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Research Article

The Italian peninsula hosts a divergent mtDNA lineage of the water vole, *Arvicola amphibius s.l.*, including fossorial and aquatic ecotypes

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Abstract

We characterized eighteen water voles, *Arvicola amphibius* (*s.l.*), from five populations along the Italian peninsula by means of mtDNA cytochrome b (Cytb) sequences. The samples included aquatic voles and one fossorial population from northern Italy. The standard karyotype of four voles from one central Italian population was also analysed and was identical to the one found in other populations outside Italy. Phylogenetic analyses, including vole Cytb haplotypes from the entire range, indicated the existence of a wellsupported and highly divergent Italian lineage (4.3%), sister to all the other haplotypes. The fossorial voles are not genetically differentiated from the aquatic voles from a nearby population and belong to the same taxon. Given the high Cytb divergence and the results of previous investigations on allozymes and hybrid fertility, we believe that the Italian population of water voles belongs to a distinct species, *Arvicola italicus* Savi, 1838, with the type locality near Pisa, although a morphological assessment of the entire skull is necessary to define it.

Introduction

Water voles (genus *Arvicola* Lacépede, 1799) are characterized by an extraordinary morphological and ecological plasticity. Two ecological types (ecotypes) can be distinguished, the aquatic and the fossorial, each with different living habits (Meylan, 1977). These ecotypes are often morphologically distinct (Meylan, 1977) and this variability has caused a long-standing dispute concerning the species limits within the genus. The two extreme positions were exemplified by the clumping of the different forms under one nominal species *A. terrestris* (Linnaeus, 1758) (Ellerman and Morrison-Scott, 1951) versus the recognition of seven species (for western Europe alone) by Miller (1912) .

The more recent revision by Musser and Carleton (2005) divided the genus into three species. Two of them are aquatic: *A. sapidus* (2n=40) (Miller, 1908), endemic to the Iberian Peninsula and France, and *A. amphibius* (2n=36), stretching from western Europe to the River Lena in eastern Siberia (Shenbrot and Krasnov, 2005; Batsaikhan et al., 2008). The third species, the fossorial *A. scherman* (2n=36) (Shaw, 1801), is mainly distributed in the upland regions of northern Spain, the Pyrenees, the Alps, the mountains of central Europe and the Carpathians, but is also recorded in plains and hilly areas (Cagnin , 2008).

A recent morphological and molecular phylogenetic study including fossorial and aquatic water voles from various regions of their European and Asiatic range (Kryštufek et al., 2015) lay the basis for a profound change in the taxonomy of *Arvicola*, which cannot be summarized better than the title of the work itself: "Fossorial morphotype does not make a species in water voles". Briefly, the mtDNA phylogenetic reconstructions of the extended sample failed to identify two reciprocally monophyletic groups matching *A. amphibius* and *A. scherman*. Instead they revealed a major clade, hereafter called *A. amphibius sensu lato* (*s.l.*) subdivided into two lineages. One lineage is distributed from Europe to western Siberia (herein called Euroasiatic clade) and includes both aquatic and fossorial water voles. The second lineage includes a few populations of strictly fossorial Swiss voles (herein called Western European clade) (Fig. 1). This pattern renders *A. scherman* sensu Musser and Carleton (2005) undeserving of species status.

In this context, the taxonomic position of Italian *Arvicola* populations was neglected. Aquatic voles are distributed with scattered populations throughout the Italian Peninsula. A previous genetic analysis based on cytochrome b (Cytb) mtDNA genes indicated the presence of divergent haplotypes in the peninsula (Taberlet et al., 1998). However the exact location of the sampled population was not indicated and the relative sequences were not deposited in public databases.

The need of more precise genetic information on Italian voles is shown by the current taxonomy, which considers the aquatic voles living in Italy as belonging to two endemic taxa (Gippoliti, 2012) deemed to be subspecies (Cagnin , 2008): "*A. amphibius italicus*" Savi, 1839, restricted to the central-northern part of Italy, and "*A. amphibius musignani*" de Sélys Longchamps, 1839, thought to be distributed in central-southern Italy. Strictly fossorial voles ("*A. scherman*") are known only from a few localities in north-eastern Italy (Lapini and Paolucci, 1994)

