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Phylogenetic signal and functional significance of incisor enamel microstructure in *Arvicola* (Rodentia, Arvicolinae)

Signal phylogénétique et signification fonctionnelle de la microstructure de l'émail des incisives chez Arvicola (Rodentia, Arvicolinae)

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ABSTRACT

Many genera of arvicolid rodents (voles) contain species that spend a considerable amount of time underground. Incisors provide a considerable effort in the digging process and a difference in the enamel microstructure of incisors is expected between species that spend most of their life above ground and those who have greater specializations for a subterranean habitat. The ratio between the thicknesses of the two layers composing the enamel of the incisors has proved to be an effective proxy to infer fossorial activity of extinct forms of *Arvicola* but this ratio exhibited no phylogenetic signal (although lifestyle was in a greater extent related to phylogeny). We were able to infer ancestral lifestyles (of extinct populations and of nodes of the phylogeny) from enamel morphologies. Inclusion of fossils did not solve the uncertainties associated with the lifestyle for the last common ancestor of extant *Arvicola* species and it did not change the inferred lifestyle of several other, less inclusive, clades.

RÉSUMÉ

De nombreux genres de campagnols (Rodentia, Arvicolinae) incluent des espèces au mode de vie clairement fouisseur et chez lesquelles les incisives jouent un rôle actif lors du fouissement. Cette situation permet d'envisager que la microstructure de l'émail des incisives pourrait révéler des différences entre espèces qui vivent surtout en surface et celles qui ont un mode de vie souterrain marqué. Ce travail montre que le rapport des épaisseurs des deux couches de l'émail des incisives est un bon d'indicateur du caractère fouisseur des formes actuelles et qu'il pourrait s'appliquer aux formes éteintes d'*Arvicola*. Ce rapport n'est pas corrélé à la phylogénie (bien que le mode de vie soit dans une grande

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mesure relié à la phylogénie). L'inclusion des populations éteintes n'améliore pas les incertitudes sur les inférences des modes de vie ancestraux pour le dernier ancêtre commun des espèces actuelles d'*Arvicola* et elle ne change pas les états ancestraux des clades moins inclusifs.

1. Introduction

Paleoclimatic and paleoenvironmental reconstructions inferred from small mammal communities are quite common in paleontology (Chaline et al., 1995; Marquet, 1989; van Dam, 1997). However, these reconstructions are often limited by the use of communities predominantly composed of taxa having closely related extant representatives, whose environmental preferences are well known. Any insight into extinct taxon lifestyle would be of extreme interest for studies on small fossil mammals. *Arvicola* is one of the few rodents displaying iterative evolution to a semi-aquatic and fossorial lifestyle, which makes it a very interesting model to test the rhythm and tempo of adaptive phenotypic changes to those special environments. In addition, *Arvicola* is nested within a clade where these changes have also occurred several times in closely related forms (e.g., in *Ondatra*, *Myodes*, *Microtus*, etc.), which makes lifestyle of fossil *Arvicola* or pre-*Arvicola* forms difficult to estimate without using a morphological or geochemical inferential approach.

Burrowing taxa, such as fossorial water voles (Airoldi et al., 1976; Laville, 1989; Laville et al., 1989), use incisors as picks for digging, whether or not they are the primary digging apparatus. Skull adaptations to the digging activity (Cubo et al., 2006), as well as modifications of incisor enamel microstructure (Stein, 2000), have been documented. However, very little is known about the relationships between enamel microstructure and lifestyle in rodents, though already in the mid 19th century Tomes (1850) showed that rodents have two layers of enamel, in contrast to lagomorphs, which have only one layer. As mammalian enamel in general, rodent incisor enamel is composed by hydroxyapatite crystallites (called enamel prisms) that are organized in a variety of structures that originate at the enamel–dentine side and run through the entire thickness of the tissue to the outer enamel surface. The Radial Enamel forms the external layer (*Portio Externa*). It is the simplest of the three main types of tooth enamel (prisms are arranged parallel to one another, radiate from the enamel–dentine junction towards the occlusal surface, and intersect the occlusal surface at approximately 90°). The inner layer (*Portio Interna*) of rodent incisors is usually composed of Hunter-Schreger Bands (the prisms originating at the dentine–enamel border for a given layer are oriented in one way, while those of the surrounding layers are oriented in the other direction) so that, at a high magnification analysis, the surface appears banded, being the bands formed by zones of enamel prisms with different orientations. Enamel is the easiest material to retrieve in the fossil record, due to its high degree of mineralization, and it is rich in information about function and phylogeny (Koenigswald, 1980; Koenigswald, 2004 and references therein). Many studies (Koenigswald, 2004; Martin, 2005; Rensberger et al., 1984) have been published about phylogeny of mammal groups inferred from their tooth enamel structure, also known under the name Schmelzmuster (Koenigswald, 1980). Yet, only a few studies are known about the relationship of enamel microstructure to incisor function in rodents. In particular, Vieytes et al. (2007) have shown that in some South American caviomorph rodents belonging to the family Ctenomyidae, which are fossorial, the thickness of the external Radial Enamel portion differs according to genus and to hardness of the soil. In particular, Vieytes et al. (2007) demonstrated that the higher values of External Index of *Ctenomys talarum* compared with *C. australis* are consistent with the former digging in harder soils. Likewise, among North American geomyid burrowing rodents, higher External Indices (Buzas-Stephens and Dalquest, 1991) are positively correlated with greater tooth-digging specialization, from the primarily scratch-digger *Geomys*, in which Radial Enamel represents only 20% of the total thickness, to the relatively more specialized tooth-diggers *Cratogeomys*, in which it represents 25%, and especially *Thomomys*, adapted to dig in hard soils and for which the thickness of the *Portio Externa* reaches up to 40% in the incisors.

