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1 **Genetic structure, ecological versatility, and skull shape differentiation**
2 **in *Arvicola* water voles (Rodentia, Cricetidae)**

3
4 **Short running title:** *Arvicola* phylogeography and morphometry

5
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23
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Abstract

Water voles from the genus *Arvicola* display an amazing ecological versatility, with aquatic and fossorial populations. The Southern water vole (*A. sapidus*) is largely accepted as a valid species, as well as the newly described *A. persicus*. In contrast, the taxonomic status and evolutionary relationships within *A. amphibius sensu lato* had caused a long-standing debate. The phylogenetic relationships among *Arvicola* were reconstructed using the mitochondrial cytochrome *b* gene. Four lineages within *A. amphibius s. l.* were identified with good support: Western European, Eurasiatic, Italian, and Turkish lineages. Fossorial and aquatic forms were found together in all well-sampled lineages, evidencing that ecotypes do not correspond to distinct species. However, the Western European lineage mostly includes fossorial forms whereas the Eurasiatic lineage tend to include mostly aquatic forms. A morphometric analysis of skull shape evidenced a convergence of aquatic forms of the Eurasiatic lineage towards the typically aquatic shape of *A. sapidus*. The fossorial form of the Western European lineage, in contrast, displayed morphological adaptation to tooth-digging behavior, with expanded zygomatic arches and proodont incisors. Fossorial Eurasiatic forms displayed intermediate morphologies. This suggest a plastic component of skull shape variation, combined with a genetic component selected by the dominant ecology in each lineage. Integrating genetic distances and other biological data suggest that the Italian lineage may correspond to an incipient species (*A. italicus*). The three other lineages most probably correspond to phylogeographic variations of a single species (*A. amphibius*), encompassing the former *A. amphibius*, *A. terrestris*, *A. scherman* and *A. monticola*.

53 Introduction

54

55 The extension of phylogeographical studies has led to the increasing recognition that many
56 species traditionally identified based on morphological traits encompass several genetic
57 distinct forms that constitute “cryptic species” [e.g. (Bryja et al., 2014; Mouton et al., 2017)].
58 Slow morphological divergence, as a probable consequence of stabilizing selection, may be
59 responsible for the limited phenotypic signature of these cryptic species. Yet, morphology,
60 inclusive osteological traits, varies according to ecological conditions, including diet
61 (Michaux, Chevret, & Renaud, 2007) but also way-of-life such as digging behavior, which
62 exerts strong functional demands on the skull (Gomes Rodrigues, Šumbera, & Hautier, 2016).
63 As a consequence, ecological versatility may lead to morphological convergence blurring the
64 signature of genetic divergence between species. Assessing the evolutionary units involved in
65 such cases is crucial to understand the selective context driving the genetic and morphological
66 divergence.

67 Water voles of the genus *Arvicola* constitute an emblematic example of the controversies that
68 may arise regarding ecological forms. Fossorial and semi-aquatic forms have been described
69 as species (*A. terrestris*, Linnaeus, 1758, type locality Uppsala, Sweden and *A. amphibius*,
70 Linnaeus, 1758, type locality England) already by Linnaeus in 1758. Later on, up to seven
71 species have been described (Miller et al., 2012). By combining chromosomal and ecological
72 data, only three species were thereafter proposed (Heim de Balsac & Guislain, 1955): the
73 Southern water vole *A. sapidus*, Miller, 1908, with $2n = 40$, *A. terrestris* for semi-aquatic
74 forms with $2n = 36$, and *A. scherman*, Shaw, 1801, for fossorial forms with $2n = 36$.

75 The status of *A. sapidus* was subject to little debate but controversy persisted regarding the
76 aquatic and fossorial forms *A. terrestris/A. scherman*: considered as a single polytypic species
77 (Wilson & Reeder, 1993), or valid distinct species: *A. amphibius* and *A. scherman* (Wilson &
78 Reeder, 2005). More recently, the Italian water vole was proposed as a separate species (*A.*
79 *italicus*, Savi, 1839) (Castiglia et al., 2016), while the aquatic and fossorial forms remained
80 considered as separate species with distinct geographic distribution, under the names of *A.*
81 *amphibius* and *A. monticola*, de Selys Longchamps, 1838 (Pardiñas et al., 2017). Even more
82 recently, Mahmoudi, Maul, Khoshyar, & Darvish (2019) identified in Iran another species, *A.*
83 *persicus*. Hence, the genus *Arvicola* currently includes five species: *amphibius*, *italicus*,
84 *monticola*, *sapidus* and *persicus* (Mahmoudi et al., 2019; Pardiñas et al., 2017).

85 Yet, an increasing genetic sampling brought new fuel in the debate, showing that the
86 ecological forms were not systematically associated with distinct clades. Fossorial and aquatic

87 forms have been found to coexist in the Italian water vole (Castiglia et al., 2016) and in *A.*
88 *scherman* (Kryštufek et al., 2015). The limited sampling of *A. monticola* precluded to reliably
89 assess the variation in this presumed fossorial clade (Mahmoudi et al., 2019).

90 The present study therefore aims at a clarification of the phylogenetic pattern within European
91 water voles, by compiling published and new cytochrome b sequences, with the aim to
92 improve the geographic coverage and representation of the two ecological forms. This genetic
93 approach was complemented by a morphometric analysis of skull shape variations of aquatic
94 and fossorial forms, in order to assess the patterns of morphological differentiation and
95 possible convergences.

96

97 **Material and Methods**

98

99 The terminology used thereafter is the following. The Southern water vole, *A. sapidus*, and
100 the Iranian water vole, *A. persicus*, were named by their Latin name. The other water voles
101 were designed as “water voles” or *Arvicola amphibius* considered *sensu lato*, hence including
102 both fossorial and aquatic water voles (including specimens labelled as *amphibius*, *monticola*,
103 *scherman* and *terrestris*). The status of the Italian water vole will be discussed, but the name
104 “*italicus*” was tentatively retained.

105

106 *Material for genetics*

107 The genetic sampling original to this study included 143 tissue samples of *Arvicola amphibius*
108 *s. l.* from Belgium, Denmark, France, Germany, Great Britain and Spain, two specimens
109 identified as *A. scherman* from Spain, as well as two samples of *Arvicola sapidus* (Table S1).
110 Most of the samples were attributed to fossorial or aquatic forms, based on field evidences:
111 fossorial forms were trapped in tumuli. It was completed with sequences available in
112 GenBank for *A. amphibius* (89), *A. scherman* (2), *A. sapidus* (12) and *A. persicus* (14) (Table
113 S1). The complete dataset for *A. amphibius s. l.* comprised 236 sequences from 102 localities
114 (Table S1, Figure 1A).

115

116 *Material for morphometrics*

117 The material available for morphometric studies (Table S2) corresponded to 223 skulls. It
118 included various fossorial populations from France and Western Switzerland. The Eurasiatic
119 lineage (*sensu* (Castiglia et al., 2016)) was sampled by aquatic populations from Finland,
120 Denmark, and Belgium. Water voles from Ticino in Southern Switzerland were labelled as

121 *italicus* and represented the Italian lineage (Brace et al., 2016; Castiglia et al., 2016).
122 Specimens from various localities in France and Spain represented the sister species *A.*
123 *sapidus*.

