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ARTICLE

MIDDLE MIOCENE REMAINS OF *ALYTES* (ANURA, ALYTIDAE) AS AN EXAMPLE OF THE UNRECOGNIZED VALUE OF FOSSIL FRAGMENTS FOR EVOLUTIONARY MORPHOLOGY STUDIES

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ABSTRACT—Fragmentary anuran remains (an ilium and radioulna) from the middle Miocene of Moratilla 2 (Teruel Province, Spain) are identified, using qualitative characters and geometric morphometrics, as belonging to a new unnamed species of midwife toad, of the extant anuran genus *Alytes* (Alytidae). The Moratilla 2 fossils of *Alytes* are dated to ca. 16–17 Ma, prior to the early splits that resulted in the current *Alytes* diversification. Our biometric study of the fossil radioulnar fragment, an element usually considered uninformative, has revealed convergent adaptive trends in forearm locomotor performance within the genus. This finding would have remained hidden otherwise, because neither molecular approaches nor the comparative osteology of living forms would have detected it. A model for the evolutionary history of midwife toads is proposed, as a case example of how molecular phylogeographic results can be combined with morphological and paleontological studies at the genus level. Historical models of morphological adaptation at low taxonomic and anatomical levels now seem feasible using quantitative reconstructions of fossil fragments. In the future, these models can be compared with independently derived data based on environmental history.

SUPPLEMENTAL DATA—Supplemental materials are available for this article for free at www.tandfonline.com/UJVP

INTRODUCTION

A combination of circumstances converge in the study of the European Neogene anuran fauna. In the first place, the known fossil record is almost completely composed of living lineages at the genus or species level; only a few residual extinct palaeobatrachid and pelobatid taxa are known (Böhme and Ilg, 2003; Rage and Roček, 2003; Martín and Sanchiz, 2013). Secondly, extensive molecular biological research on the phylogeny of European amphibians has been conducted in recent years. Therefore, evolutionary and phylogeographic analyses, which provide evolutionary models of relationships that incorporate a time component, are now available for most living groups (e.g., Fromhage et al., 2004; Stöck et al., 2006, 2008; Zangari et al., 2006; Akin et al., 2010; Recuero et al., 2012). Finally, large quantities of amphibian and reptile fossil fragments have been obtained as by-products of micromammalian paleontological research in Europe. These isolated and fragmentary fossils are seldom studied and frequently remain unsorted, likely as a consequence of the reduced number of currently active specialists, who appear to prefer working with samples from older periods or with articulated skeletons.

Modern morphometric approaches are able to accurately reconstruct ancient morphologies using bone fragments (Gunz et al., 2009; Bastir et al., 2011; Benazzi et al., 2011; Neubauer et al., 2012). We will show that reconstructed morphologies can be synergically integrated into molecular evolutionary frameworks, providing polarity clues necessary for understanding the process of adaptation. To test this procedure under non-optimal conditions, our case study uses an element with low anatomical information content, the radioulnae from the midwife toads of the genus *Alytes*. This example is also significant because this group of primitive anurans were previously not unequivocally detected in the pre-Quaternary fossil record.

In this study, we not only provide an example of the usefulness of fragments in order to complete the amphibian fossil record, but also show that the study of ancient morphologies, even at the level of single anatomical elements, can provide important information that is currently impossible to obtain by other methods. Furthermore, using the midwife toad as a test case, we argue that paleontological data should be more frequently utilized for the study of morphological change at low taxonomic levels, taking advantage of the phylogenetic and dating results obtained from other biological disciplines.

MATERIALS AND METHODS

Comparative Material

One extant species of each European anuran genus and all species of the genus *Alytes* were selected for comparison. The comparative material belongs to the herpetological collections of the Museo Nacional de Ciencias Naturales, CSIC (Madrid, Spain), abbreviated MNCN, and the Museum Ţării Crişurilor (Oradea, Romania), abbreviated MTC. Specimens used for the radioulnar morphometric analyses are listed in Supplementary Data. The genus *Bufotes*, formerly the *Bufo (viridis)* species group, is very similar to *Bufo* in radioulnar morphology, and was not analyzed morphometrically. Comparative material of extinct *Latonia gigantea* (Alytidae) comprises uncataloged fossils from the sites La-Grive-Saint-Alban (probably outcrop 'M') and Sansan (France), in the collection of the Museum National