The aims of this study were to determine possible differences in the External Index between semi-aquatic and fossorial populations of *Arvicola*, and if these differences were significant, to use this index as a proxy to infer ecological preferences of extinct forms of this genus. Analyses were performed on eight extant taxa, four fossorial and four semi-aquatic, and on fossil specimens of three extinct populations (one from the Middle and two from the Late Pleistocene). This research constitutes a part of an ongoing project on morphometrics, evolution and phylogeny of extinct and extant representatives of *Arvicola*. In addition to address issues regarding the lifestyle and phenotypic evolution of *Arvicola*, we also provide a review of the current knowledge about *Arvicola* taxonomy and phylogeny in order to give an historical framework for the evolution of lifestyles and incisor enamel evolution in that group.

2. Phylogeny and taxonomy of *Arvicola*

From the 1960s on, it has been generally considered that *Arvicola* includes two extant species (see e.g. Reichstein, 1963; Reichstein, 1982): the south-western water vole, *A. sapidus*, which is found in the Iberian Peninsula and much of France (Baudoin, 1984; Saucy, 2002; Ventura, 2007), and the northern water vole, *A. terrestris*, which extends widely throughout the Palaearctic region (Corbet, 1978). While *A. sapidus* is a semi-aquatic form, it is considered that *A. terrestris* presents two ecological forms: semi-aquatic and fossorial (Kratochvíl, 1983). Musser and Carleton (2005), following Panteleyev (2001) and Zagorodnyuk (2001) reviews on the genus *Arvicola*, have demonstrated that the two long established ecological forms of *A. terrestris* are different taxonomic units, reserving the name of *A. amphibius* (European water vole) for the semi-aquatic populations and *A. scherman* (montane water vole) for the fossorial ones. Nevertheless, positive results on the hybridization between *terrestris* and *scherman* (Bernard, 1961; Müller-Böhme, 1936) and certain molecular studies (Piertney et al., 2005; Taberlet et al., 1998) do not support this taxonomic configuration. Additionally, although the specific name *A. amphibius* has nomenclatural priority over *A. terrestris* (Brink et al., 1967; Musser and Carleton, 2005), the latter seems to have been maintained to avoid "confusion and ambiguity" (see Corbet, 1978). In spite of these controversies we have followed in this study the new taxonomic proposal by Musser and Carleton (2005) in order to contrast it with the results obtained here and give new insights onto this taxonomic problem. The extinct representatives of *A. amphibius,* which were widespread in Europe from the Middle to the Late Pleistocene, have been traditionally considered semi-aquatic as well as its possible ancestor *A. mosbachensis*. Such a statement, however, is uncertain and the ancestor of *A. sapidus*, *A. scherman*, and *A. amphibius* may also have been fossorial since reverse evolution is attested at least for *A. amphibius.* Maul et al. (1998) considered *A. cantianus* as a synonym of *A. mosbachensis*. However, the type material is highly fragmentary and we prefer to consider *Arvicola cantianus* as a *nomen dubium.* This name should therefore not be employed for the taxonomy of fossil specimens.

For extant taxa, Taberlet et al. (1998) found evidence for a sister group relationship among the Italian *A. amphibius* populations (*A. a. italicus*) and all other European populations belonging to *A. amphibius* and *A. scherman*. Later on, Piertney et al. (2005) showed that European populations are structured into two clades: *A. amphibius korabensis*, *A. amphibius terrestris* and *A. amphibius amphibius* on the one hand, and *A. scherman cantabriae*, *A. scherman monticola*, *A. scherman exitus*, *A. scherman scherman* and *A. amphibius reta* on the other hand. These results strongly suggest that *A. amphibius*, as currently recognized, is not monophyletic. The phylogenetic structure of the last group of populations may partly reflect the northward colonization routes of these populations after the end of Marine Isotope Stage (MIS) 2 (approximately after 11 ky BP).

We followed Cuenca-Bescós et al. (2001) for the 400 ky BP divergence time between *A. sapidus* and the other *Arvicola* species. Recently, Centeno-Cuadros et al. (2009), based on a molecular clock, dated the same split at about 250 ky Bp. This result, however, needs a revision because there is paleontological evidence of *A. sapidus* in the early Middle Pleistocene of Iberian Peninsula.