124 In three populations (Chapelle d'Huin, La Grave, and Prangins), sex information was
125 available and allowed to test for sexual dimorphism. Almost all specimens corresponded to
126 adult and sub-adult specimens. The specimens are stored in the collections of the Centre de
127 Biologie et Gestion des Populations (Baillarguet, Montferrier-sur-Lez, France), the Muséum
128 d'Histoire Naturelle (Geneva, Switzerland) and the Université de Liège (Belgium).

129

130 *Genetic analysis*

131 DNA was extracted from ethanol-preserved tissue of *Arvicola*, using the DNeasy Blood and
132 Tissue kit (Qiagen, France) following the manufacturer instructions. The cytochrome *b* gene
133 sequence was amplified using previously described primers L7 (5'-
134 ACCAATGACATGAAAATCATCGTT-3') and H6 (5'-
135 TCTCCATTTCTGGTTTACAAGAC-3') (Montgelard, Bentz, Tirard, Verneau, & Catzeflis,
136 2002). PCRs were carried out in 50 µl volume containing 12.5 µl of each 2 mM primers, 1 µl
137 of 10 mM dNTP, 10 µl of reaction buffer (Promega), 0.2 µl of 5 U/µl Promega *Taq* DNA
138 polymerase and approximately 30 ng of DNA extract. Amplifications were performed using
139 one activation step (94°C/4 min) followed by 40 cycles (94°C/30 s, 50°C/60 s, 72°C/90 s)
140 with a final extension step at 72°C for 10 min. PCR products (1243 bp long) were send to
141 Macrogen (Seoul, Korea) for sequencing.

142 The sequences generated were visualized and analyzed using Seqscape (Applied Biosystems)
143 or CLC Workbench (Qiagen) and aligned with Seaview v4 (Gouy, Guindon, Gascuel, &
144 Lyon, 2010). All the new sequences were submitted to GenBank: accession number
145 LR746349 to LR746495. The final alignment comprised 264 sequences of *Arvicola*, 147 new
146 ones and 117 retrieved from GenBank. Sequences of *Microtus arvalis*, *Myodes glareolus*,
147 *Eothenomys melanogaster*, and *Ellobius tancrei* were used as outgroups. This resulted in a
148 final alignment comprising 268 sequences and 911 positions (Alignment S1) after the removal
149 of the sites with more than 10% of missing data. The best model fitting our data (GTR+I+G)
150 was selected with jModelTest (Darriba, Taboada, Doallo, & Posada, 2012) using the Akaike
151 criterion (Akaike, 1973). The phylogenetic tree was reconstructed using maximum likelihood
152 with PhyML 3.0 (Guindon et al., 2010) and Bayesian inference with MrBayes v3.2 (Ronquist
153 et al., 2012). Robustness of the nodes were estimated with 1000 bootstrap replicates with
154 PhyML and posterior probability with MrBayes. Markov chain Monte Carlo (MCMC)

155 analyses were run independently for 20 000 000 generations with one tree sampled every 500
156 generations. The burn-in was graphically determined with Tracer v1.6 (A. Rambaut, Suchard,
157 Xie, & Drummond, 2014). We also checked that the effective sample sizes (ESSs) were
158 above 200 and that the average standard deviation of split frequencies remained <0.05 after
159 the burn-in threshold. We discarded 10% of the trees and visualized the resulting tree with
160 Figtree v1.4 (Rambaut, 2012). MEGA 7 (Kumar, Stecher, & Tamura, 2016) was used to
161 estimate K2P distance between and within lineages. We use POPART (Leigh & Bryant,
162 2015) to reconstruct a median joining network of haplotypes (Bandelt, Forster, & Rohl,
163 1999). This analysis was restricted to the 236 sequences of *A. amphibius*, and the
164 corresponding haplotypes were determined with DnaSP v6 (Rozas et al., 2017).

165

166 *Morphometric analyses*

167 Each skull was photographed in ventral and lateral views using a Canon EOS 400D digital
168 camera. The ventral view was described by a configuration of 22 landmarks and 13 sliding
169 semi-landmarks along the zygomatic arch. The lateral view was described using a
170 configuration of 24 landmarks and 39 sliding semi-landmarks (Figure S1). All landmarks and
171 sliding semi-landmarks were digitized using TPSdig2 (Rohlf, 2010). The sampling included
172 189 skulls in ventral view and 186 ones in lateral view (Table S2).

173 The configurations of landmarks and semi-landmarks were superimposed using a generalized
174 Procrustes analysis (GPA) standardizing size, position and orientation while retaining the
175 geometric relationships between specimens (Rohlf & Slice, 1990). During the
176 superimposition, semi-landmarks were allowed sliding along their tangent vectors until their
177 positions minimize the shape between specimens, the criterion being here bending energy
178 (Bookstein, 1997). A Principal Component Analysis (PCA) was performed on the resulting
179 aligned coordinates. Relationships between difference groups were investigated using a
180 Canonical Variate Analysis (CVA) which aims at separating groups by looking for linear
181 combinations of variables that maximize the between-group to within-group variance ratio.
182 By standardizing within-group variance, this method may be efficient for evidencing
183 phylogenetic relationships (Renaud, Dufour, Hardouin, Ledevin, & Auffray, 2015). In order
184 to reduce the dimensionality of the data, the CVA was performed on the set of Principal
185 Components (PC) totaling more than 95% of the variance.

186 Skull size was estimated using the centroid size (square root of the sum of the squared
187 distances from each landmark to the centroid of the configuration). Size differences were
188 tested using analyses of variance (ANOVA).

189 Geometric differences between groups and regression models were investigated using
190 procedures adapted for Procrustes data (Procrustes ANOVA). Using this approach, the
191 Procrustes distances among specimens are used to quantify explained and unexplained
192 components of shape variation, which are statistically evaluated via permutation (here, 9999
193 permutations) (Adams et Otárola-Castillo 2013). Allometric variations were investigated,
194 investigating skull shape as a function of skull size. The effect of a grouping variable
195 combining genetic and ecological information (“GxE”) was also included (Table S2),
196 allowing to test if the allometric slopes were different between the groups. Visualization were
197 obtained using the Common Allometric Component (CAC) derived from this allometry
198 analysis (Adams et al. 2013). Procrustes superimposition, PCA and Procrustes ANOVA were
199 performed using the R package geomorph (Adams & Otárola-Castillo, 2013). The CVA was
200 computed using the package Morpho (Schlager, 2017).

