in a growth series of this salamander, from larvae to adults.

MATERIALS AND METHODS

Biological Material

We used a growth series of larvae from hatching (10 days post-fertilization) to metamorphosis (4-month-old larvae), 5- and 12-month-old juveniles, and 3-year-old adults of *P. waltl*, a caudate amphibian routinely bred in our laboratory. The specimens were deeply anaesthetized (MS222) and sacrificed according to the guidelines of French Ethics Committee.

Histology

Depending on animal size, either entire heads, or dissected lower jaws were fixed. They were immersed for 2 hr, at room temperature, in a mixture containing 1.5% glutaraldehyde and 1.5% paraformaldehyde in PBS buffer (pH 7.4). Then, the samples were demineralized, either for 3 days (early larvae), 7 days (late larvae), 15 days (juveniles), or 21 days (adults), at 4°C in the same fixative, to which 5% EDTA was added. The samples were post-fixed for 2 hr, at room temperature, in 1% osmium tetroxide in PBS buffer, dehydrated using a graded series of ethanol, and embedded in Epon 812. Transverse or longitudinal 1–2 μm thick

sections were obtained with an ultramicrotome (Leica-OMU3, Leica, Germany), then stained with toluidine blue, examined in a binocular microscope and photographed.

Amelogenin Gene Sequences

Six amphibian *AMEL* sequences were found in NCBI database (http://www.ncbi.nlm.nih.gov/). Four anurans, *Rana pipiens* (NCBI accession No AY695795), *Xenopus laevis* (AF095570), *Xenopus tropicalis* (BC157232) and *Litoria cloris* (DQ069788), and two caudates *Plethodon cinereus* (DQ069790) and *Ambystoma mexicanum* (DQ069791). For comparison, we used *AMEL* sequence of humans (*Homo sapiens*: NM_182680), snake (*Elaphe quadrivirgata*: AF118568), and crocodile (*Paleosuchus palpebrosus*: AF095568). Nucleotide sequences were translated into protein sequences and aligned using Se-Al v2.0a11.

P. waltl Amelogenin

The lower jaw of a juvenile specimen was immersed in liquid nitrogen, reduced to a thin powder, and RNAs extracted (RNeasy Midi kit, Qiagen Les Ulis, Essonne, France) and aliquoted. We identified phylogenetically conserved regions from the alignment of mammalian, reptilian, and amphibian *AMEL* sequences, in order to design a primer set using Primer 3 (v.0.4.0) software (http://frodo.wi.mit.edu/). A fragment of 180 bp corresponding to *AMEL* exons 3–6 was targeted using the following primers: sense, 5′-TATATCAACTTCAGTTACGAGGT-3′; anti-sense, 5′-CCCATGATGCCCCAGCA-3′. Full length *AMEL* transcripts were obtained from cDNA using PCR followed by 5′ and 3′ RACE PCR.

PCR

cDNAs were obtained by RT-PCR (RevertAid H Minus First Strand cDNA Synthesis kit, Fermentas MBI) and routinely amplified in presence of GoTaq DNA Polymerase (Promega, Madison, WI, USA) and the two primers. Each PCR was performed in a total volume of 50 µL containing 500 ng of cDNA, 0.2 µM of sense and antisense primers, 1× of GoTaq reaction buffer, 0.2 mM of dNTPs, and 1.25 U of GoTaq DNA Polymerase (Promega). Amplification was performed in a thermal cycler (G-Storm GS1; GRI, UK) for 35 cycles, each cycle consisting of 1 min of denaturation at 94°C, 1 min of annealing at 58°C, and 1 min of extension at 72°C. The final extension was during 20 min at 72°C. Amplified fragments were sent to GATC Biotech SARL for sequencing.

5′ and 3′RACE-PCR

Rapid amplification of cDNA ends (RACE) was used to complete the *AMEL* sequence of *P. waltl* upstream and downstream the regions obtained with PCR, including the 3′ and 5′ untranslated regions (UTRs). Using Primer 3, four inner primers were designed from the *AMEL* sequence obtained after PCR: GSP1 (gene specific primer) for the first 5′ RACE run and NGSP1 (nested gene specific primer) for the second run; GSP2 for the first 3′ RACE run and NGSP2 for the second run. 5′ RACE: GSP1, antisense, 5′-ATTCCACATATGCAGCCCCAT-3′ then NGSP1, 5′-AACCAGTGGGTGGATGGCTGCAGAG-3′; 3′ RACE: GSP2, sense, 5′-AACCAGTGGGTGGATGGCTGCAGA-3′ then NGSP2, 5′-GATGCCCCAGCAGCATTTTCCACAC-3′.

For each PCR the mixture (50 µL) was composed of 34.5 µL PCR-grade water, 5 µL 10× Advantage 2 PCR buffer, 1 µL dNTP mix (10 mM), 1 µL 50× Advantage 2 polymerase mix (Clontech), 1 µL 3′ or 5′ RACE primers (GSP1 or GSP2), 5 µL universal mix primer, and 2.5 µL RACE cDNA. We used a specific touch down thermal cycling program for the RACE reactions as follows: 5 cycles (94°C for 30 sec and 72°C for 3 min); 5 cycles (94°C for 30 sec, 70°C for 30 sec, and 72°C for 3 min); and 20 cycles (94°C for 30 sec, 68°C for 30 sec, and 72°C for 3 min). The first run was always followed by a nested PCR. Sequencing was performed by GATC Biotech SARL.