Maul et al. (1998) and Masini et al. (2003) suggested that the divergence between "peninsular Italian arvicolas" (=*A. amphibius musignani*, according to recent systematics, Cagnin, 2008) and the other clade including *Arvicola scherman* + *Arvicola amphibius* occurred between 250 and 200 ky BP. No data exist to set the timing for the divergence node between *A. scherman* and *A. amphibius* (excluding Italian populations); for this reason we considered the results from Maul et al. (1998) that place the differentiation between *Arvicola amphibius terrestris* and the European *Arvicola scherman* at about 125 ky BP. They based this conclusion on the Schmeltzband Differentierungs Quotient (SDQ) ratio, the ratio between enamel thickness measured in the posterior and anterior cutting edges of molar triangles. This age constitutes a lower age limit, but we cannot exclude younger divergence age for this node.

Three extinct populations were placed in the phylogenetic tree according to recent phylogenies and according to their skull and molar morphologies: *Arvicola mosbachensis* from Mosbach (West Germany) and dated at 500Ka BP (Maul et al., 2000), fossil *Arvicola amphibius* from Grotta Breuil (central Italy) dated at about 34Ka BP (Alhaique et al., 1998; Kotsakis, 1991) and fossil *Arvicola scherman* coming from Bouziès (southern France) dated at about 17ka BP (Aguilar et al., 2008). *Arnicola mosbachensis* is generally considered to be the sister-group of all other species of *Arvicola*. Since Signor Lipps effects (sampling bias in the fossil record) may have occurred (Signor and Lipps, 1982), we opted for two strategies concerning the initial splits in *Arvicola* phylogeny. In a first attempt, the split between *A. mosbachensis* and all other *Arvicola* species was dated 100 Ky before the first occurrence *A. mosbachensis,* that is 605 Ky. This phylogenetic scenario (phylogenetic scenario 1) is possible because the first occurrence of *Arvicola* sp. is from

Fig. 1. Location map of the populations of *Arvicola* used in the text. 1. *A. scherman cantabriae. 2. A. sapidus. 3. A. scherman monticola. 4. A. scherman exitus. 5. A. scherman scherman. 6. A. amphibius italicus. 7. A. amphibius musignani. 8. A. amphibius terrestris. 9. A. mosbachensis extinct (Mosbach, Germany). 10. A. scherman* extinct (Bouziès, France). 11. *A. amphibius* extinct (Grotta Breuil, Italy).

Fig. 1. Carte indiquant l'origine des populations citées dans le texte. *1. A. scherman cantabriae ; 2. A. sapidus ; 3. A. scherman monticola ; 4. A. scherman exitus ; 5. A. scherman scherman ; 6. A. amphibius italicus ; 7. A. amphibius musignani ; 8. A. amphibius terrestris ; 9. A. mosbachensis* extinct (Mosbach, Allemagne) ; 10. *A. scherman* extinct (Bouziès, France) ; 11. *A. amphibius* extinct (Grotta Breuil, Italy).

la Isernia La Pineta in central Italy (Sala, 1996). The second scenario considered that *A. mosbachensis* was the ancestor of all other *Arvicola* species (therefore, it is considered as not divergent from that ancestor). The two scenarios are presented in Fig. 2. Based on skull and teeth morphology, the population of *Arvicola* from Bouziès (France) referred here to *A. scherman*, was placed as the sister group of all other extant *A. scherman*, while the Italian extinct population coming from Grotta Breuil and attributed to *A. amphibius* was considered as the sister taxon of the clade (*A. amphibius musignani* + *A. amphibius italicus*).

3. Materials and methods

3.1. *Material*

Our sample includes eight extant and three extinct populations. Lower incisors of thirty two specimens coming from three extant populations of *Arvicola amphibius*, four extant populations of *A. scherman* and one extant population of *Arvicola sapidus* (Fig. 1) were analyzed in order to assess differences, if any, in the external index value (see below). These specimens belong to the following subspecies: *A. amphibius terrestris* (5 specimens; Sleneset Islands, Norway), *A. amphibius musignani* (7 specimens, Sila Grande, Italy), *A. amphibius italicus* (3 specimens; Agno, Switzerland), *A. scherman scherman* (3 specimens; Prangins, Switzerland), *A. s. exitus* (3 specimens; Ally, France), *A. scherman monticola* (3 specimens; Aran Valley, Spain), *A. scherman cantabriae* (3 specimens; Ribadesella, Spain), *A. sapidus* (5 specimens; Ebro River delta, Spain). Moreover, we analyzed the lower incisors of eleven fossil specimens belonging to *A. amphibius* from Grotta Breuil (3 specimens), *A. scherman* from Bouziès (3 specimens), and *A. mosbachensis* from Mosbach (5 specimens).