201

202 **Results**

203

204 *Genetics*

205 On the phylogenetic tree (Figure 2, see Figure S2 for the complete phylogeny) the three
206 groups corresponding to *A. sapidus*, *persicus* and *amphibius s. l.* were well supported
207 ($PP \geq 0.93$, $BP \geq 82$) with *A. amphibius* more closely related to *A. persicus* ($PP=0.93$, $BP=72$)
208 than to *A. sapidus*. The phylogenetic tree as well as the network (Figure 1B) evidenced four
209 lineages within *A. amphibius s. l.*. Most of the sequences of *amphibius s. l.* belonged to two
210 lineages: Lineage 1 (L1) and Lineage 2 (L2). L1 was present in France, Spain, Switzerland
211 and the North of Great Britain and comprises mostly fossorial forms. This lineage was
212 divided into two sub-groups: the first one with samples from France and Spain and the second
213 one with samples from Great Britain, Switzerland and France. The two specimens identified
214 as *A. scherman* belonged to this lineage. L2 had a large repartition area from the south of
215 Great Britain to Russia (East), Finland (North) and Romania (South) and it comprised more
216 aquatic than fossorial forms. The two remaining lineages were restricted to Turkey, Lineage 3
217 (L3) with aquatic forms only and Italy, Lineage 4 (L4) with both aquatic and fossorial forms
218 (Figure 2, Figure S2 and Figure 1C). In several French localities (Chapelle d’Huin, Doubs;
219 Val d’Ajol, Vosges; Vauconcourt, Haute-Saône; Vigeois, Corrèze), a co-occurrence of
220 lineages 1 and 2 was documented. In all cases, L1 was dominant and the population mostly
221 fossorial.

222 Regarding the amount of genetic divergence, *A. sapidus*, *A. persicus* and *A. amphibius s. l.*

223 appeared well differentiated (K2P distances ≥ 7.2). L3 was closely related to L2 (K2P = 2.9)
 224 whereas L4, which correspond to *A. italicus*, was the most divergent ($4.4 < \text{K2P} < 5.1$) within
 225 *A. amphibius s. l.* (Table 1).

226

227 *Morphometrics*

228 Sexual dimorphism. – Sexes displayed very similar skull size and shape. No difference was
 229 detected for the size of the skull in ventral view (ANOVA on Ventral Centroid Size: Chapelle
 230 d’Huin P = 0.3031; La Grave P = 0.8706; Prangins P = 0.9799) and in lateral view (ANOVA
 231 on Lateral Centroid Size: Chapelle d’Huin P = 0.1358; La Grave P = 0.3575; Prangins P =
 232 0.9576). Similarly, skull shape was not different between sexes, for the skull in ventral view
 233 (Procrustes ANOVA on ventral skull shape: Chapelle d’Huin P = 0.6130; La Grave P =
 234 0.4556; Prangins P = 0.8309) as for the skull in lateral view (Procrustes ANOVA on lateral
 235 skull shape: Chapelle d’Huin P = 0.1918; La Grave P = 0.4337; Prangins P = 0.7164). All
 236 animals were therefore pooled in subsequent analyses.

237 Skull size. – The different groups of water voles significantly differed in skull size (ANOVA
 238 on CSventral and CSlateral: $P < 0.0001$). Skulls of water voles, being fossorial or aquatic,
 239 were smaller than those of *A. sapidus* (Figure 3). Important size variation occurred within *A.*
 240 *amphibius s.l.* The aquatic populations of L2 were especially variable in size, the Belgian one
 241 being almost as large as *A. sapidus* whereas skulls from Finnish *A. amphibius* were among the
 242 smallest. Important size variation also occurred within populations belonging to L1.

243 Skull shape in ventral view. – The variation of skull shape in ventral view was structured in
 244 two groups on the first two axes of a PCA on the aligned coordinates (Figure 4A). These two
 245 groups opposed aquatic forms (*A. sapidus* and specimens belonging to L4 and L2) to fossorial
 246 forms belonging to L1. The L2 fossorial population from Alsace plotted between these two
 247 main groups, whereas the Slovakian specimens, presumably also belonging to L2 given the
 248 geographic extension of this lineage, plotted within the range of variation of fossorial L1. The
 249 fossorial specimens from Western Switzerland (Arzier and Prangins), presumably belonging
 250 to L1, and the population from Chappelle d’Huin, characterized by a genetic mixing of L1
 251 and L2, shared the same range of variation as fossorial L1. The shape change from negative to
 252 positive PC1 scores, and hence from aquatic to fossorial forms, mostly involved a lateral
 253 extension of the zygomatic arch and a forward displacement of the incisor tip.

254 Both fossorial and aquatic groups displayed an important variation along PC1 and PC2. This
 255 was related to an important allometry (Figure 4B) (Procrustes ANOVA on aligned
 256 coordinates: shape ~CS: $P < 0.001$). A Procrustes ANOVA including as factors centroid size

257 and the GxE grouping indicated a significant influence of both factors ($P < 0.001$) but
258 supported the hypothesis of parallel slopes. These parallel trends in the different groups were
259 visualized along the CAC (Figure 4B), which involved discrete shape changes with a slight
260 backward shift of the incisor tip, and a compression of the posterior part of zygomatic arch
261 (Figure 4C). For similar CAC scores, aquatic forms tended to display larger skulls, especially
262 those of *A. sapidus*.

263 The PCA on the aligned coordinates was further used to reduce the dimensionality of the data.
264 The first 23 axes totaled 95% of the total variance and were used in a CVA, the grouping
265 factors being the geographical groups (Figure 4D). As the PCA, the CVA tended to separate
266 aquatic and fossorial forms; but it more clearly isolated *A. sapidus* and to a lesser extent the
267 population from Ticino corresponding to the genetically well-differentiated L4. Despite their
268 ecological heterogeneity, populations affiliated to L2, including the fossorial population from
269 Alsace and the Slovakian population of unknown ecology, tended to share negative CVA1
270 scores. All other fossorial populations, affiliated to L1 or with a mixing of L1 and L2, plotted
271 towards positive CVA1 scores.

272 The morphological differences between some groups means were further visualized (Figure
273 4C). The change from the aquatic *A. sapidus* and to the typical fossorial water voles from the
274 L1 mostly involved a lateral expansion of the zygomatic arch, a posteriorly compressed brain
275 case and a forward shift of the incisor tip. The lateral expansion of the zygomatic arch is also
276 observed in the shape change from aquatic to fossorial ecology within L2. This change within
277 L2 is however of a lesser magnitude than the change between the well differentiated units *A.*
278 *sapidus* and fossorial L1.

279 Skull shape in lateral view. – The PCA on the aligned coordinates of the skull in lateral view
280 (Figure 5A) provided a less clear structure than the one of the skull in ventral view. Aquatic
281 and fossorial forms tended to segregate along PC1, but with a considerable overlap. The shape
282 changes along this axis involved a proodont shift of the incisor, a ventral expansion of the
283 zygomatic arch, and a curvature of the brain case, but these shape changes corresponded both
284 to a difference between aquatic and fossorial forms, and to an extensive variation within each
285 ecological form.

286 This extensive variation is largely due to allometry (Procrustes ANOVA: shape ~ CS: $P <$
287 0.001). As for the ventral view, the different groups had parallel allometric slopes which were
288 shifted between groups (Figure B; shape ~CS: $P < 0.001$, ~GxE: $P < 0.001$). The common
289 allometric trend corresponded to a flattening of the brain case and a slight backward shift of
290 the incisor tip (Figure 5C).

291 The first 29 axes of the PCA totaled 95% of variance and were used in a CVA (Figure 5D). *A.*
292 *sapidus* and the Ticino population from L4 appeared as well divergent along the first CVA
293 axis, explaining most of the variation. All other populations were close to each other.
294 Whatever their ecology, populations attributed to L2, including that from Slovakia, were
295 tightly clustered towards CVA1 scores close to zero. All fossorial populations belonging to
296 L1, or where lineages 1 and 2 co-occur, were clustered towards negative CVA1 scores.
297 The shape change between aquatic and fossorial group means (Figure 5C) allowed to better
298 assess the shape changes related to ecology. The difference between *A. sapidus* and the
299 fossorial L1 clearly showed the proodont shift of the incisor. This shift is also characteristic,
300 although at a lesser magnitude, in the transition from aquatic to fossorial forms within L2; this
301 was associated with a downward shift of the zygomatic arch.