Probe

The 180-bp fragment of the coding *P. waltl AMEL* sequence, that is, from exon 3 to end of exon 6, was amplified using the two primers designed above for the PCR. One microgram of PCR product was ligated to pCR 2.1-TOPO (Invitrogen) plasmid vector by the TA-cloning method, and used to transform competent *E. coli* TOP10F bacteria. The plasmids were purified and sequenced to check the identity of the amplicon. The antisense and sense (control) RNA probes labelled with Digoxigenin UTP (Roche Applied Science, Penzberg, Upper Bavaria, Germany) were synthetized (Ribo Probe Combination System SP6/T7, Promega) and purified (Probe Qant G 50 micro columns, GE Healthcare Velizy-Villacoublay, Yvelines, France).

In Situ Hybridization (ISH)

Immediately after dissection the samples were immersed in Formoy's solution (10% acetic acid, 30% formalin, and 60% ethanol) overnight at 4°C, demineralized in 1 M acetic acid for 4 days (larvae), 9–15 days (juveniles), and 21 days (adults). Then, the samples were dehydrated in a graded series of ethanol, immersed shortly in toluene and embedded in Paraplast (Sigma Aldrich, St Louis, MO, USA). Transverse and longitudinal 10 µm-thick sections were obtained using a microtome (Lemardeley, Paris, France) and deposited on superfrost-plus slides. Sections were dewaxed in toluene and rehydrated in a decreasing series of ethanol. After digestion in PBS/proteinase K (0.6 µg/mL) at 37°C for 5 min, the slides were fixed for 30 min in 4% paraformaldehyde. After two successive baths of PBS and SSC2X, the probe was hybridized overnight in a wet chamber at 65°C in SSC2X.

The following day, the slides were rinsed in a buffer containing 50% formamide, once for 30 min and twice for 1 hr at 65°C, then rinsed with MABT for 30 min at room temperature. After 2 hr in the blocking solution containing 20% inactived goat serum and 2% blocking reagent (Roche), 0.1% anti-Dig (Roche) was added to the solution and 300 µL of this mixture was deposited on the sections overnight, at room temperature.

The next day, the samples were rinsed four times for 30 min with MABT, once with TMN during 30 min at room temperature,

then the signal revealed with NBT (0.45 μL/mL, Roche) and BCIP (3.5 μL/mL, Roche). After the rise of the signal, the slides were rinsed and mounted in Gel Mounting Medium (Dako Cytomation). DIC images were acquired using an Olympus BX61 microscope equipped with a Q-imaging camera and using the software Image Pro Plus (Mediacybernetics, Bethesda, MD).

RESULTS

P. waltl AMEL

We obtained the full-length sequence of *P. waltl AMEL* cDNA. Additional transcripts that would have resulted from alternative splicing were not identified. The 772 bp sequence was validated through alignment with tetrapodan *AMEL* sequences, and deposited in Genbank (accession No. JX508595). The gene is composed of six exons (1, 2, 3, 5, 6, and 7). Exon 4, which is found in some mammalian *AMELs*, was not present (Fig. 1). 5′ UTR consists in exon 1 (78 bp) and the beginning of exon 2 (22 bp) (not shown). The rest of exon 2 (54 bp) encodes the 16 amino acid-long signal peptide and the first two residues of the protein, as in other tetrapodan *AMELs*. Exons 3 (48 bp) and 5 (45 bp) have the same length as in other *AMELs* while exon 6 (375 bp) is shorter. Exon 7 encodes the single last residue of the protein, while the rest of this exon (>150 bp) constitutes the 3′ UTR (not shown). The putative

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                        1        .       18 .         .   34     .        49.         .      67
                       |       exon2      |      exon3     |     exon5     |       exon6
Pleurodeles waltl       MRPWILLTCLLGVTFAMP LPPHPNHPGYINFSYE VMTPLKWYQSMMRQQ YPSYAYEPVGGWLQSQIM
Ambystoma mexicanum     .G.........SASC... ................ ............... ....G...M.......M.
Plethodon cinereus      ..TL.......SA..... .....S.......... ............... ....G...IS......V.
Xenopus tropicalis      ..L.VM..A.T.AA.SV. .....Q....V..... ILS.........KH. ..N.G...MS....NPVV
Xenopus laevis2         ...LVM..A.I.AA.SL. ...Q.Q....V..... ILS.I.......KN. ..N.G....S.....PMI
Litoria chloris         .KSC.M..S.I.AA.SI. ..L..Q....V.L... IL.........LKH. ....G...MS...KNPVA
Rana pipiens            .KS..I..S.I.AA.CL. .....Q....V.L... IL..........KH. ...FG...MS....NPMI
Elaphe quadrivirgata    .ET.T.VM...STA..I. ..Q.---......... .......F..IIGH. ..R.G...M...MHHN--
Paleosuchus palpebrosus .EG.M.I.....A...I. .....H....V..... .L........L...P .S..G...M....HQPML
Homo sapiens            .GT...FA....AA.... ......G......... .L........-I.PP ....G...M....HH..I
                        #        #       # ##  # #### # ###    # ## ##      #    ###  ##
                        T  S                                  I                    T      L