3.2. *Enamel External Index (EI)*

Teeth have been first cut at half of their length, then ground to expose a flat surface and polished. Specimens were then dehydrated with alcohol and etched for 4–5 s with 10% hydrochloric acid to accentuate topographic detail. Afterwards they were sputter-coated with graphite. Photographs were taken using a Philips Scanning Electron Microscope. Total enamel thickness (ET) and Radial enamel thickness or *Portio Externa* (PE) have been measured, according to Vieytes et al. (2007). An External Index (EI) has been calculated as the ratio between PE and ET (Vieytes et al., 2007) and averaged for each species. Prior to making a comparative analysis of EI values and lifestyle we opted to check whether there was a phylogenetic signal in characters and in that case to use ancestral character reconstruction in order to orient subsequent comparative analyses.

3.3. *Reconstruction of ancestral states and phylogenetic signal*

The phylogeny defined above and presented in Fig. 2 has been used to estimate ancestral state values of Enamel Index as well as for lifestyle for the root and inner nodes of the tree. Lifestyle was considered as varying between two extremes: fossorial *vs.* semi-aquatic. In order to appraise intermediary lifestyle, we attributed a value of 0 for an exclusively semi-aquatic population and a value of 1 for an exclusively fossorial population. We considered all extant species as exclusive fossorial or semi-aquatic, although we are aware that it approximates the reality, and that semi-aquatic forms can perhaps spend time digging burrows using their teeth. The two behaviours appear not to be mutually exclusive; we therefore considered possible continuous variation between these two extremes. Estimation of ancestral character states were obtained first only

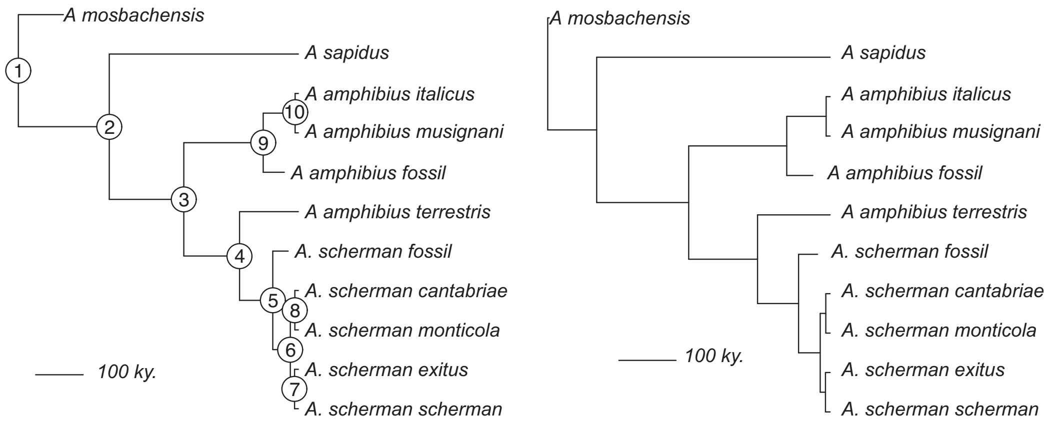

Fig. 2. Phylogenetic relationships among the taxa analyzed in this study based on molecular data (Piertney et al., 2005; Taberlet et al., 1998). Branch lengths were assigned using the paleontological literature (Cuenca-Bescós et al., 2001; Masini et al., 2003; Maul et al., 1998) and are proportional to geological time, in m.y. The two-presented phylogenies differ in the placement of *A. mosbachensis* (on the left: phylogenetic scenario 1, on the right: phylogenetic scenario 2). Nodes labels are used in Table 2.

Fig. 2. Relations phylogénétiques entre les taxons analysés dans cette étude basée sur des données moléculaires (Piertney et al., 2005 ; Taberlet et al., 1998). Les longueurs des branches ont été calculées utilisant la littérature paléontologique (Cuenca-Bescós et al., 2001 ; Masini et al., 2003 ; Maul et al., 1998) et sont proportionnelles au temps géologique en millions d'années. Les deux phylogénies présentent deux scénarios différents concernant la position de *A. mosbachensis* (à gauche le scénario phylogénétique 1 ; à droite, le scénario phylogénétique 2). La numérotation des noeuds est reprise dans le Tableau 2.

using extant taxa, since lifestyles of extinct species cannot be observed. Prior to performing estimations of ancestral values, we tested several evolutionary models in order to assess the best model for character evolution. Possible models are Brownian motion model where the variance of a character is proportional to branch length; Ornstein-Uhlenbeck, that fits a model of random walk with a central tendency with a strength proportional to a given parameter *alpha*; Pagel's *lambda* multiplies all internal branches of the tree by a given parameter *lambda*, leaving tip branches as their original length; Pagel's *kappa*: it raises all branch lengths to the power *kappa*; when kappa approaches zero, the model becomes speciational; Pagel's *delta*; raises all node depths to the power *delta*; if delta is less than one, evolution is concentrated early in the tree; *delta* greater than 1 concentrates evolution towards the tips; ACDC models: set by the "*eb*" parameter, it fits a model where the rate of evolution increases or decreases exponentially through time, under the model $r(t) = rho \times \exp(r \times t)$, where ro is the initial rate and r is the rate change parameter; white noise model: no phylogenetic signal.