302

303 Discussion

304

305 *Phylogeny evidenced widespread ecological versatility*

306 The molecular data confirmed the separation of *A. sapidus* and *A. persicus* and other water
307 voles as in Mahmoudi et al. (2019). They further evidenced four lineages within the
308 “European water vole” *A. amphibius s. l.*: (1) L1, with a Western European distribution
309 (Castiglia et al., 2016) and a dominance of fossorial forms. This lineage was found mostly in
310 France, the neighboring Western Switzerland, and in Northern areas of Great Britain. (2) A
311 widespread Euroasiatic L2 (Castiglia et al., 2016; Kryštufek et al., 2015), present from
312 Belgium and Germany to the West up to Eastern parts of Russia. This lineage showed a
313 dominance of aquatic forms. Note that the co-occurrence of lineages 1 and 2 in Great Britain
314 has been shown to be the consequence of a colonization in two waves, the second partly
315 replacing the first *ca* 12-8 kyr BP (Brace et al., 2016; Searle et al., 2009). (3) Related to the
316 Eurasiatic L2, a third lineage (L3) was found, up to now, in Turkey (Kryštufek et al., 2015).
317 (4) L4, characteristic of Italy and the neighboring Southern Switzerland (Ticino) (Brace et al.,
318 2016; Castiglia et al., 2016). It was the most divergent of the lineages *within A. amphibius s. l.*
319 and corresponded to the proposed species *A. italicus*.

320 Confirming recent results (Castiglia et al., 2016; Kryštufek et al., 2015), the present study
321 undermined the interpretation of fossorial and aquatic forms as distinct genetic units. Instead,
322 ecological versatility was evidenced within at least three out of four lineages, aquatic and
323 fossorial forms being mixed in the lineages 1 (Western Europe), 2 (Euroasiatic) and 4
324 (Italian). The reduced sampling of L3 (Turkey) precluded any conclusions regarding this

325 lineage. Clearly, aquatic and fossorial forms do not constitute separate species in water voles
326 *A. amphibius s. l.* (Kryštufek et al., 2015).

327 The genetic distances separating the lineages typically fell within a “grey zone”, where values
328 typical for intraspecific divergence and those associated with interspecific divergence overlap
329 ($\sim 3 < K2P < \sim 6$) (Barbosa, Pauperio, Searle, & Alves, 2013). With K2P values between 4 and
330 5, they typically corresponded to the range of differentiation between phylogenetic lineages
331 within rodent species (Michaux, Magnanou, Paradis, Nieberding, & Libois, 2003; Paupério et
332 al., 2012), and slightly below values corresponding to the differentiation between species
333 (Amori, Gippoliti, & Castiglia, 2009; Kohli et al., 2014; Vallejo & González-Cózatl, 2012).

334

335 *A two-fold morphological signature*

336 The morphological differentiation among water voles was assessed using two widely used
337 methods in morphometrics: PCA and CVA. These methods provided different structures
338 between populations in the corresponding morphospaces, the PCA emphasizing the
339 morphological differences related to ecology, opposing aquatic and fossorial voles whatever
340 their phylogenetic background, while the CVA retrieved a signal more related to the
341 phylogenetic structure. This discrepancy is related to the properties of the methods. The PCA
342 decomposes the total variance, and therefore is highly impacted by extensive within-group
343 variation related to ontogenetic and ecological changes. In contrast, by expressing between-
344 group differences while standardizing within-group variance, the CVA can put forward more
345 discrete traits characterizing different lineages. It is confirmed here as an efficient tool for
346 showing phylogenetic relationships (Renaud et al., 2015).

347

348 *Ecological forms and their adaptive morphological signature on skull shape*

349 The chisel-tooth digging behavior is known to exert strong physical loads on the skull, and
350 thus to constitute a strong selective pressure leading to morphological convergence in skull
351 shape across different rodent families (Gomes Rodrigues et al., 2016; Samuels & Van
352 Valkenburgh, 2009). Accordingly, an important skull shape differentiation opposed aquatic to
353 fossorial groups. Chisel-tooth digging especially requires powerful masseter muscles to move
354 the mandible into occlusion. This muscle originates along the zygomatic arch and inserts on
355 the angular process of the mandible. As a consequence, the expanded zygomatic arch is
356 typical for fossorial rodents (Samuels & Van Valkenburgh, 2009). Proodont incisors are also
357 a typical trait for fossorial rodents, favoring the process of biting in the substrate (Samuels &
358 Van Valkenburgh, 2009). The signal found in *Arvicola* skulls agrees with these general

359 ecomorphological characteristics: fossorial populations display an expanded angular
360 processes on the mandible (Durão, Ventura, & Muñoz-Muñoz, 2019), especially visible in
361 ventral view, and proodont incisors, a trait that is best traced in lateral view. Altogether, the
362 morphometric differentiation between fossorial and aquatic water voles documents an
363 integrated adaptive response to the functional demand of tooth digging.

364 Opposite to fossorial water voles, the skulls of *A. sapidus* display extreme skull shapes,
365 without overlap with other water vole populations, even aquatic ones. This pronounced
366 morphological differentiation [(Durão et al., 2019); this study] is likely the combined result of
367 a genetic divergence supportive of a valid species, and of the absence of ecological versatility
368 in this taxon, always displaying a semi-aquatic way of life.

369 Within *A. amphibius s.l.*, fossorial and aquatic populations tended to be well differentiated.
370 This was especially true for the fossorial populations of the Western European L1
371 (dominantly fossorial) and the aquatic populations of the Euroasiatic L2 (mostly aquatic). The
372 Alsatian fossorial population of L2 was shifted towards the fossorial populations of L1, but
373 still displayed an intermediate morphology between aquatic and fossorial forms. This suggests
374 that the genetic divergence between the two lineages was enough to accumulate adaptations to
375 the dominant ecology. However, the persistent ecological versatility triggers local adaptation
376 in case of a switch to the alternative strategy. This response in skull shape probably include a
377 plastic component, since bone permanently remodel in response to mechanical stress
378 produced by muscular activity, including in the context of digging activity (Durão et al.,
379 2019; Ventura & Casado-Cruz, 2011) .