                       68 .         .         .         . exon6   .         .         .        140
                        PxxPxxPxx  Pxx   Pxx Pxx       PxxP   xx      Pxx      Pxx    PxxPxx  Pxx
Pleurodeles waltl       PGSPMMPQQHFPHMHTIPHMQPHH-------PMLP---HQQMM---PQHQQLLQQPGQQ---PLLPQQ--PTH
Ambystoma mexicanum     ..........---.QP..Q.....PMMLPQQ....---.G...PL-.......P...H.PMQ.MI...--.S.
Plethodon cinereus      .......P..L..L.AV.Q.....PMMLPQQ....---.P-VLPM-.....P.PL..H----.M...A--...
Xenopus tropicalis      .AP......QH.SQ.VV.KLP...PFLIPQQ.VV.VP-.P-VIPLT...THQ.-K.TYL---SN-.D---SAN
Xenopus laevis2         .VP...Q..QL.SQNAV.KLPS..PLLIPQQ.LV.VPV.HPLIPLT...THQ.-K.IYL---FN-SDGQY..N
Litoria chloris         .M.......PLS.P.A..KLPL..PMLMPQP..VSAHG.HSLFP--.PPSHQI-N.TYL---KN-.ESQ-...
Rana pipiens            .L.......QL..P.A..KLP...SVLIPQQ..I.LSP.HPLLP--..A-NPS-N.TYL---TN-.ESQY..N
Elaphe quadrivirgata    -AG...HPP..HGV.A.-.-PAL.QKQ-PQQ--L.QN---------..L..PGLN-AFVPLNGHNALIPHYQ.
Paleosuchus palpebrosus ---.I-A...P-PIQ.L-TPHHQIPFLSPQH.LMQMPGPH...PI-..-..PSL.MPV.E-PVQPQAGEH.SP
Homo sapiens            ---.VLS...P-PT..L-QPHH.IPVV.AQ..VI.QQPMMP-VPG--..SMTPI.HH.PNLP.PAQ.P-YQPQ
                           #