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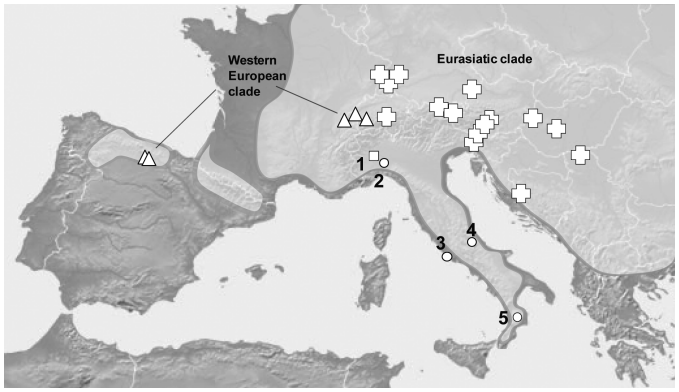

Figure 1 – The distribution of *Arvicola amphibius s.l.* in Europe (light grey). Triangles and crosses indicated the distribution of the Western European clade and of the Euroasiatic clade respectively. White square and dots indicated the Italian respectively fossorial and acquatic samples analysed in this study.

and recently in Piedmont, north-western Italy (Capizzi and Santini, 2007; Bertolino S., unpublished data).

In this study we characterized populations of *Arvicola amphibius s.l.* along the Italian peninsula through molecular (mtDNA Cytb) and cytogenetic markers. The samples included aquatic voles and the newly found fossorial vole population from Piedmont. The aim was to assess the phylogenetic position and the genetic and karyotypic divergence of Italian voles with respect to the other European lineages. Moreover, we analyzed the morphology of the Italian fossorial population to deepen the phenetic diversity of ecotypes taking into account their genetic relationships.

Methods

Specimens and morphometry

Eighteen voles were trapped in five populations along the Italian Peninsula (Fig. 1, Tab. 1), namely four aquatic (localities 2-5) and one fossorial population (locality 1). Notwithstanding the low number of sampled populations, their locations should represent the main genetic lineages occurring in the peninsula (Randi, 2007).

Aquatic voles were captured with Sherman live-traps set irregularly in the study areas. Individuals of the aquatic populations were captured along the banks of canals or other waterways. The fossorial population in Piedmont was sampled in an apple orchard by means of live traps placed at the entrance of the openings to their burrows. A tissue sample was collected from the ear flap with a biopsy punch and stored in 95% ethanol. Other fossorial animals were provided by farmers, sampled for DNA analysis and stored at -20°C. When captured alive, individuals were set free after tissue sample collection.

In order to better characterize the fossorial voles found in Italy the length of head and body (HB), the tail length (TL), the hind foot length (HF) and maxillary tooth row length (MxT) and incisor orientation were compared with data available in literature. Linear measurements were taken on eight fossorial specimens from Piedmont using a dial caliper to the nearest 0.1 mm.

Kryštufek et al. (2015) summarized the morphological studies performed so far: the aquatic voles usually shows orthodont upper incisors and attains larger dimensions (H&B, length of head and body > 165 mm; HF, Hind foot length $\geqslant$ 28 mm; MxT, maxillary tooth row length $\geqslant$ 9 mm) and a longer tail (TL > 98 mm) with respect to the fossorial one. The fossorial ecotype was always found to be smaller (H&B $\leqslant$ 160 mm, HF $\leqslant$ 27 mm, MxT $\leqslant$ 9 mm), with a shorter tail (TL $\leqslant$ 98 mm) and protruding (proodont) incisors.

DNA methods

Total genomic DNA was extracted from tissue preserved in 80% ethanol using the DNeasy tissue kit (Qiagen) according to the manufacturer's recommendations.

Table 1 – Details of the specimens used for the analysis: localities, morphotypes and length of the sequenced Cytb fragment.

Locality – n° on map	N	Morphotype	Karyotype	Cytb
Verzuolo (Piedmont) – 1	8	Fossorial	–	1081bp
San Genuario (Piedmont) – 2	2	Aquatic	–	647bp
Fondi (Latium) – 3	4	Aquatic	yes	647bp
Popoli (Abruzzo) – 4	2	Aquatic	–	1081bp
Sila Grande M.ts (Calabria) – 5	2	Aquatic	–	1081bp