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d'Histoire Naturelle (Paris, France), and Devínska Nová Ves (Slovakia), in the collection of the Naturhistorisches Museum Wien (Vienna, Austria). The three localities are of Astaracian age: Sansan and Devínska Nová Ves belong to the European Neogene Mammal Zone MN6 and La-Grive-Saint-Alban belongs to the MN7+8 zone. The rare and endangered *Alytes muletensis* is represented by a late Pleistocene or early Holocene sample from Cova de la Barxa (Majorca, Spain), in the collection of the Instituto Mediteráneo de Estudios Avanzados IMEDEA (Palma de Mallorca, Spain).

Sexual dimorphism is known to occur in the forelimbs of several anuran groups, particularly in neobatrachian lineages. Dimorphism is mainly observed in the humerus rather than the radioulna. Nevertheless, when possible, we selected the samples in a balanced way to control for gender bias. All of the specimens were adults; no juveniles were used in this study.

Osteological nomenclature for the radioulna follows Bolkay (1919) and Ecker (1889), and that for the ilium follows Bolkay (1919) and Sanchiz (1998) concerning the orientation system of the element through a main longitudinal axis. Taxonomy and systematic arrangements follow Frost (2013).

Definitions of Landmarks and Measurements

Landmarks (sensu lato) used in the study of the radioulna are numbered L1–L8 and were taken on two-dimensional (2D) projections of the bones as represented by their silhouettes on digital photographs. The bones were oriented by maximizing the observable area of the facies caudalis, using plasticine as a support substrate. A repetition of the orientation of the same element by 20 different individuals not related to this study, quantified by the mean coefficient of variation (CV) of all the 28 interlandmark distances present on a complete bone, has given a mean parallax error of 3.11%. The mean CV obtained by one of us (B.S.) on the same test (20 repetitions) was 1.58%. Anatomical definitions are given in Table 1, and further details are provided in Figure 1 and in the text below.

Linear distances between landmarks were named according to their numbers. For example, D(2–3) indicates the length of a straight line between landmarks L2 and L3. Radioulnar landmarks names are listed in Table 1. Using a caudal (posterior) view, the landmarks are as follows: (a) landmark L1 (distal radioulna) is defined as the most distal point of the fused edges of the radius and ulna; (b) a main longitudinal axis is devised passing through L1 and the middle part of the radioulnar column (collum antibrachii), i.e., through the middle of the narrowest part of the bone outline; (c) the tangent intercepts of the bone outline with the closest parallel line to the main axis, at both lateral and medial sides of the bone, define the maximum ulnar diaphyseal curvature (L3) and maximum radial diaphyseal curvature (L7); (d) the intercepts of the proximal bone outline, anterior with respect to the collum, with the farthest parallel line to the main axis, at both sides of the bone, define the most laterally projecting point on the olecranon (L4, lateral olecranon) and the most medially projecting point on caput radii (L6, caput radii); (e) similarly, the intercepts of the distal outline with the farthest parallel line to the main axis, at both lateral and medial sides of the bone, define the most lateral and distal ulnar point (L2, caput ulnae) and the most distal and medial radial point (L8, distal radius); and (f) the tangent intercept of a perpendicular line to the main axis with the most proximal outline defines the most proximal point on the olecranon (L5, proximal olecranon), which is also the farthest point from L1 (distal radioulna).