                       141        .         .         .   exon6 .         .         .        210
                                    PxxP  xx       Pxx                                      | ex7
Pleurodeles waltl       -VQQT-AQ-AIQPILP--HP-------PQQ---SGQTIYQVQQ-QPPLLPEAPLEPWQPVDKANQEEQ D* (175)
Ambystoma mexicanum     -.L..-..-.NH.LQ.--.Q-------..H---...P..P...Q......DM.......A..T..... .* (185)
Plethodon cinereus      -...V-..-PNHAMQ.--.L-------..---PS.PM.PS.P-....Q.DV..........T..... .* (182)
Xenopus tropicalis      ------T.----.LE.-FK--------.DHENKN.HPMFPL.P-L...FE.R.Q....AAGNT....L .* (179)
Xenopus laevis2         ------T.----LLE.-SK--------.DHESQN..PTFPLHP-L...VE.R.Q....EAGNDK...L .* (184)
Litoria chloris         ------T.----.MEL-PK--------.DLSHQP..P..PN.P-....VE.KT....P..S.TQL..L .* (181)
Rana pipiens            ------T.----..EL-PK--------.DILNKP..PLFPI.P-....VE.R.Q....AS.TKL..L .* (181)
Elaphe quadrivirgata    ---PFQP.AGEH..QSL---SPVNPMN...PGNAN.P..P----L..VI.DT...S.-.A..TKK..V .* (178)
Paleosuchus palpebrosus P..PQQPGHPNP.MQ.QLPGSPHPPMR...PGIPNPPM.PM.P-L.....DM.....R.M..TK...I .* (199)
Homo sapiens            P..PQ.P-HQ--.MQ.Q-P.-VH.PMQ.LPPQPPLPPMFPM.P-L..M..DLT..A.PST..TKR..V .* (191)
                                                   #                 ##       # #        ##  #
```

Figure 1. Alignment of *Pleurodeles waltl* AMEL sequence with amphibian, human, snake (*Elaphe quadrivirgata*), and crocodile (*Paleosuchus palpebrosus*) sequences. Our alignment comprises 210 positions. (175) = sequence length; | = exon limits;.: residue identical to the *P. waltl* AMEL residue; —: indel; *: stop codon; #: unchanged position. Signal peptide is squared. Amino acids that are important for AMEL function in mammals (Delgado et al., 2005a) are indicated in gray background on the human sequence. In humans the five positions substituted with the residue in bold (T, S, I, T, L) lead to *amelogenesis imperfecta*. The 16 triplet repeats identified in *P. waltl* AMEL are indicated (Pxx). Accession numbers in NCBI: *Rana pipiens* (AY695795), *Xenopus laevis* (AF095570), *Xenopus tropicalis* (BC157232), *Litoria cloris* (DQ069788), *Plethodon cinereus* (DQ069790), *Ambystoma mexicanum* (DQ069791), *Homo sapiens* (NM_182680), *Elaphe quadrivirgata* (AF118568), *Paleosuchus palpebrosus* (AF095568), and *P. waltl* (JX508595).

encoded protein is composed of 175 amino acids, which means that the *P. waltl* sequence is the shortest *AMEL* sequence reported in the literature to date (Fig. 1).

Comparison to Other AMEL Sequences

The *P. waltl* AMEL sequence was aligned to the six other amphibian sequences known to date, and to two reptilian and one mammalian sequences (Fig. 1). The divergence time between these amphibian species and *P. waltl* is large, even when considering the other caudate species, from which *P. waltl* is supposed to be more than 150 Ma evolutionary distant (Fig. 2).

The N-ter (residues 1–64 in our alignment) and C-ter (residues 150–175) AMEL regions are well conserved in tetrapods while the central region is highly variable and difficult to align accurately, even within caudates (Fig. 1). Out of 34 unchanged positions revealed from our alignment, 26 (76%) are located in the highly conserved N-ter region. Remarkable AMEL motifs such as the phosphorylation site (SYE) encoded by the 3′ extremity of exon 3, and the tyrosyl-rich domain encoded by the 5′ extremity of exon 6 (YPSYAY) are conserved. Interestingly, out of the five positions (M^1, W^4, T^{51}, P^{70}, and H^{77}) currently reported to lead to *amelogenesis imperfecta* (AI) in humans when substituted, only three are unchanged in amphibian AMELs. The two other are substituted, but with another residue than that reported in human AI (Fig. 1). The intraexonic splicing site reported in the 3′ region of some mammalian exon 6 was not found in *P. waltl* and in other amphibian *AMELs*. The proteolytic site known in mammalian AMEL, MGGW (residues 58–61), is replaced with VGGW in *P. waltl*. At least 16 triplet repeats (Pxx) similar to those described in mammalian and reptilian AMEL were identified in the central, variable region of the protein (Fig. 1). As reported in other tetrapods, *P. waltl* AMEL is rich in proline and glutamine. The amount of proline (18.28%) is less important than in crocodilian and mammalian AMEL (in average 25%), while in contrast glutamine (18.85%) is better represented than in amniotes (10–13%). However, the hydrophobic pattern of this central region was similar for all the sequences used in our alignment (not shown).

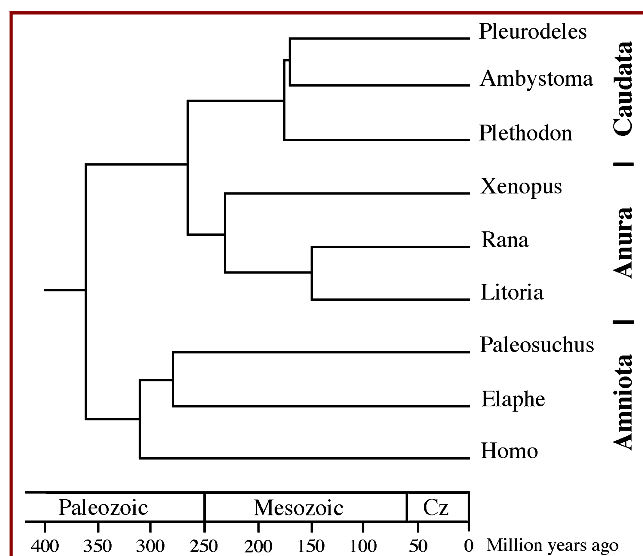

Figure 2. Timetree of the species used for AMEL sequence comparison. Note the large evolutionary distance between *P. waltl* and other caudate species of this study, *Ambystoma mexicanum* and *Plethodon cinereus*. Cz, Cenozoic. Divergence times from Bossuyt and Roelants (2009), Canatella et al. (2009), and Vieites et al. (2009).

Amelogenin Gene Expression During Amelogenesis Through *P. waltl* Ontogeny

Various developmental stages of tooth formation were identified in the jaws of larvae, juveniles and adults, and serial sections allowed to choose appropriate section levels for studying *AMEL* expression using ISH.