The "GEIGER" package for R (Harmon et al., 2008) has been used to estimate the best fit among various model using Akaike Information Criterion (AIC) and the parameters for each model. The original tree was eventually transformed according to the parameters of the best model found. On this transformed tree EI ancestral states were estimated via maximum likelihood procedure using "ape" package for R v. 2.5-3 (Paradis et al., 2004).

3.4. Comparative analyses

Results of this previous analysis oriented our selection for a comparative analysis including the phylogeny, transforming the phylogeny, or excluding the phylogeny (if no phylogenetic signal was detected in the traits). In this latter case (no phylogenetic signal), we applied a simple generalized linear model of lifestyle on EI in order to check whether there was an association between the behavioural trait and the morphological one.

4. Results

4.1. Enamel External Index (EI)

Mean and standard deviation for EI values among populations are shown in Table 1. Mean EI values were below 22 for all semi-aquatic extant species or populations and always above 25 for all living fossorial populations or species (Fig. 3). The range of mean EI values is wider for fossorial (25 to 40) than for semi-aquatic populations (18 to 22). Within populations, variation in EI value can be important, especially for *A. scherman cantabriae*, which also shows the largest EI values. When all living populations are considered together (semi-aquatic and terrestrial), interpopulation variation is significantly larger than intra-population variation (F(10.33) = 7.3; $P < 0.001$). However, when lifestyle is taken into account, interspecific variation is significantly larger than intraspecific variation only for fossorial populations (F(3.8) = 11.1; $P < 0.005$) and no longer in semi-aquatic populations (F(3.16) = 0.3, $P = 0.79$). Moreover, intrapopulation variation was found to be similar between semi-aquatic and fossorial populations (F(16.8) = 1.21, $P = 0.8$), while interpopulation variation was greater for fossorial populations (F(3.3) = 26.7, $P = 0.011$). If we consider population as a random effect, the variation of EI values is significantly explained by the lifestyle (F(1.6) = 7.85, $P = 0.03$). The EI value of the *A. scherman* extinct population from Bouziès (Fig. 4A) equals to 35.97; therefore it is similar to that observed in the fossorial *A. s. cantabriae*. In contrast, the *A. amphibius* extinct population from Grotta Breuil has a very low EI (18.87) similar to the lowest values among extant semi-aquatic populations. However, the Grotta Breuil specimens are slightly eroded in their external layer and the real value could be slightly larger. Finally, EI value of the extinct population *A. mosbachensis* (the oldest population), equals 23.66. *A. mosbachensis* (Fig. 4B) has therefore an EI value intermediate between semi-aquatic and fossorial populations (Table 1); it should also be noted that for this taxonomic unit, EI values were variable among individuals (ranging from 18.43 to 33.96).

Table 1
Sampling size (*n*), mean and standard deviation (sd) for EI values, and lifestyle for living populations (1: fossorial, 0: semi-aquatic).

Tableau 1
Taille de l'échantillon (*n*), moyenne et écart-type (sd) pour les valeurs du EI, et mode de vie des populations actuelles (1 : fouisseur, 0 : semi-aquatique).

Species	*n*	EI (mean)	EI (sd)	Lifestyle
A. sapidus	5	19.76	5.01	0
A. amphibius italicus	3	21.86	1.96	0
A. scherman cantabriae	3	39.62	6.23	1
A. scherman scherman	3	25.46	1.69	1
A. scherman exitus	3	26	2.27	1
A. scherman monticola	3	25.03	2.66	1
A. amphibius terrestris	5	18.9	2.75	0
A. amphibius musignani	7	20.15	4.53	0
A. scherman fossil	4	35.97	9.13	–
A. mosbachensis fossil	5	23.66	6.31	–
A. amphibius fossil	3	18.87	1.64	–

4.2. Ancestral states reconstructions.

The AIC comparison between the different models of character evolution demonstrated that the White Noise model was the best for EI evolution along the phylogeny including only extant forms. This suggests that there is no phylogenetic signal in EI (that this trait is very labile). The same result was found when extinct forms were included using the first phylogenetic scenario; on the other hand, the best model was Pagel's *lambda* with *lambda* equals 0.160 for the second phylogenetic scenario. Since Pagel's *lambda* is low, this model shows that most of the character evolution is concentrated on terminal branches rather than on internal branches (i.e., that we are not far from a White Noise model). The result obtained with the second scenario can also be interpreted as an artifact coming from errors concerning the divergence time of *A. mosbachensis* from other *Arvicola* species. Evolution of lifestyle followed a Brownian Motion model in the tree with only extant taxa.