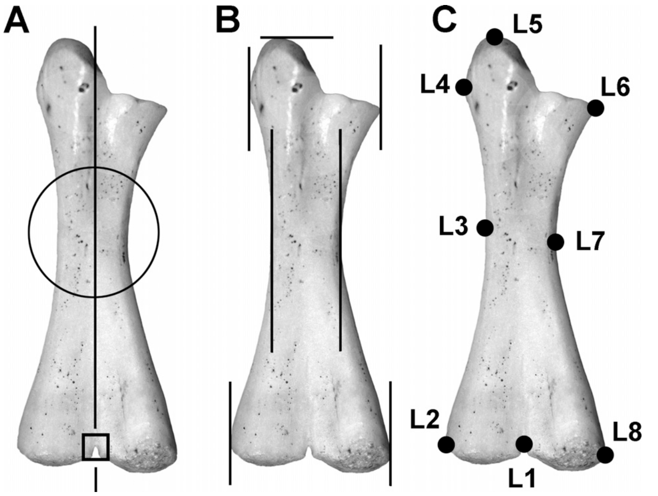

FIGURE 1. Radioulnar landmarks. Left radioulna of *Latonia gigantea* in posterior view. The geometric protocol for obtaining landmark coordinates is described in the text. **A**, a circle shows the region of the collum antibrachii, and a square points to the position of landmark L1, which jointly establish the main orientation axis; **B**, parallel lines, nearest and farthest to the main axis, tangent to the bone outline in the proximal and distal sectors; **C**, position of landmarks L1–L8.

Inter- and intraobserver errors of landmark coordinates were assessed by a repetition of the complete procedure made by 10 different colleagues, who were unrelated to this study. The results indicate that the percentage of Procrustes distance (defined below) in which interobserver error contributed to the total variance was small (1.7%), indicating a high level of repeatability for the process for landmark allocation and thus a high accuracy and repeatability in radioulna shape descriptors.

TABLE 1. Landmarks and their anatomical definitions on anuran radioulnae.

Landmark	Name	Definition
L1	Distal radioulna	Most distal point on radioulnar fusion edges
L2	Caput ulnae	Most lateral and distal point
L3	Ulnar diaphysis	Lateral maximum curvature
L4	Lateral olecranon	Most lateral projection of olecranon
L5	Proximal olecranon	Most proximal projection of olecranon
L6	Caput radii	Most medial and proximal point
L7	Radial diaphysis	Lateral maximum curvature
L8	Distal radius	Most distal and medial point

Morphometric Procedures

Procrustes Geometric Morphometrics—Geometric morphometric approaches use a partial Procrustes superimposition (Dryden and Mardia, 1998) of landmark configurations from anatomical structures, which translates, rotates, and scales (to unit centroid size) all specimens relative to the sample mean. Thus, Procrustes registration removes information unrelated to shape from the original coordinate configurations (Kendall, 1989). Procrustes shape coordinates (or principle components scores for each specimen) and a scaling factor (centroid size) are obtained as variables of shape and size, respectively (Bookstein, 1991; O'Higgins, 2000; Zelditch et al., 2004). After projection into the space tangent to Kendall's shape space, the shape data can be analyzed by linear multivariate statistics and variations represented using either transformation grids computed from thin plate splines (TPS) or other visualization techniques (Bookstein, 1991; O'Higgins, 2000; Zelditch et al., 2004). The metric of these configurations is Procrustes distance (d), computed as the square root of the summed squared distances between homologous landmarks of Procrustes-registered landmark configurations. Alternatively, TPS can be also used to provide a set of shape variables in the form of partial warps that can be used in statistical tests without any further adjustment of the degrees of freedom (Bookstein, 1991, 1996; Rohlf, 1996; Rohlf et al., 1996; Zelditch et al., 2004). This study used both types of shape variables.

Missing Data Reconstruction—Several methods can be used for missing data estimation, including statistical, geometric, and anatomical reconstructions (reviewed in Gunz et al., 2009; see also Neeser et al., 2009; Bastir et al., 2011; Benazzi et al., 2011; Neubauer et al., 2012). Among the statistical approaches to reconstruction, multiple multivariate regressions proved preferable to replacement of missing landmarks by the mean when larger reference populations were available. The geometric approach to reconstruction uses thin plate splines (TPS) to estimate missing landmarks by minimizing the bending energy in the transformation of a complete reference configuration into the (incomplete) target configuration. This method is particularly useful in the presence of densely spaced semilandmarks or if only one morphologically close and well-preserved specimen is available as reference (Gunz et al., 2009). However, it has been recommended to use several different reconstruction methods in order to improve the overall assessment of missing data (Gunz et al., 2009; Benazzi et al., 2011). Also, anatomical knowledge should always control the appraisal of quality of estimation. In this study, we used regression and TPS methods for missing data estimation; these were carried out using Morpheus et al. software (Slice, 2000).