The comparison of ISH labeling on sections (Fig. 3c, d, f–h, j–l, n, o, q) to similar developmental stages obtained in routine histology (Fig. 3b, e, i, m) through ontogeny allowed to describe amelogenesis as taking place in two steps in all stages studied: first, the enamel matrix is deposited and mineralizes (Fig. 3a–h), then maturation occurs (Fig. 3i–o). Control probe provided negative results indicating that our *AMEL* probe labeled *AMEL* transcripts only (Fig. 3p).

In all ontogenetic stages studied *AMEL* expression was never detected prior to matrix deposition (Fig. 3q). *AMEL* mRNAs were always identified in the ameloblasts around the forming teeth once enameloid in larvae (Fig. 3a–d) or predentin in juveniles (Fig. 3f, g) and adults (Fig. 3e, h) had started to be deposited, and before the pedicel (tooth base) had formed. During matrix deposition and mineralization, *AMEL* is detected in ameloblasts around the tooth tip where enameloid/enamel matrix is deposited (Fig. 3a–h). We never observed *AMEL* expression in the ameloblasts elsewhere along the tooth base surface.

In larvae, juveniles and adults, at the time when the maturation has started at the tooth tip *AMEL* transcripts were no longer detected in the ameloblasts facing this region; in contrast *AMEL* expression was still obvious in the ameloblasts located along the enamel matrix that is not maturing (Fig. 3j–l). Then, *AMEL* expression totally disappeared in the ameloblasts once enamel is entering in the last maturation phase (Fig. 3m–o).

In larvae, the teeth are monocuspid. Therefore, on longitudinal (Fig. 3a, b, d, j, o) and transverse (Fig. 3c, m, n, q) sections the outlines of the teeth are roughly similar. In contrast, juvenile and adult teeth being bicuspid, on longitudinal sections the major cusp can be observed at the lingual side and the minor cusp at the labial side (Fig. 3e, f, g) and on transverse sections the major and minor cusps cannot be distinguished (Fig. 3h, i, k, l, p). In bicuspid teeth, enamel matrix is deposited first on the major cusp, around which *AMEL* transcripts are strongly expressed in ameloblasts (Fig. 3g).

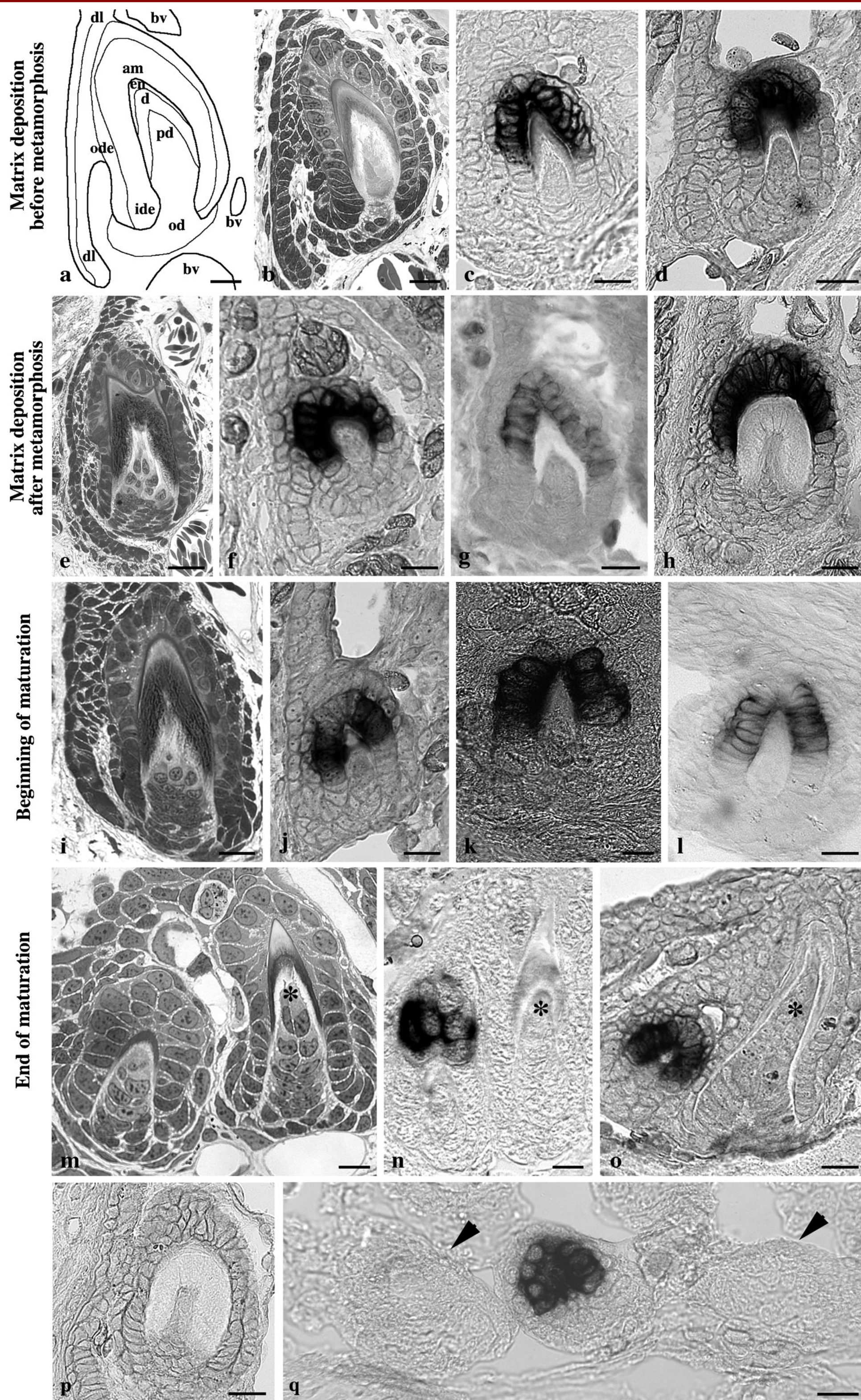

Figure 3. Continued.

AMEL expression is weaker in the ameloblasts facing the minor cusp.

Through *P. waltl* ontogeny, *AMEL* expression was never found in the pulp cells nor in odontoblasts nor in the cells locacted along the pedicel/root at any stage of tooth development.

DISCUSSION

P. waltl AMEL, the Shortest Tetrapodan AMEL Sequence

P. waltl AMEL displays a similar gene structure as described in mammals, reptiles (Toyosawa et al., '98; Delgado et al., 2005a, 2006) and amphibians (Toyosawa et al., '98; Wang et al., 2005; Diekwisch et al., 2009). The protein is encoded by five exons—exon 4 is specific to mammals and was recruited in placentals (Sire et al., 2012), and the N- and C-ter regions are well conserved. A total of 34 positions are unchanged in our alignment, among which 26 are located in the N-ter region. Such conservation indicates that these residues play a role in AMEL structure and/or function for more than 360 million years (Sire et al., 2007). However, some putatively important positions previously identified as being unchanged during mammalian lineage evolution (Delgado et al., 2005a) are found to be substituted in *P. waltl* and in other amphibian AMELs, which indicates that the amino acid substitution on these positions probably occurred a long time ago in the amphibian lineage. This also suggests that these particular residues are not as crucial for AMEL functions in anurans and caudates as reported in mammals. A weaker functional pressure on these positions compared to mammals could explain the enlarged range of residue possibility observed at these positions. This lower constraints could be related to two main differences between amphibian and mammalian dentition, that is, polyphyodonty (permanent tooth renewal) versus mono- or diphyodonty, and lack versus presence of occlusion, respectively. The lack of occlusion probably reduces wear while polyphyodonty does not need resistance to break and abrasion for years. In contrast, the positions that remained unchanged in all tetrapodan AMELs for 360 Ma are essential for the correct protein functions.

The central region of *P. waltl* AMEL is the shortest described so far among tetrapodan sequences. However, as already reported in reptiles and mammals, this region is characterized by the presence of repeated amino acid triplets (16 Pxx repeats identified), which indicates that this feature was present in an ancestral sarcopterygian, prior to tetrapod diversification. This AMEL region has evolved differently in the three tetrapod lineages with living representatives and it is particularly large (25 Pxx repeats) in mammalian and lizard AMELs (Delgado et al., 2005a, 2006). The Pxx repeat region is not subjected to local functional constraints, as demonstrated by its high variability in tetrapods and it was considered a hot spot of mutation (Delgado et al., 2005a). The presence of enameloid during the first month of life seems, however, not related to a particular change in the central region of AMEL because