The Cytb sequences were isolated with universal primers L14723 and H15915 (Irwin et al., 1991). A shorter fragment was amplified from more problematic samples with a new designed reverse primer H15408M arv (5' – TGA AAG GG ATT TTA TCT GC – 3') used together with 14727-SP (5' – GAC AGG AAA AAT CAT CGT TG – 3') (Jaarola and Searle, 2002) for amplification. Amplifications were performed by polymerase chain reaction (PCR) in a Biometra Thermocycler machine. The resulting sequences ranged from 647 to 1081 bp (see Tab. 1, accession numbers LT546145-62, European Nucleotide Archive - ENA)

Phylogenetic analyses

In order to build a comprehensive phylogenetic tree, we downloaded 68 Cytb sequences of *A. amphibius s.l.* from GenBank: KM004997-KM005047 (Kryštufek et al., 2015), HQ728467-78 (Schlegel et al., 2012), JX457750-51 (Barbosa et al., 2013), AY332708-9 (Pfunder et al., 2004), GU954310 (Fink et al., 2010). Three sequences belonging to *Arvicola sapidus* (FJ539342), *Myodes rutilus* (KJ789562) and *Chionomys nivalis* (AY513848) were also downloaded and used as the outgroup.

The alignment of 1081bp includes 90 sequences (with 19 Cytb sequences new to Italy). The aligned sequences were used to build maximum likelihood (ML) and Bayesian phylogenetic trees under the assumption of a HKY model of sequence evolution considering gamma rate of substitutions and the proportion of invariable sites. This model was chosen among 54 evolutionary models using the software ModelGenerator v85 (Keane et al., 2006). The ML phylogenetic tree was obtained with PhyML 3.0 (Guindon et al., 2010). Node support was obtained by means of bootstrap re-sampling (1000 replicates). The Bayesian tree was obtained with the software MrBayes v3.2.1 (Ronquist and Huelsenbeck, 2003). Two independent runs were performed (5,000,000 generation sampling every 1000 generations). The 10,000 retained topologies were used to obtain a consensus tree after the first 25% of topologies were discarded.

Karyotype

The karyotypic analysis was performed on four specimens captured in Fondi (central Italy, locality 3). Somatic metaphase plates were obtained from cell lines established from ear clippings by conventional procedures. Photographs of the metaphases were captured with a Photometrics Sensys 1400 digital camera and IPLab software (Scanalytics, Inc).

Results

Phylogenetic analyses

The resulting phylogenetic tree (Fig. 2) revealed three main clades within *A. amphibius s.l.* The first clade, highly supported by both bootstrap and posterior probabilities, clusters all the specimens from Italy (eleven haplotypes) including both the aquatic and fossorial ecotypes. This "Italian clade" can be divided into two additional lineages, the first including both fossorial and aquatic voles from Piedmont and the second clustering haplotypes from central Italy and Calabria (Fig. 2).

The other two clades are reciprocally monophyletic and include all the other voles from outside Italy. The topology obtained for non-Italian voles mainly corresponds to the one found by Kryštufek et al. (2015). The first clade includes European, Turkish and Siberian indi-