Statistical Analyses—For assessing the morphometric affinities of the fossils, several methods were employed. First, geometric morphometric analyses were performed on the full sample and on six landmarks (those preserved in the Moratilla 2 (M2) fragment and shared with the remaining sample). We used analysis of variance (ANOVA) for comparing the centroid size of M2 with the mean centroid size of the remaining 15 species. Principal components analysis and Procrustes distances between different species group means and Moratilla 2 were used to address overall shape affinities. Then, regression analyses were carried out to address possible allometric factors on shape variation and to size-standardize the comparative sample, by downscaling species by species, to the shapes that correspond to the centroid size of Moratilla 2.

Once the size and shape affinities were established, we used multivariate regression and TPS reconstruction methods to estimate the two missing landmarks at the distal diaphysis of M2. With these methods several possible M2 reconstructions were produced. Using these different reconstructions and following standard recommendations (Gunz et al., 2009; Benazzi et al., 2011), we analyzed the effect of the reconstruction methods on the final landmark configuration of the fossil by principal components analysis (PCA) and by canonical variates analysis (CVA) (Nolte and Sheets, 2005). Analyses were carried out using Statistica 6.0 (StatSoft Inc., 1999), MorphoJ (Klingenberg, 2011), Morpheus et al. (Slice, 2000), and IMP series (Standard6, CVAGen7a) (Sheets, 2001).

PALEONTOLOGICAL ANALYSIS

Materials and Geologic Setting

The materials studied are fragments of one right ilium (Fig. 2) and one left radioulna (Figs. 3, 4), collected from the Moratilla 2 site during the 1984 joint excavations between the Museo Nacional de Ciencias Naturales (MNCN; Spain) and the University of Groningen (The Netherlands). The fossils are deposited in the Department of Paleobiology at the MNCN (Madrid, Spain) under catalog numbers MOR2-2000 (radioulna) and MOR2-2001 (ilium).

FIGURE 2. *Alytes* sp. Right ilium fragment from Moratilla 2 (MNCN Mor2-2001). **A**, lateral view; **B**, medial view, viewed as a mirror image to facilitate comparison. Scale bar equals 1 mm.

Locality Moratilla 2 is situated in the Ramblian type area (Daams et al., 1987; Van der Meulen and Daams, 1992), in the Lechago-Navarrete part of the Calatayud-Montalban Basin, 5.5 km north of Navarrete del Río (Teruel Province, eastern-central Spain). The fossiliferous sediments are represented by red silts with limestone concretions. The faunistic assemblage of Moratilla 2 (Van der Meulen and Daams, 1992) belongs to the biozone Aragonian Db, and to the European Neogene Mammal Zone MN5. Based on biostratigraphic correlation to the magnetostratigraphical dated type area of the Aragonian (Villafeliche area, Calatayud-Daroca Basin; Daams et al., 1999a, 1999b), the site could be dated to 15.78 Ma (Van der Meulen et al., 2003; Van Dam et al., 2006). However, unresolved general correlation problems that affect chronology in the European MN5 zone between the Iberian and North Alpine regions (e.g., Abdul-Aziz et al., 2010; Van der Meulen et al., 2011) suggest that the age of Moratilla 2, although not younger than 15.8 Ma, could be as old as ca. 17 Ma.

In comparison with other Spanish localities of middle Miocene age, the associated ectothermic vertebrate fauna of Moratilla 2

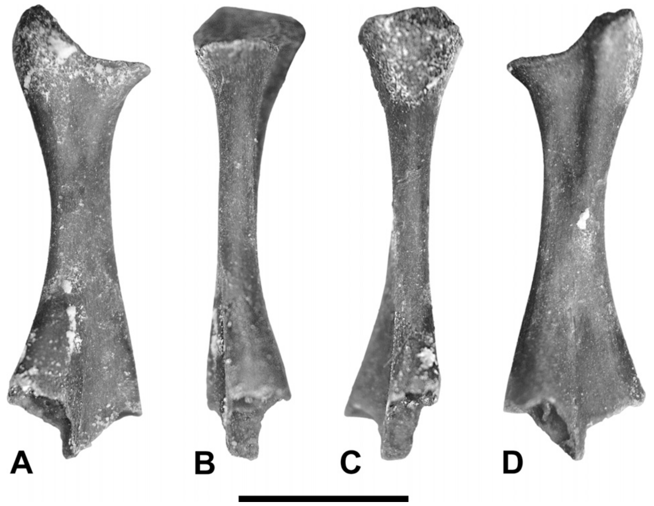

FIGURE 3. *Alytes* sp. Left radioulna from Moratilla 2 (MNCN Mor2-2000). **A**, facies caudalis; **B**, facies medialis; **C**, facies lateralis; **D**, facies cranialis. Scale bar equals 2 mm.