enameloid is no longer present after metamorphosis. However, the central region of AMEL is supposed to play an important role in structuring enamel through its high amount of proline and glutamine residues. Proline is a non-polar amino acid that inhibits the helical structure and forms peptide bonds that fold back upon themselves in regions that form turns. Glutamine is a polar and hydrophylic amino acid, which is almost always found at the protein surface, and can function as a chain crosslinker via hydrogen bond formation. In *P. waltl*, the percentage of proline and glutamine residues in the central region of AMEL differs slightly from that observed in reptiles and mammals (18% vs. 25% and 19% vs. 10–13%, respectively; Sire, personal data). However, although in this region the amino acid sequences vary between species their hydrophobicity is roughly similar. Therefore, these residues probably have a similar function in all sequences but the amount of proline and glutamine could modify slightly enamel microstructure. Indeed, a correlation was shown between the presence of increased proline tripeptide repeats (Pxx) in AMEL and sophisticated enamel structures in vertebrates (Jin et al., 2009).

Figure 3. The two steps of amelogenesis (matrix deposition and maturation) selected in similar stages of tooth formation in 21-day-old (second generation teeth) **(m, n)** and 3 month-old larvae (third generation teeth) **(a–d, j, o, q)**, 5-month-old (fourth generation teeth) **(f, k)** and 12-month-old juveniles (sixth generation teeth) **(g, l)**, 3-year-old adult **(e, h, i, p)** of *P. waltl*. (a–d) Matrix deposition before metamorphosis; (e–h) matrix deposition after metamorphosis; (i–l) early stages of enamel maturation; (m–o) late stage of tooth formation, prior to tooth attachment. (n,o) *AMEL* is no longer expressed when the tooth starts the attachment process (* on the figure) while it is already well expressed in the early stages of the neighbour replacement tooth (on the left on the figure). (p) Control using AMEL sense probe. (q) Tooth buds at different stages of amelogenesis; AMEL is not expressed in pre-ameloblasts surrounding early tooth buds (arrows) while *AMEL* transcripts are obvious in differentiated ameloblasts during matrix deposition (central bud). (a) Interpretative drawing of Figure 3b, with indication of the main cells and tissues: am, ameloblasts; bv, blood vessels; d, dentin; dl, dental lamina; en, enamel; ide, inner dental epithelium; od, odontoblasts; ode, outer dental epithelium; pd, predentin. (b, e, i, m) 2 μm-thick epon sections of representative stages for the four selected steps of amelogenesis; toluidine blue staining. (c, d, f, g, j–l, n, o, q) in situ hybridized 10 μm-thick paraffin sections with *AMEL* anti-sense probe. (p) in situ hybridized 10 μm-thick paraffin sections with *AMEL* sense probe. Scale bars: a, b, k, m, n = 10 μm; c–j, l, o–q = 25 μm.

These authors found smaller nanospheres in species with numerous Pxx repeats than in those with fewer repeats, suggesting that the length of the Pxx region could determine the supramolecular enamel matrix assembly (Jin et al., 2009). The large percentage of glutamine residues is also of interest because glutamine is the second residue, after proline, that is present in the left-handed polyproline II helical conformations (PPII) and its role in the repeat sequence is essential for compaction of PPII and hence for crystal growth (Jin et al., 2009). Therefore, we could expect that the nanospheres would be larger and more compacted in *P. waltl* than in mammals, and that the supramolecular assembly would have a consequence on enamel microstructure. Unfortunately, to our knowledge there are no comparative data on enamel microstructure that could support this hypothesis and no experiments have been performed in order to compare the resistance of amphibian, reptilian, and mammalian enamel to breaks and wears.

AMEL Expression During Amelogenesis Is Similar in Tetrapods

This is the first study of *AMEL* expression during amphibian amelogenesis using a specific RNA probe. Previous studies dealt with immunohistochemical detection of AMEL in the frog (*R. pipiens*) enamel using a mouse antibody, and showed the protein expression in the ameloblasts and in the enamel matrix (Wang et al., 2005).

During *P. waltl* amelogenesis, whatever the ontogenetical stages studied from larvae to adults, *AMEL* was found exclusively expressed in teeth and was not identified in the other regions of the upper and lower jaws, or of the head. Transcripts location resembled to that described during mammalian and reptilian tooth development (Snead et al., '88; Bleicher et al., '99; Delgado et al., 2005b, 2006). More precisely, *AMEL* expression was restricted to ameloblasts. This finding is in contrast with previous immunohistochemical descriptions of the protein not only in ameloblasts but also in odontoblasts, dental pulp cells, or cells located along the root in rodents (Aoba et al., '92; Papagerakis et al., 2003; Veis, 2003) and in squamates (Wang et al., 2005; Handrigan and Richman, 2011). These contrasted results could be related to the different methods used in order to detect AMEL expression, that