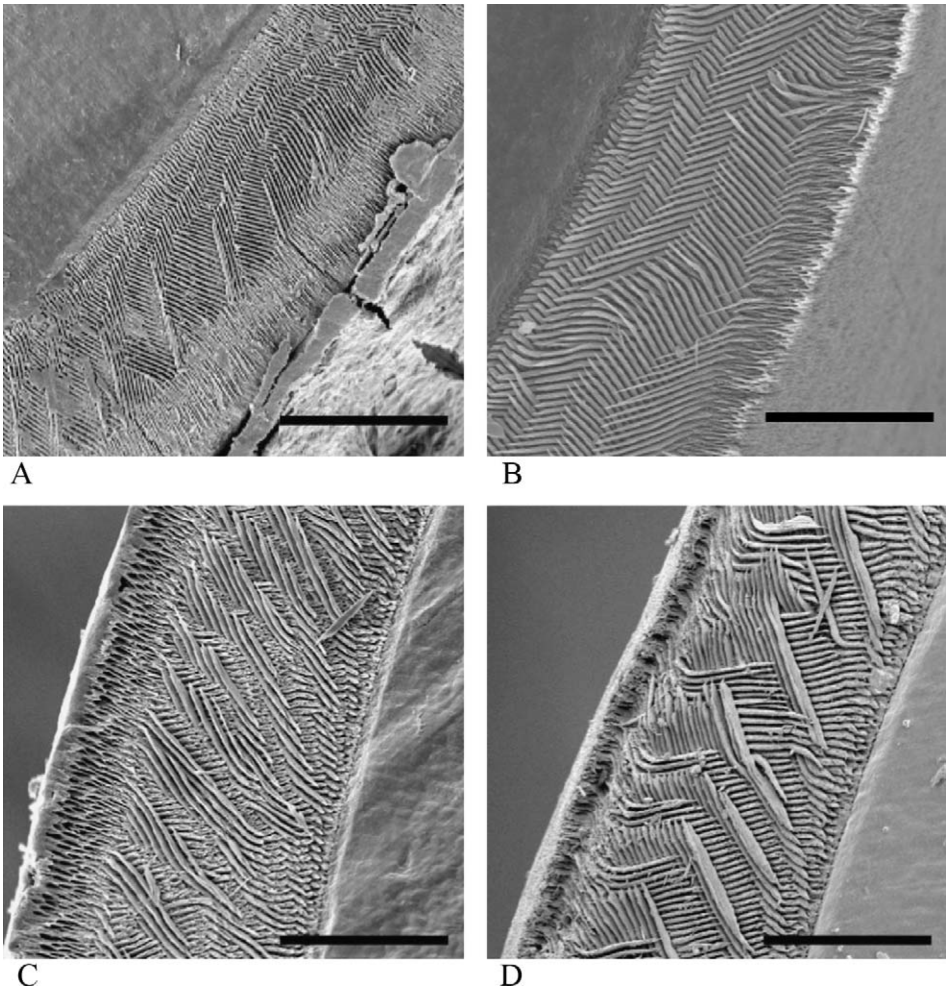

Fig. 3. SEM photographs of enamel microstructure of extant *Arvicola* lower incisors (cross sections in A and B, outer tooth surface at right, enamel–dentine junction at left; in C and D: outer tooth surface at left, enamel-dentine junction at right). **A**: *A. scherman cantabriae*; **B**: *A. amphibius musignani*; **C**: *A. scherman scherman*; **D**: *A. sapidus*. Scale bar represents 50 μm.

Fig. 3. Photographies de sections transversales d'incisives inférieures d'*Arvicola* actuels (surface externe de la dent à droite, jonction émail - dentine à gauche dans A et B; surface externe de la dent à gauche, jonction émail - dentine à droite dans C et D) obtenues par microscopie électronique à balayage. **A** : *A. scherman cantabriae* ; **B** : *A. amphibius musignani* ; **C** : *A. scherman scherman* ; **D** : *A. sapidus*. L'échelle représente 50 μm.

4.3. Comparative analyses

The inference for extinct lifestyle can be made only using a model based on extant taxa. No phylogenetic signal was found for the EI indices using a phylogeny including only extant taxa or our first phylogenetic scenario including fossils. We therefore tentatively opted to check whether lifestyle could be predicted by EI indices of populations using a generalized linear model (glm). We used a binomial family for the response because living forms were considered as exclusively fossorial or semi-aquatic. The model was highly significant with a residual deviance of nearly 0 ($P < 0.001$). Lifestyles were predicted using this logistic model as corresponding to scores on a zero to one scale (0 corresponding to exclusive semi-aquatic populations, and 1 to exclusive fossorial populations). On this scale, values of 0.958, 1, and 0 were found for populations of *A. mosbachensis*, *A. scherman* from Bouziès, and *A. amphibius* from Grotta Breuil. While Grotta Breuil fossils can be considered as semi-aquatic and Bouziès as fossorial with some confidence, uncertainties remain for *A. mosbachensis*. Indeed, one should consider that there is a great uncertainty with *A. mosbachensis* because no living populations were sampled with values between 0.22 and 0.25, the interval that separates semi-aquatic from fossorial populations. There is therefore a large uncertainty on the inflexion of the logistic curve. We therefore also interpolated the lifestyle score of *A. mosbachensis* using a linear interpolation between the semi-aquatic and fossorial populations that got the closest EI indices of that extinct populations. This gave a 0.567 score for that population, which may better reflect the aforementioned uncertainty. In order to incorporate inferences from fossil specimens in the phylogeny, we used their lifestyle estimates and we predicted ancestral