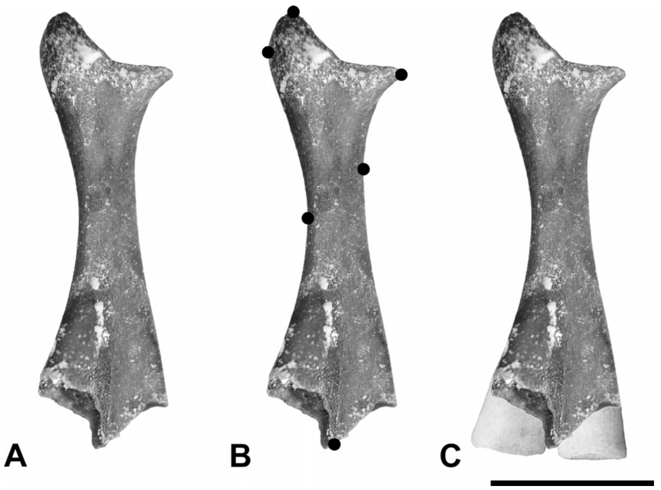

FIGURE 4. *Alytes* sp. Left radioulna from Moratilla 2 (MNCN Mor2-2000). **A**, fossil fragment in posterior view; **B**, position of landmarks directly observable; **C**, element reconstruction. Scale bar equals 1 mm.

is relatively diverse and, excluding the fossils described here, includes 11 additional taxa: *Latonia* sp. (Alytidae), Amphisbaenidae indet., Cordylidae indet., Scincidae spp. 1 and 2, Lacertidae spp. 1, 2, and 3, *Ophisaurus* sp. (Anguidae), Emydidae indet., and indeterminate snakes (Böhme and Ilg, 2003). The scincomorph reptiles are the most abundant ectothermic fossils, whereas fishes and urodeles are lacking in the sample. This assemblage suggests a relatively open environment with well-drained and oxygenated soils near nonpermanent water bodies.

Anatomical Description and Identification

Ilium—The available fragment (Fig. 2) lacks the dorsal extreme of the pars ascendens, the ventral part of the acetabulum, most of the pars descendens, and the anterior part of the iliac shaft. Nevertheless, the ilium is a highly informative element for anuran identification, and the Moratilla 2 fossil preserves enough features to be taxonomically located with confidence (Fig. 5). For example, following the criteria detailed by Böhme (1977) and Bailon (1999), the absence of a proximal dorsal crest rules out its assignment to discoglossines (*Discoglossus*, *Latonia*) or ranids, whereas the presence of a conspicuous tuber superius confirms that it is not a member of pelobatid (*Eopelobates*, *Pelobates*) or pelodytid (*Pelodytes*) taxa. Only the most dorsal part of the pars descendens is preserved, but its anterior dorsal origin (lower preacetabular zone) does not show the peculiar shape found in European hylids (*Hyla*). The presence of a pars descendens rules out its referral to Palaeobatrachidae, a family in which this structure is absent. European bombinatorids (*Bombina*), unlike the Moratilla 2 ilium, have a small tuber superius that is always located above the acetabulum, under the orientation system defined by Sanchiz (1998). The shape of the tuber superius makes the Moratilla 2 fossil different from European bufonids, other than some *Bufo* (sensu stricto), because it is long, low, and devoid of tubercles, unlike those of *Bufo verrucosissimus* and species in the genera *Bufotes* and *Epidalea*.