380 Regarding size, fossorial forms of *A. amphibius s.l* were mentioned to be smaller than the
381 aquatic forms in the Euroasiatic region (Kryštufek et al., 2015) but the reverse in Italy
382 (Castiglia et al., 2016). The present study did not evidence any clear trend between lineages or
383 forms, to the exceptions of the clearly larger *A. sapidus*. The hypothesis that burrowing would
384 favor small-sized animals, because of reduced digging costs (Durão et al., 2019) is therefore
385 not supported within *A. amphibius s. l.*, although local ecological conditions may be involved
386 in the important geographic differences in skull size even within the same lineage. The age
387 structure of the sampled populations may explain at least partly differences in the size
388 distribution, depending whether or not young animals were dominant at the time of trapping
389 (Renaud, Hardouin, Quéré, & Chevret, 2017). Whatever its cause, size variation was
390 associated to allometric variation of skull shape. *A. sapidus*, and fossorial and aquatic forms
391 of *A. amphibius s.l.* shared parallel allometric trajectories, fossorial forms showing more
392 “adult-like” morphologies than aquatic ones for a given size. In that respect, the evolution of

393 fossorial forms may be seen as heterochronic (Cubo, Ventura, & Casinos, 2006). However,
394 the morphological signal directly related to allometry was of limited amount and did not
395 match the differences related to ecology. Adaptive and plastic response to the functional
396 demand of chisel-tooth digging thus appears a more likely explanation of the morphological
397 differences between groups. The corresponding morphological characteristics seem to appear
398 early in life, with a conservation of the ontogenetic trajectory in aquatic and fossorial water
399 voles.

400

401 *Fossorial vs. aquatic: an oversimplified classification*

402 The Italian lineage (L4) was represented in the morphometric study by a sole population from
403 Ticino in Switzerland. This population was within the range of aquatic populations in PCA
404 morphospaces, in agreement with its dominant ecology. In the CVA morphospaces, it clearly
405 departed from the other lineages of *A. amphibius s. l.*, suggesting a morphological signature of
406 the Italian lineage.

407 Similarly, populations belonging to L2 appeared clustered in the CVA plots, especially in
408 lateral view, suggesting a morphological signature for this lineage as well. However, in the
409 PCA morphospaces, these populations ranged from a typically aquatic to a fossorial
410 morphology (considering the Slovakian population as likely belonging to this lineage), with
411 the Alsacian population being intermediate in shape. This illustrates the ecological versatility
412 of water voles when facing environmental changes. Furthermore, if some forms are attached
413 all year to water, and some inhabit dry areas, some animals switch between both habitats
414 during the year (Wust-Saucy, 1998). In front of this ecological versatility even on very short
415 time scales, the expectation of discrete fossorial and aquatic morphotypes may be inadequate
416 (Kryštufek et al., 2015). Typical aquatic voles from the dominantly aquatic L2 and typical
417 fossorial voles from L1 might represent endmembers of a phenotypic continuum, the skull
418 morphology being dependent both on the genetic background and the ecological conditions of
419 growth.

420

421 *Taxonomic implications*

422 The strength of the present study relies on the extensive sampling of water voles across
423 Europe. However, the taxonomic conclusions are only based on a mitochondrial gene
424 (cytochrome *b*) and the pattern of morphological divergence of the skull. Nuclear data would
425 be required to validate these conclusions, but the only data available so far, based on the
426 Interphotoreceptor Retinoid Binding Protein (IRBP) gene, remained inconclusive for *Arvicola*

427 *amphibius s.l.* (Mahmoudi et al., 2019).

428 In agreement with previous studies, genetic and morphometric results support the specific
429 status for the Southern water-vole *A. sapidus* Miller, 1908 (type locality Santo Domingo de
430 Silos, Burgos Province, Spain). Its genetic divergence from the other lineages ($K2P > 7$) was
431 close to what is observed between other valid rodent species (Amori et al., 2009; Barbosa et
432 al., 2013). The genetic divergence was also very high for the species described in Iran: *A.*
433 *persicus* ($K2P > 9$) (Mahmoudi et al., 2019). Nuclear and mitochondrial data support the
434 specific status of these two species (Mahmoudi et al., 2019).

435 The Italian lineage is the most differentiated within *A. amphibius s.l.* ($K2P \geq 4.4$).

436 Reproductive isolation has been evidenced between animals from the north and south sides of
437 the Swiss Alps, populations that can be nowadays attributed to the Western European L1 and
438 the Italian L4 [(Morel, 1979) in (Castiglia et al., 2016)]. This supports the Italian lineage as an
439 incipient species: *A. italicus* (type locality Pisa, Italy).

440 The other lineages (Western European L1, Euroasiatic L2, and Turkish L3) correspond
441 partially to entities that have been even recently proposed as separate species: *A. amphibius*
442 and *A. monticola* (Mahmoudi et al., 2019). In Mahmoudi et al. (2019), *A. monticola* was
443 proposed for fossorial voles from Western Europe (Switzerland and Spain), which are
444 included in our Lineage 1, whereas *A. amphibius* include Siberian (aquatic) and European
445 (aquatic and fossorial) voles, which are included in our Lineage 2. The genetic divergence
446 between lineages 1, 2 and 3 was rather low ($K2P = 2.9-4.1$), hence they are most likely
447 phylogenetic lineages related to repeated isolations in glacial refugia during the Quaternary
448 climatic fluctuations (Michaux, Chevret, Filippucci, & Macholan, 2002; Taberlet, Fumagalli,
449 Wust-Saucy, & Cosson, 1998). Furthermore, our extensive sampling evidenced that the two
450 main lineages (1 and 2) can co-occur in the same localities at the fringe of their respective
451 distribution area. In the population of Chapelle d'Huin, (Doubs, France), showing a co-
452 occurrence of lineages 1 and 2, specimens display a skull shape typical of L1. This suggests
453 that exchanges between the two lineages occur at the nuclear level. The three lineages, which
454 present low genetic divergence, should thus be attributed to a single species: *A. amphibius*
455 (Linné, 1758) (including the former recognized species *amphibius*, *monticola*, *sherman* and
456 *terrestris*).

457

458

459

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635

636 **Figure legends**

637

638 Figure 1. Distribution of the ecological forms in the sampled localities (A), genetic network
639 (B) and distribution of the genetic lineages (C).

640

641 Figure 2. Simplified Bayesian phylogeny of *Arvicola* water voles. The support is indicated as
642 follow: Posterior Probability (MrBayes) / Bootstrap Support (PhyML). The different lineages
643 are indicated on the right side of the phylogeny. For each lineage, the dominant ecotype is
644 indicated in brackets, with its percentage of occurrence based on the number of sequences in
645 the tree attributed to this ecotype.

646

647 Figure 3. Variation of skull centroid size between populations of water voles (above, ventral
648 side; bottom, lateral side). Each dot represents a specimen.

649

650 Figure 4. Skull shape in ventral view. (A). Morphospace corresponding to the first two axes of
651 a PCA on the aligned coordinates. (B). Allometric relationship, represented by the Common
652 Allometric Component in the “GxE” groups (CAC_{GxE}), as a function of centroid size. (C).
653 Visualization of shape changes, as arrows pointing from a first to a second item. From top to
654 bottom: Shape changes along the first PC axis; allometric shape change, from minimum to
655 maximum centroid size along the CAC_{GxE} ; change between the mean morphology of *A.*
656 *sapidus* and fossorial lineage 1; change between the mean morphology of aquatic and
657 fossorial forms within lineage 2. (D). Morphospace corresponding to the first two axes of a
658 CVA on the PC axes totaling 95% of shape variance.