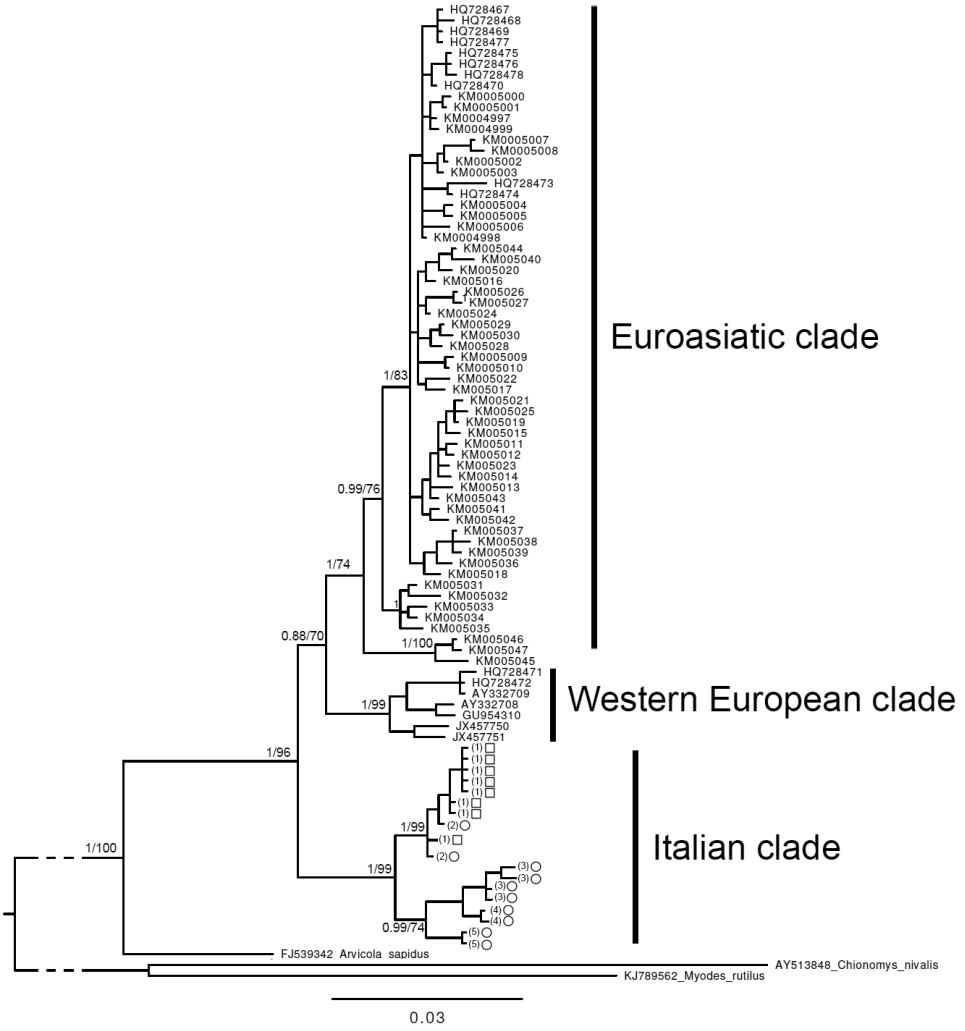

Figure 2 – Maximum likelihood phylogenetic tree of water vole Cytb sequences. The three main clades are indicated. In the Italian clade, voles with aquatic and fossorial morphotypes are indicated by squares and circles respectively; sampled locality (see map in Fig. 1) is indicated in brackets. Numbers at nodes indicate Bayesian posterior probability and ML bootstrap values respectively.

viduals (Euroasiatic clade), while the second includes Swiss specimens and two haplotypes from Spain previously not included in the phylogenetic tree by Kryštufek et al. (2015) (Western European clade).

The genetic divergence (between group mean p-distances) is 4.3% between the Italian and its sister clade and 4.0% between the Euroasiatic and Western European clades. The genetic divergence between the two Italian lineages is 2.3%.

Karyotype

The standard karyotype of the four specimens from Fondi (central Italy) consists of 36 chromosomes (Fig. 3). The autosomal complement includes 13 metacentric/submetacentric pairs (nos. 1–13), and four acrocentric/subtelocentric pairs (nos. 14–17). The first eleven pairs of meta- and submetacentric autosomes are large or medium-sized, whereas two metacentric autosomal pairs (nos. 12, 13) are clearly smaller. One subtelocentric pair of autosomes is medium-sized, while the other acrocentric/subtelocentric autosomes includes small elements. The X and Y chromosomes were identified as a medium-size submetacentric and a small acrocentric respectively.

Morphometrics

All the fossorial voles from Piedmont showed unexpected orthodont upper incisors, typical of the aquatic ecotypes. In Tab. 2, the morphometric characters for these specimens and for other Italian aquatic voles retrieved from literature are reported. The fossorial ecotype found in Italy shows larger average values for two linear measurements taken (HB=172–188 mm; HF=27–32 mm) with respect to the Italian aquatic ecotype. However, if we consider the range of the morphometric characters investigated, the aquatic voles can reach much larger values than the fossorial ecotype. The tail of the fossorial ecotypes was very short (range 52–89 mm) similar to the one characterizing the strictly fossorial voles (TL$\leqslant$98 mm) with an average ratio TL/HB of 41.77%.

Discussion

The Cytb phylogenetic analysis confirms the previous indication (Taberlet et al., 1998) of a well supported and highly divergent Italian lineage. The genetic divergence between the Italian clade and the sister clade (4.3%) is slightly higher than that between the other two clades (Euroasiatic and Western European). The overall phylogenetic pattern suggests early isolation of the Italian population from the other European populations of *Arvicola amphibius s.l.*

The biochemical polymorphism pattern reported by Saucy et al. (1994) strongly agrees with the distinctiveness of the Italian voles indicated by our mtDNA phylogeny. The genetic distances (calculated over 36 loci) between populations from Italy and ones from central and western Europe (range 0.029–0.038) are, on average, at least twice as large as the largest genetic distances found among aquatic and fossorial populations from central and western Europe (0.015). This suggests that the genetic distinctiveness of the Italian lineage goes beyond the matrilinear lineage, mainly related to the time of divergence, and is rather scattered along the entire genome.