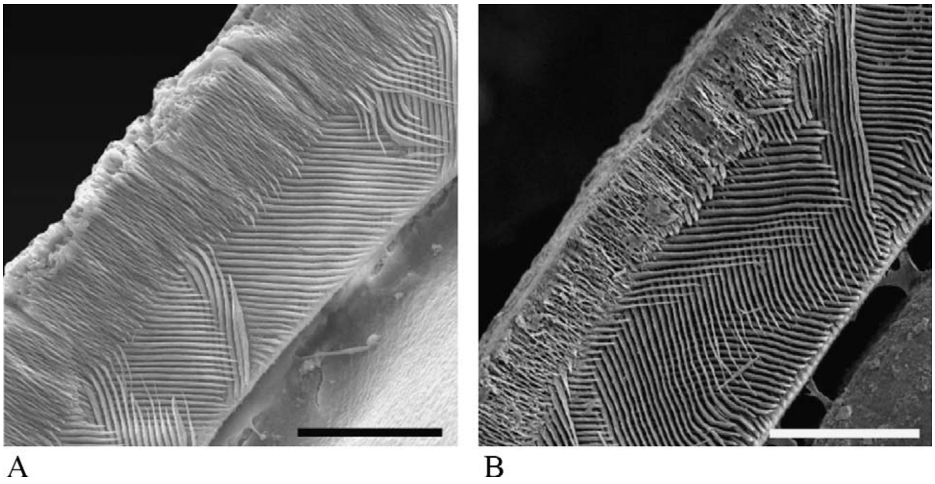

Fig. 4. SEM photographs of enamel microstructure of fossil *Arvicola* lower incisors (cross sections, outer tooth surface at left, enamel–dentine junction at right). **A**: *Arvicola scherman* extinct (Bouziès, France); **B**: *A. mosbachensis* extinct (Mosbach, Germany). Scale bar represents 50 μm.

Fig. 4. Photographies de sections transversales d'incisives inférieures d'*Arvicola* fossiles (surface externe de la dent à gauche, jonction émail – dentine à droite) obtenues par microscopie électronique à balayage. **A** : *Arvicola scherman* extinct (Bouziès, France) ; **B** : *A. mosbachensis* extinct (Mosbach, Germany). L'échelle représente 50 μm.

Table 2

Ancestral character reconstructions using different phylogenetic scenarios and estimates of *A. mosbachensis* lifestyle. AC1: ancestral character estimation of lifestyle using living taxa only. AC2: ancestral character estimation using both living and fossil taxa according to the first phylogenetic scenario and the logistic model for inferring *A. mosbachensis* lifestyle. AC3: Same as AC2, but using phylogenetic scenario 2. AC4: same as AC2 but using a linear interpolation for *A. mosbachensis* lifestyle. AC5: same as AC4 but using phylogenetic scenario 2. 0 corresponds to an exclusive aquatic lifestyle, while 1 corresponds to an exclusive fossorial lifestyle. 95% confidence half intervals are provided.

Tableau 2

Reconstruction des modes de vie ancestraux utilisant différents scénarios phylogénétiques et estimations du mode de vie de *A. mosbachensis*. AC1 : Reconstruction des modes de vie ancestraux utilisant seulement des taxons actuels. AC2 : Reconstruction des modes de vie ancestraux utilisant des taxons actuels et fossiles et assumant le scénario phylogénétique 1 et le modèle logistique pour inférer le mode de vie de *A. mosbachensis.* AC3 : comme dans AC2, mais utilisant le scénario phylogénétique 2. AC4 : comme dans AC2, mais utilisant une interpolation linéaire pour inférer le mode de vie de *A. mosbachensis.* AC5 : comme AC4, mais utilisant le scénario phylogénétique 2. 0 correspond à un mode de vie exclusivement aquatique, tandis que 1 correspond à un mode de vie exclusivement fouisseur. Des intervalles de confiance 95 % sont fournis.

Node labels	AC1	AC2	AC3	AC4	AC5
1		0.80 ± 0.26	0.96 ± 0.00	0.57 ± 0.17	0.57 ± 0.00
2	0.18 ± 0.40	0.46 ± 0.30	0.54 ± 0.81	0.21 ± 0.93	0.30 ± 0.81
3	0.25 ± 0.30	0.37 ± 0.25	0.29 ± 0.47	0.26 ± 0.47	0.27 ± 0.47
4	0.43 ± 0.22	0.52 ± 0.19	0.50 ± 0.17	0.49 ± 0.18	0.50 ± 0.17
5		0.90 ± 0.12	0.99 ± 0.03	0.99 ± 0.03	0.99 ± 0.03
6	0.97 ± 0.08	0.98 ± 0.07	0.99 ± 0.01	0.99 ± 0.01	0.99 ± 0.01
7	0.99 ± 0.06	0.99 ± .05	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
8	0.99 ± 0.06	0.99 ± .05	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
9		0.05 ± 0.10	0.00 ± 0.05	0.00 ± 0.05	0.00 ± 0.05
10	0.00 ± 0.06	0.02 ± 0.06	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