659

660 Figure 5. Skull shape in lateral view. (A). Morphospace corresponding to the first two axes of
661 a PCA on the aligned coordinates. (B). Allometric relationship, represented by the Common
662 Allometric Component in the “GxE” groups (CAC_{GxE}), as a function of centroid size. (C).
663 Visualization of shape changes, as arrows pointing from a first to a second item. From top to
664 bottom: Shape changes along the first PC axis; allometric shape change, from minimum to
665 maximum centroid size along the CAC_{GxE} ; change between the mean morphology of *A.*
666 *sapidus* and fossorial lineage 1; change between the mean morphology of aquatic and
667 fossorial forms within lineage 2. (D). Morphospace corresponding to the first two axes of a
668 CVA on the PC axes totaling 95% of shape variance.

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Supporting Information

Figure S1. Examples of water vole skulls in ventral and lateral view, with the location of the landmarks (red dots) and sliding semi-landmarks (blue dots along the black lines).

Figure S2. Phylogenetic tree reconstructed with the cytochrome *b* mitochondrial gene. For the main nodes, the support is indicated as follow: posterior probability (MrBayes) / bootstrap support (PhyML). The different lineages are indicated on the right side of the phylogeny. The color code of the sequence names represents the ecology: in orange fossorial; in blue aquatic.

Table S1. Sampling for the genetic study. Abbreviations: JPQ = Jean-Pierre Quéré, JRM = Johan R. Michaux, RGU = Rainer G. Ulrich.

Table S2. Sampling for the morphometric study.

GxE: grouping variable combining genetics and ecology. Nventra/Nlateral: number of skulls measured in ventral/lateral view. Abbreviations: JPQ = Jean-Pierre Quéré; JRM = Johan R. Michaux, CBGP: Centre de Biologie et Gestion des Populations (Baillarguet, Paris), MHN: Muséum d'Histoire Naturelle (Geneva, Switzerland), ULG = Université de Liège (Belgium).

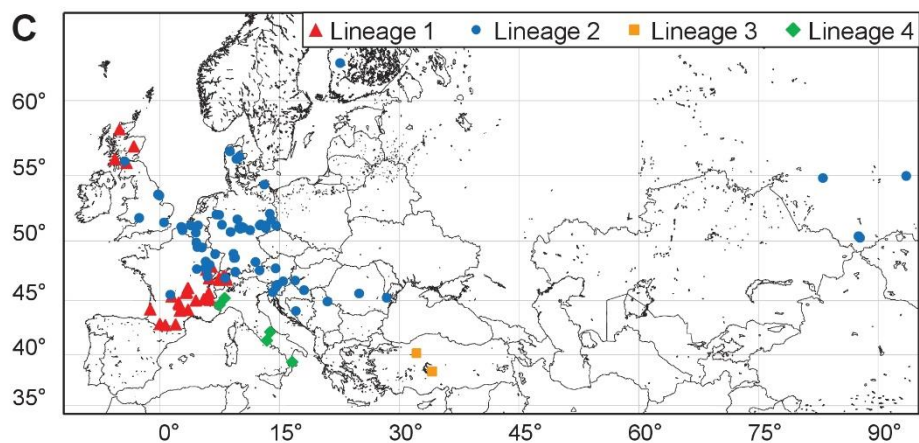
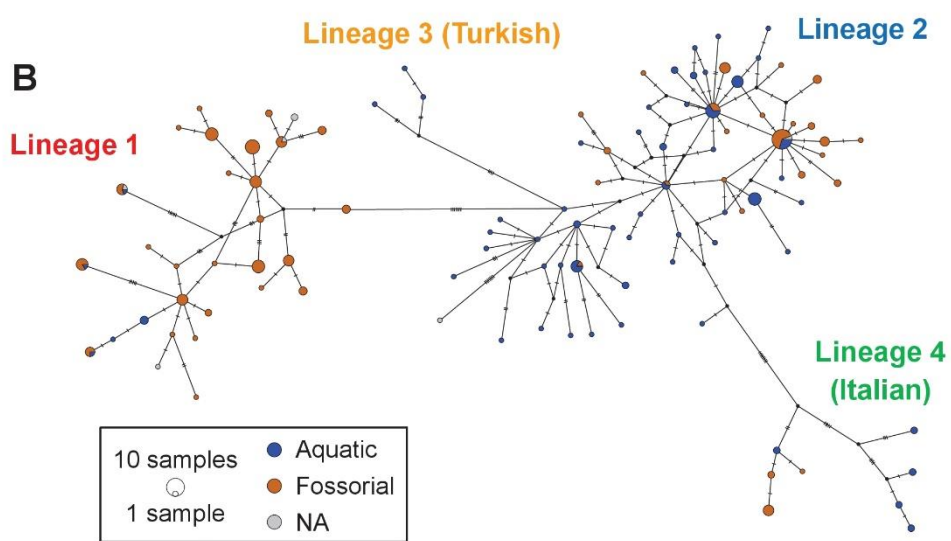
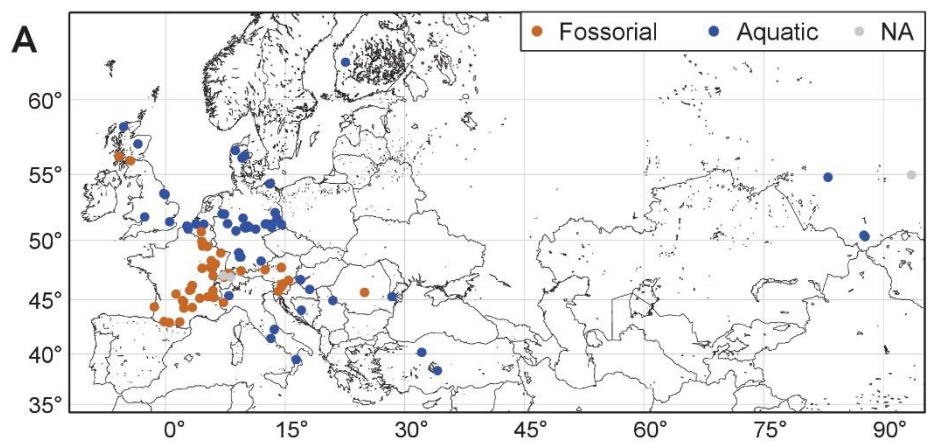
Alignment S1. Alignments of Cytb sequences used in the present study.

692 Table 1. K2P distances and standard error between (below the diagonal) and within (on the diagonal)
 693 lineages.

| | Lineage 1 | Lineage2 | Lineage 3 | Lineage 4 | <i>A. sapidus</i> | <i>A. persicus</i> | Outgroup |
|--------------------|------------|------------|------------|------------|-------------------|--------------------|------------|
| Lineage 1 | 1.3 ± 0.2 | | | | | | |
| Lineage 2 | 4.1 ± 0.6 | 1.2 ± 0.2 | | | | | |
| Lineage 3 | 3.8 ± 0.6 | 2.9 ± 0.5 | 0.6 ± 0.2 | | | | |
| Lineage 4 | 5.1 ± 0.8 | 4.4 ± 0.7 | 4.8 ± 0.8 | 1.6 ± 0.3 | | | |
| <i>A. sapidus</i> | 7.5 ± 1 | 7.2 ± 0.9 | 7.6 ± 1 | 8.1 ± 1.1 | 0.9 ± 0.2 | | |
| <i>A. persicus</i> | 9.4 ± 1.2 | 10.1 ± 1.2 | 9.8 ± 1.2 | 9.2 ± 1.1 | 10 ± 1.2 | 1.2 ± 0.3 | |
| Outgroup | 19.2 ± 1.5 | 18.5 ± 1.4 | 18.3 ± 1.5 | 17.5 ± 1.5 | 17.8 ± 1.4 | 19 ± 1.5 | 17.2 ± 1.4 |