The observed genetic differences seem to be restricted to the gene level whereas we did not find any evidence of large genomic changes at the chromosomal level. Indeed the diploid number (2n=36) and the

Table 2 – Morphometry, mean±ds (range), of fossorial voles from Piedmont compared to aquatic and fossorial voles from peninsular Italy. The morphometric distinction between aquatic and fossorial ecotype followed Kryštufek et al. (2015). Length of head and body (H&B), tail length (TL), hind foot length (HF), maxillary tooth row length (MxT). References: 1, present data; 2, Cagnin (2008); 3, Lapini and Paolucci (1994); 4, Kryštufek et al. (2015).

	Ref.	H&B	TL	HF	MxT
Fossorial (Piedmont) (N=8)	1	177.75±7.89 (172–188)	74.25±12.71 (52–89)	29.62±1.60 (27–32)	9.2±0.44 (8.6–10.0)
Aquatic (Italy) (N=137)	2	159.66±20.08 (115–201)	93.87±11.86 (62–120)	29.18±1.95 (23–37)	–
Fossorial (North Eastern Italy) (N=1)	3	109.6	55	23.4	7.3
Aquatic ecotype	4	>165	>98	>28	⩾9
Fossorial ecotype	4	⩽160	⩽98	⩽27	⩽9

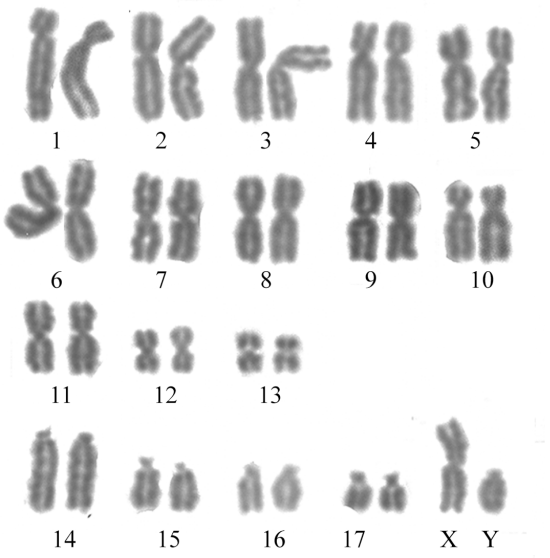

Figure 3 – The standard karyotype of a water vole (female) from Fondi (Latina).

autosomal morphology of specimens representative of the Italian clade from central Italy (Fondi, locality 3) were identical to those found thus far in populations belonging to *A. amphibius s.l.* The Y chromosome of the Italian population is small, similar to the one recorded in other European populations, as well as in western Siberia and Azerbaijan (reviewed in Özkurt et al., 1999; Arslan et al., 2011), and different from the larger Y chromosome found in Russia and northern Spain (Diaz de la Guardia and Pretel, 1979).

Baker and Bradley (2006) suggested that taxa having a genetic distance between allopatric or parapatric phylogroups equal or greater to the mean value found for sister species belonging to the same genus or family should be considered cryptic new species. The level of Cytb genetic divergence between the Italian lineage and the other *Arvicola amphibius s.l.* (4.3%) is lower than the divergence between *A. sapidus* and *A. amphibius s.l.* (7%, present work) but comparable to the divergence commonly found between sister species in other genera of Arvicolinae. More specifically, Amori et al. (2009) found a mean genetic divergence of 5.7% in 11 pairs of sister species in voles of the allied genus *Microtus*. Similar values have been found in five sister species of *Myodes* and *Alticola* (mean 4.7%, range 2.6–6.9, Tab. 1 in Kohli et al., 2014).

Moreover, laboratory crosses between fossorial animals from northwestern Switzerland (possibly belonging to the Western European clade) and aquatic water voles from south of the Alps (possibly belonging to the Italian clade) showed a certain level of reproductive isolation. In a study on fertility of laboratory reared hybrids Morel (1979) showed that only few couples (5/24) formed by parental individuals gave rise to litters. Moreover, most of the hybrid males (7/8) showed incomplete spermatogenesis with absence of spermatozoans in the testes and the epididymis. Finally, almost all the pairs (16/17) including one F1 hybrid and one parental individual (i.e., backcross) did not produced litters .