is, a *P. waltl* specific probe (ISH, present study) versus immunohistochemistry, which could have detected various epitopes. In squamates, Handrigan and Richman (2011), using the same frog AMEL antibodies as Diekwisch et al. (2006) in amphibians, detected epitopes in squamate preameloblasts, preodontoblasts and odontoblasts, and described in the cap stage a signal weaker than in the bell stage, during which specialized cells differentiate. Diekwisch et al. (2006) suggested that AMELs could be involved in epithelial–mesenchymal signaling or mineral induction. Using ISH in various steps of *P. waltl* amelogenesis, we did not detect *AMEL* transcripts neither in preameloblasts prior to enamel matrix deposition nor in preodontoblasts and in differentiated odontoblasts. These findings indicate that *AMEL* expression is specific to ameloblasts as previously reported in lizards (Delgado et al., 2006).

Although repeated sequencing of cDNA was done, we did not find any *AMEL* splice products. However, splice variants were reported many times in rodents (e.g., Li et al., 2010) and recently in a lizard (Wang et al., 2012). In mammals, among the numerous splice products reported, one of them, the Leucine Rich Amelogenin Peptide has been considered a signalling molecule (Veis, 2003; Veis et al., 2010). In lizard the transcript variant possesses an additional exon between exons 5 and 6 (Wang et al., 2012). To date, this new exon was only reported in a single squamate and this is the only report of alternative splicing of AMEL in a non-mammalian species. Such an alternative splicing could be related to the recruitment of the new exon, and could result in a lower expression of the transcript containing this exon, as reported in mammalian AMEL for exon 4 (Sire et al., 2012).

In rodents *AMEL* expression during amelogenesis is well described but there are contrasted results concerning the earliest transcript location. At mouse E10.5 and E14, high sensitivity RT-PCR detected *AMEL* transcripts but they were not identified using ISH (Couwenhoven and Snead, '94; Papagerakis et al., 2003). After birth *AMEL* expression is detected in preameloblasts prior to enamel matrix deposition, then during amelogenesis in secretory, transition and early maturation ameloblasts (Snead et al., '88; Hu et al., 2001). Along the crown, *AMEL* expression ceases at the cemento-enamel junction, where the inner and outer dental epithelium cells join to form the Hertwig's epithelial root sheath (Hu et al., 2001).

In lizards, although enamel is prismless (Sander, 2001) and the mode of cusp formation differs (Handrigan and Richman, 2011), during amelogenesis *AMEL* expression was found roughly similar to that described in mammals (Delgado et al., 2006).

In *P. waltl*, in contrast to rodents and lizards AMEL transcripts were not identified prior to enamel matrix deposition, but during amelogenesis they were similarly identified as described in mammals and lizards: first at the tooth tip, then along the crown. As soon as maturation stage begins at the tooth tip, *AMEL* is no longer expressed in the facing ameloblasts as indicated by the fading signal then its disappearance. The limit between *AMEL*-expressing and non-expressing ameloblasts is always clear cut as described at the cemento-enamel junction in mammals (Hu et al., 2001).

In conclusion, although the central region of *P. waltl* AMEL sequence differs from reptilian and mammalian AMELs, and despite some differences with previous findings dealing with protein expression, during *P. waltl* amelogenesis AMEL gene expression displayed a pattern close to that described in other tetrapods. This indicates that *P. waltl* could be a good model species for evo-devo studies of amelogenesis in tetrapods. However, the differences in the *P. waltl AMEL* sequence could find their importance at the microstructural level and in a lower resistance to wear of polyphyodont amphibian teeth compared to amniote teeth.

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LITERATURE CITED

Aoba T, Shimoda S, Shimokawa H, Inage T. 1992. Common epitopes of mammalian amelogenins at the C-terminus and possible functional roles of the corresponding domain in enamel mineralization. Calcif Tissue Int 51:85–91.

Bleicher F, Couble ML, Farges JC, Couble P, Magloire H. 1999. Sequential expression of matrix protein genes in developing rat teeth. Matrix Biol 18:133–143.

Bossuyt F, Roelants K. 2009. Frogs and toads (Anura). In: Hedges SB, Kumar S, editors. The Timetree of Life. New York: Oxford University Press. p 357–364.

Canatella DC, Vieites DR, Zhang P, Wake MH, Wake DB. 2009. Amphibians (Lissamphibia). In: Hedges SB, Kumar S, editors. The Timetree of Life. New York: Oxford University Press. p 353–356.

Couwenhoven RI, Snead ML. 1994. Early determination and permissive expression of amelogenin transcription during mouse mandibular first molar development. Dev Biol 164:290–299.

Davit-Béal T, Allizard F, Sire JY. 2006. Morphological variations in a tooth family through ontogeny in *Pleurodeles waltl* (Lissamphibia, Caudata). J Morphol 267:1048–1065.

Davit-Béal T, Chisaka H, Delgado S, Sire JY. 2007a. Amphibian teeth: current knowledge, unanswered questions, and some directions for future research. Biol Rev Camb Philos Soc 82:49–81.