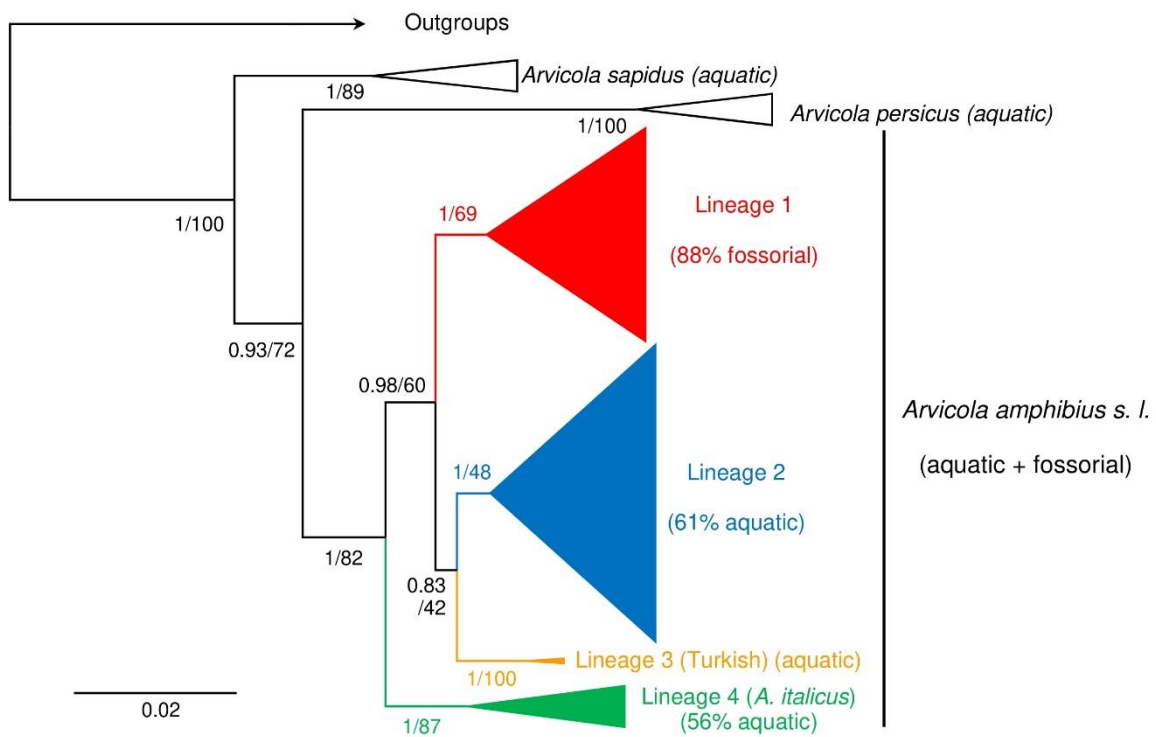
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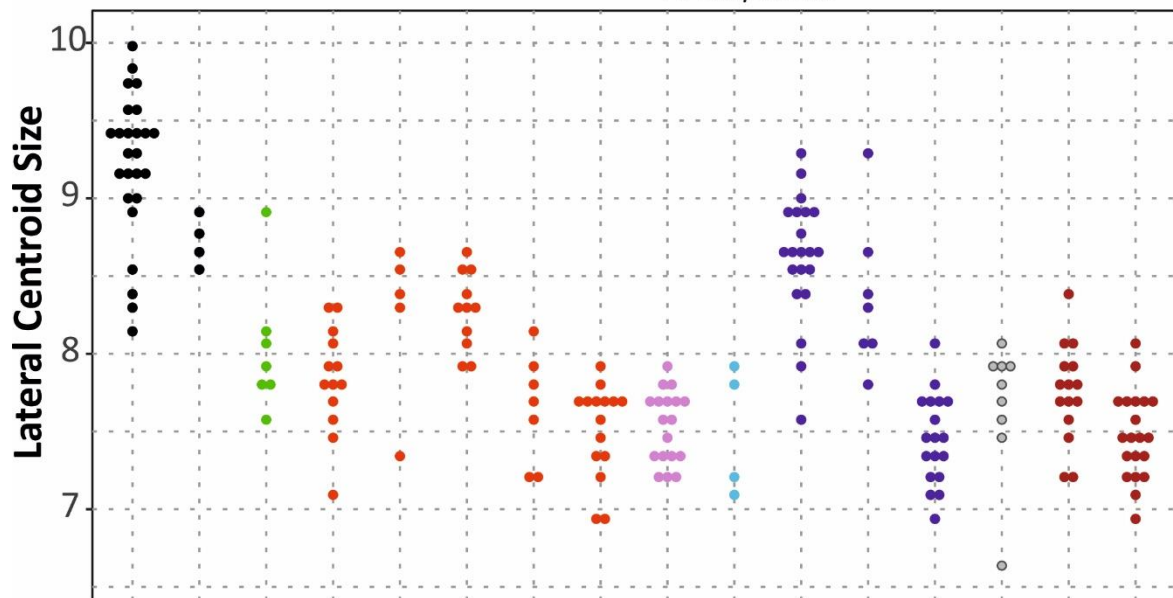
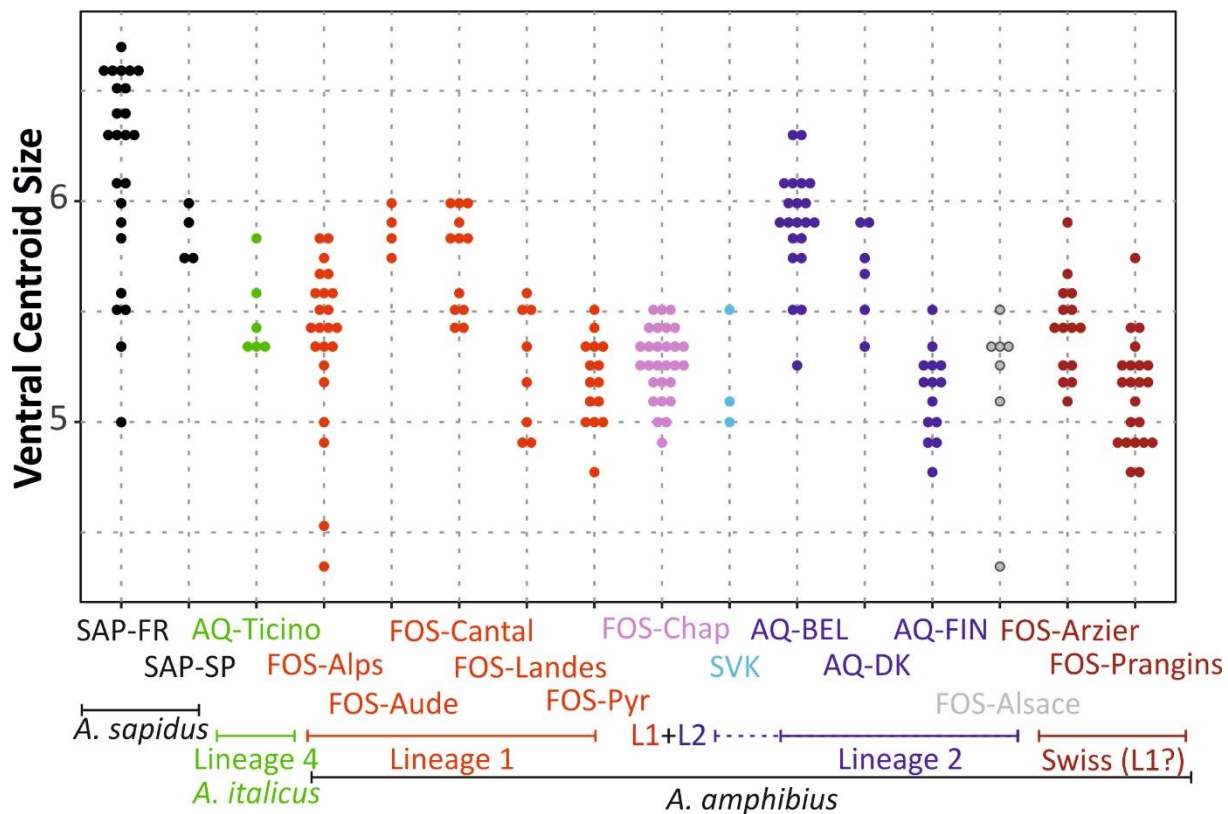
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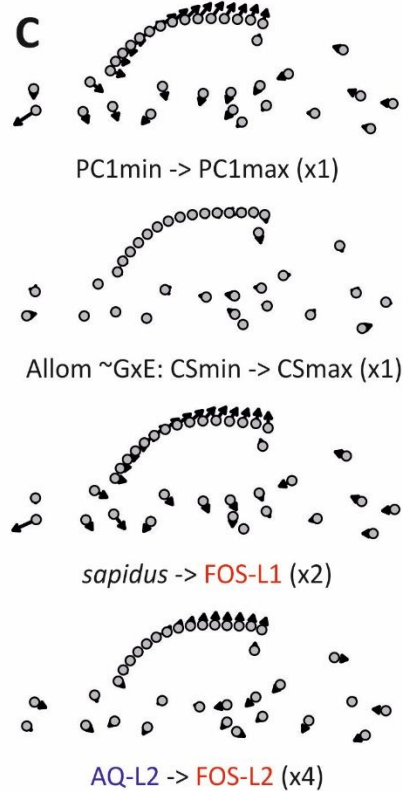