character states for nodes in the phylogenetic tree according to the different scenarios and inferences for *A. mosbachensis*. The best model using GEIGER package was the brownian model for the first phylogenetic scenario when the value of *A. mosbachensis* was 0.958, while it was Pagel's *delta* with *delta* equal to 2.999 for the second phylogenetic scenario for both values of *A. mosbachensis* and for the 0.567 value of *A. mosbachensis* with the first phylogenetic scenario. Ancestral character states for lifestyles are given in Table 2. Results show similarities with the tree obtained only with extant taxa, except for deep nodes. In all the models, the ancestral lifestyle for the deep nodes are highly uncertain while ancestral lifestyles for more recent nodes are consistent between methods and have narrower confidence intervals. The inclusion of fossils would rather favor a more fossorial ancestor for all living populations, but the uncertainties around ancestral character state are too important for clearly supporting this conclusion.

5. Discussion

Previous phylogeographic studies (Piertney et al., 2005; Taberlet et al., 1998) allowed us to place our results in a phylogenetic framework (Fig. 2). In this context, *Arvicola amphibius* (as defined by Zagorodnyuk, 2001) is paraphyletic: the last common ancestor of all subspecies of *Arvicola amphibius* is also the ancestor of all subspecies of *Arvicola scherman* (Fig. 2).

When considering only extant forms, no phylogenetic signal was found on EI, suggesting that this feature is highly labile during evolution. However, lifestyle was clearly related to phylogeny showing that this trait may evolve more slowly during evolution. In addition, we have found that despite of the different evolutionary behaviour of both characters, there is a significant relationship between the average EI values and lifestyle, fossorial populations showing significantly higher values than semi-aquatic ones: EI values show an important variation, especially in fossorial populations; in addition, there are differences in EI values among fossorial populations. We suggest that this measure can be taken as a proxy for predicting the fossorial activity of *Arvicola*. The high degree of variation within fossorial populations and the weak relation with phylogeny suggest that this character has a great phenotypic plasticity; however, this still need to be tested by ecological observations and experimentation.

Considering that there is a relationship between EI and lifestyle at the population level, we used this histological trait to infer the lifestyle of the three fossil populations included in our sample. The sample of *Arvicola scherman* coming from the fossil-bearing locality of Bouziès (Fig. 4A) has an EI of 35.97, almost as high as that of *A. scherman cantabriae* (39.62), thus suggesting that these animals were fossorial. Such a hypothesis is sustained also by observations on some cranial fragments recovered in the site showing forwardly directed upper incisors, that further support the idea of a burrowing population and an attribution of the Bouziès population to *A. scherman*. In contrast, the *A. amphibius* specimens from the Grotta Breuil have an EI of 18.87, close to the lowest values of living semi-aquatic populations, suggesting a semi-aquatic lifestyle. The EI value of the oldest fossil population of our sample (Fig. 4B), coming from the Middle Pleistocene site of Mosbach, is 23.66 and its lifestyle is more difficult to determine because this population is intermediate between the upper limit for semi-aquatic population and the lower limit for fossorial populations. Since our sampling of *Arvicola* is small at the population level, there are great uncertainties concerning the lifestyle of the Mosbach population. These uncertainties are also high because this population shows an important variation in EI index, suggesting that some individuals were digging while some other were not. Therefore, although our inferences are somewhat uncertain for this population, the lifestyle of *A. mosbachensis* may be considered as intermediate between that of extant ones, or as uncertain. It is also possible that this species may have had a lifestyle with no extant analogue (at least when one considers the population we have sampled). Whether or not fossil populations are included, the estimation of character state for lifestyle for the common ancestor of recent clades is consistent among methods: the ancestor of *A. amphibius terrestris* + populations of *A. scherman* ssp was intermediate between semi-aquatic and fossorial, while the ancestor of *A. amphibius italicus* and *A. amphibius musignani* was semi-aquatic. However the ancestral character reconstruction for deeper nodes is more uncertain: the value for the most recent common ancestor of all *A. amphibius* and *A. scherman* was intermediate between semi-aquatic and fossorial population, but in average closer to the "semi-aquatic pole". Including or not fossils did not change significantly this value. The value of the most common ancestor of all living species or population of *Arvicola* is more uncertain, but seems to be closer to semi-aquatic populations, meaning that fossorial adaptation are secondary in *A. scherman* populations. Inclusion of extinct forms did not help to reduce uncertainties, but suggested that this ancestor may have had a lifestyle intermediate between semi-aquatic and fossorial. This, however, is speculative and one would need more fossil populations and living analogues to test our hypotheses. Moreover, uncertain position of fossils and uncertainties in topologies and branch lengths still remain in the phylogeny. Including the sister groups of *Arvicola* (*Microtus*, *Myodes*, and *Ondatra*) where both lifestyles are also known to occur, could probably shed some more lights concerning the evolution of a semi-aquatic or fossorial lifestyle and its impact on enamel histology of rodents.

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