Davit-Béal T, Allizard F, Sire JY. 2007b. Enameloid/enamel transition through successive tooth replacements in *Pleurodeles waltl* (Lissamphibia, Caudata). Cell Tissue Res 328:167–183.

Delgado S, Girondot M, Sire JY. 2005a. Molecular evolution of amelogenin in mammals. J Mol Evol 60:12–30.

Delgado S, Davit-Béal T, Allizard F, Sire JY. 2005b. Tooth development in a scincid lizard, *Chalcides viridanus* (Squamata), with particular attention to enamel formation. Cell Tissue Res 319:71–89.

Delgado S, Couble ML, Magloire H, Sire JY. 2006. Cloning, sequencing, and expression of the amelogenin gene in two scincid lizards. J Dent Res 85:138–143.

Diekwisch TGH, Wang X, Fan JL, Ito Y, Luan X. 2006. Expression and characterization of a *Rana pipiens* amelogenin protein. Eur J Oral Sci 114:86–92.

Diekwisch TG, Jin T, Wang X, et al. 2009. Amelogenin evolution and tetrapod enamel structure. Front Oral Biol 13:74–79.

Fincham AG, Moradian-Oldak J, Simmer JP. 1999. The structural biology of the developing dental enamel matrix. J Struct Biol 126:270–299.

Handrigan GR, Richman JM. 2011. Unicuspid and bicuspid tooth crown formation in squamates. J Exp Zool (Mol Dev Evol) 316:598–608.

Hedges SB. 2009. Vertebrates (Vertebrata). In: Hedges SB, Kumar S, editors. The Timetree of Life. New York: Oxford University Press. p 309–314.

Hu JC, Sun X, Zhang C, Simmer JP. 2001. A comparison of enamelin and amelogenin expression in developing mouse molars. Eur J Oral Sci 109:125–132.

Jin T, Iro Y, Luan X, et al. 2009. Elongated polyproline motifs facilitate enamel evolution through matrix subunit compaction. PLoS Biol 7:1–10.

Li W, Sire JY, Takano Y, et al. 2010. Amelogenin exons 8 and 9. In: Goldberg M, editor. Amelogenins: multifaceted proteins for dental and bone formation and repair. Danvers, MA, USA: Bentham Science Publishers. p 163–173.

Moradian-Oldak J, Iijima M, Bouropoulos N, Wen HB. 2003. Assembly of amelogenin proteolytic products and control of octacalcium phosphate crystal morphology. Connect Tissue Res 44:58–64.

Paine ML, Snead ML. 2005. Tooth developmental biology: disruptions to enamel-matrix assembly and its impact on biomineralization. Orthod Craniofac Res 8:239–251.

Paine ML, Wang HJ, Snead ML. 2003. Amelogenin self-assembly and the role of the proline located within the carboxyl-teleopeptide. Connect Tissue Res 44:52–57.

Papagerakis P, MacDougall M, Hotton D, et al. 2003. Expression of amelogenin in odontoblasts. Bone 32:228–240.

Sander PM. 2001. Primless enamel in amniotes: terminology, function and evolution. In: Teaford M, Ferguson MWJ, Smith MM, editors. Development, function and evolution of teeth. New York: Cambridge University Press. p 92–106.

Simmer JP, Papagerakis P, Smith CE, et al. 2010. Regulation of dental enamel shape and hardness. J Dent Res 89:1024–1038.

Sire JY, Delgado S, Fromentin D, Girondot M. 2005. Amelogenin: lessons from evolution. Arch Oral Biol 50:205–212.

Sire JY, Davit-Béal T, Delgado S, Gu X. 2007. The origin and evolution of enamel mineralization genes. Cells Tissues Organs 186:25–48.

Sire JY, Huang Y, Li W, et al. 2012. An evolutionary story of mammalian-specific amelogenin exons 4, "4b", 8 and 9. J Dent Res 91:84–89.

Smith MM, Miles AEW. 1971. The ultrastructure of odontogenesis larval and adults Urodeles. Differentiation of the dental epithelial cells. Z Zellforsch 121:470–498.

Snead ML, Luo W, Lau EC, Slavkin HC. 1988. Spatial- and temporal-restricted pattern for amelogenin gene expression during mouse molar tooth organogenesis. Development 104:77–85.

Snead ML. 2003. Amelogenin protein exhibits a modular design: implications for form and function. Connect Tissue Res 44:47–51.

Toyosawa S, O'hUigin C, Figueroa F, et al. 1998. Identification and characterization of amelogenin genes in monotremes, reptiles, and amphibians. Proc Natl Acad Sci USA 95:13056–13061.

Veis A. 2003. Amelogenin gene splice products: potential signaling molecules. Cell Mol Life Sci 60:38–55.

Veis A, Tompkins KA, Iacob S. 2010. Role of spliced forms of amelogenins as signaling molecules. In: Goldberg M, editor. Amelogenins: multifaceted proteins for dental and bone formation and repair. Danvers, MA, USA: Bentham Science Publishers. p 143–162.

Vieites DR, Zhang P, Wake DB. 2009. Salamanders (Caudata). In: Hedges SB, Kumar S, editors. The Timetree of Life. New York: Oxford University Press. p 365–368.

Wang X, Ito Y, Luan X, Yamane A, Diekwisch TGH. 2005. Amelogenin sequence and enamel biomineralization in *Rana pipiens*. J Exp Zool B Mol Dev Evol 304:177–186.

Wang X, Deng X, Zhang X. 2012. Identification of a novel splicing form of amelogenin in a reptile, *Ctenosaura similis*. PLoS ONE 7:e45871.

Wistuba J, Greven H, Clemen G. 2002. Development of larval and transformed teeth in *Ambystoma mexicanum* (Urodela, Amphibia): an ultrastructural study. Tissue Cell 34:14–27.

Wright JT. 2006. The molecular etiologies and associated phenotypes of amelogenesis imperfecta. Am J Med Genet A 140:2547–2555.

Wright JT, Li Y, Suggs C, et al. 2011. The role of amelogenin during enamel-crystallite growth and organization in vivo. Eur J Oral Sci 119:65–69.