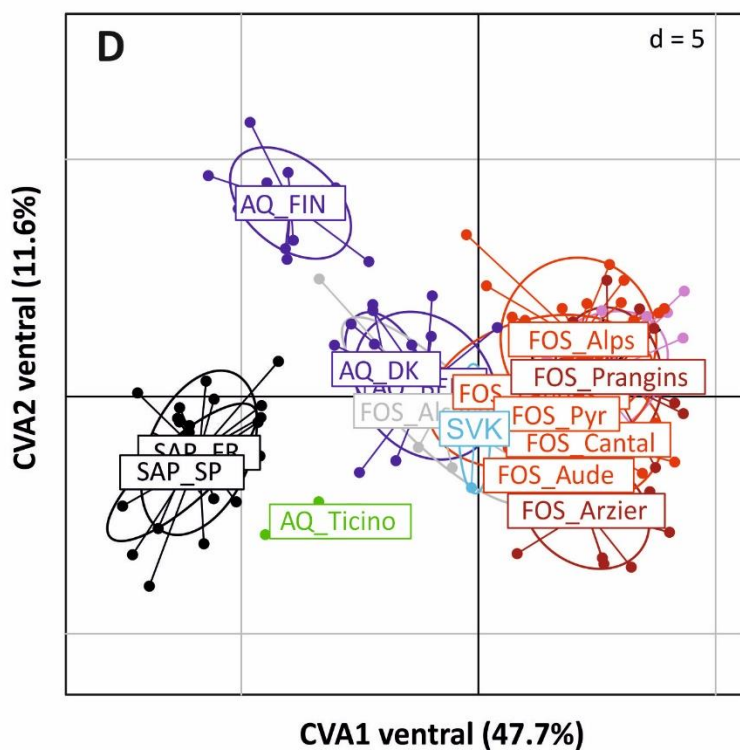
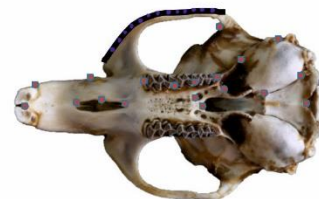
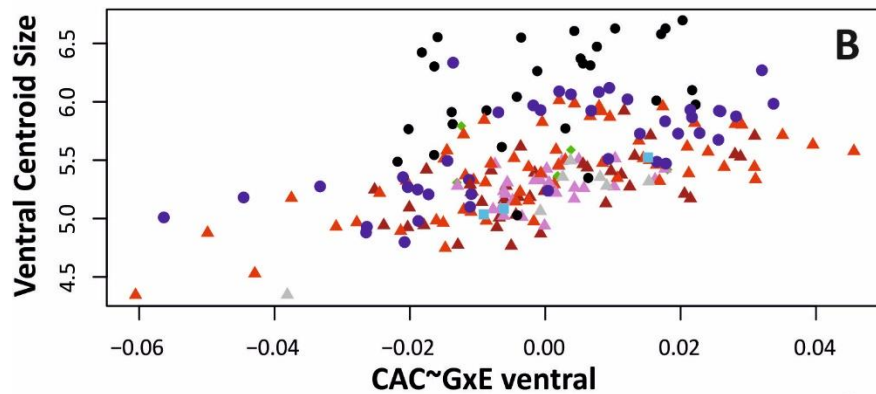
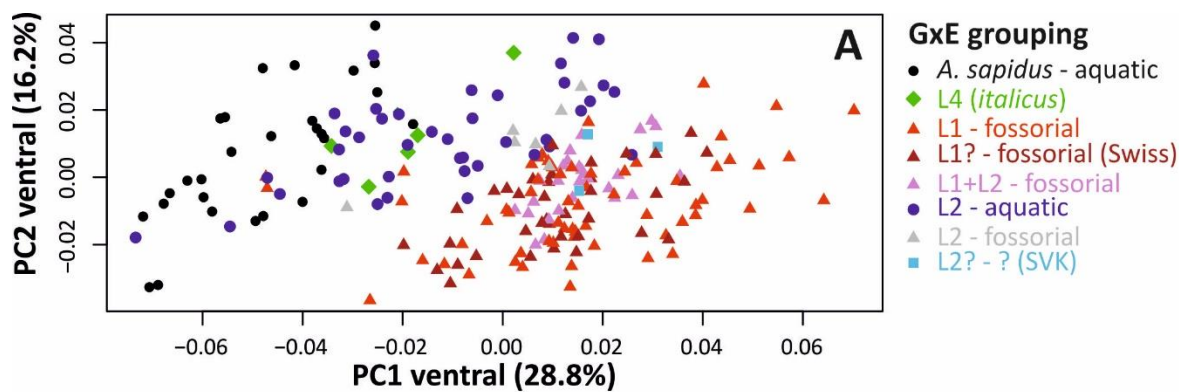


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