Given the high genetic divergence in Cytb (present data) and allozymes (Saucy et al., 1994) and the partial reproductive barrier (Morel, 1979), we believe that the Italian population of water voles belongs to a distinct species. The first available name for the Italian water vole is *Arvicola italicus* Savi, 1838, with the type locality near Pisa (Gippoliti, 2012). It should be underlined that the absence of chromosomal differentiation of the Italian lineage does not necessarily hamper a taxonomic delimitation. In fact, different rodent species do not always display different karyotypes (Castiglia, 2014) and thus the presence of the same karyotype cannot be taken as proof of conspecificity.

The new species would be endemic to Italy. This raises questions related to the conservation status, which should be re-established (Bertolino et al., 2014, 2015), and the legal protection of the species, which does not enjoy any protection at the moment (Bertolino et al., 2015). We observed a certain degree of genetic divergences within the Italian sample, with two well supported lineages (Northern and Southern-Central). However the number of sampled localities is too low to assess their distribution and this topic deserves additional studies.

The morphological distinction between aquatic voles and those from other localities has not been thoroughly analysed. Miller (1912) noted that their skull was rather similar to the one from other European localities "...but brain-case is deeper and noticeably longer in proportion to its breadth...". Recent analyses of the lower first molar yielded contrasting results. Piras et al. (2012) reported that shape variation is not phylogenetically structured and that molar morphology was mainly influenced by the climate, whereas Masini et al. (2003) found a certain morphological distinctiveness of Italian voles. There is clearly a need of additional comparative morphological analyses of Italian water voles and those from other localities.

The fossorial voles (herein from Piedmont) should be attributed to *Arvicola italicus* since they are not genetically differentiated from the two aquatic voles from a nearby population. This pattern is similar to the one found by Kryštufek et al. (2015) in which aquatic and fossorial voles were scattered along the same Euroasiatic clade. This confirms the observation that two different ecologies are expressed in related populations and that the shift in habitats occurs very rapidly in response to ecological opportunities (Panteleyev et al., 1978). The gross morphology (including a large H-B length) and the orientation of incisor of the fossorial voles from Piedmont is similar to the one found in the aquatic ones (Cagnin , 2008). By contrast, the length of the tail is exceptionally short, a typical adaptation of fossorial rodents (Prout, 1964). The only other Italian fossorial specimen which has been measured belongs to northeastern Italy (Lapini and Paolucci, 1994) and showed a considerable small size (Tab. 2). According to these observations, we found that most of the morphological characters used to distinguish the aquatic and fossorial ecotypes are not fully applicable to the Italian specimens. The only exception is the tail length, short in the fossorial ecotypes, and the HB/TL ratio that fall in the range reported by Kryštufek et al. (2015) for the fossorial ecotypes outside Italy. Kryštufek et al. (2015) argued that fossorial and aquatic water voles might be at the extremes of a phenotypic continuum, rather than two discrete morphotypes. This seems to be our case but our findings highlight also the necessity to make a review of the morphological characters, both qualitative and quantitative used to distinguish ecotypes in museum specimens.

The presence of the fossorial habits in all three main clades of *A. amphibius s.l.* (Fig. 2) suggests that the ecological plasticity of *Arvicola* is an ancient characteristic of the genus, at least present in the common ancestor of *Arvicola amphibius s.l.* This agrees with Marcolini et al. (2011) who inferred ancestral lifestyles from enamel morphologies: the ancestor at the node, corresponding here to the ancestor of all *Arvicola amphibius s.l.*, was intermediate between the aquatic and fossorial forms, suggesting a certain level of fossorial lifestyle in aquatic species.

The ecological plasticity of water voles seems exceptional among rodents and requires additional research possibly focused on the ecological or demographic factors that determined this rapid shift in ecological habits. An open issue is the reason why fossorial populations are present in some mountain areas (e.g. the Alps) and not in others (e.g. the Apennines). Moreover, we cannot rule out the possibility that these ecologically divergent populations display a certain degree of assortative mating and thus a certain degree of gene flow reduction. This opens the way to the study of genetic differentiation in sympatry and ecology-driven speciation (Rundle and Nosil, 2005).

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