As preserved, the Moratilla 2 ilium resembles, among European anurans, those of *Alytes*, from which it does not differ in any qualitative feature. It is also similar, but not identical, to the

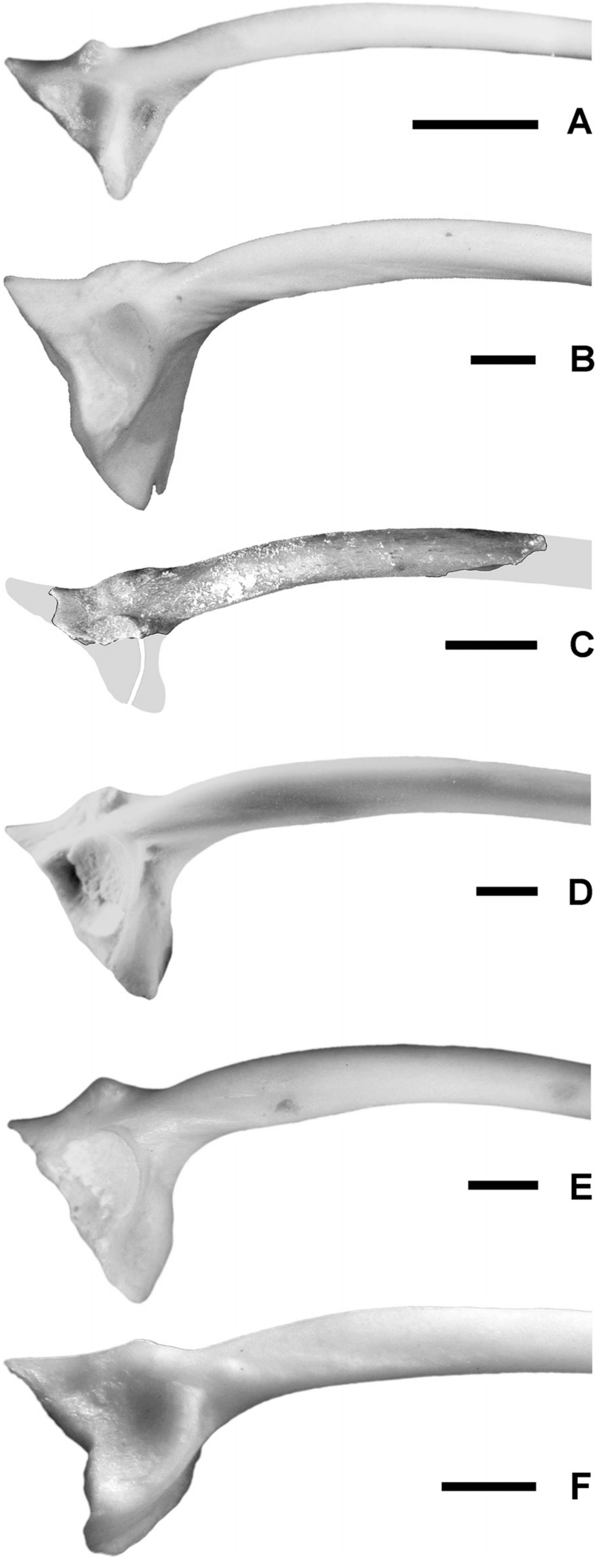

FIGURE 5. Comparative sample of right ilia in lateral view. Distal parts of the bones not shown. **A**, *Hyla meridionalis* (MNCN 19636); **B**, *Bufo bufo* (MNCN 15414); **C**, Moratilla 2 fragment (MNCN Mor2-2001), reconstructed after a generalized model of *Alytes*; **D**, *Bufotes viridis* (MNCN 40454); **E**, *Epidalea calamita* (MNCN 15477). **F**, *Bombina variegata* (MTC uncataloged). Scale bars equal 2 mm.

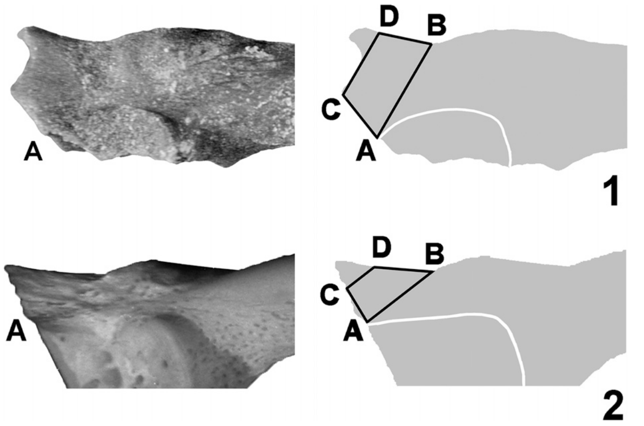

FIGURE 6. Origin of the pars ascendens, external view, in right ilia oriented following Sanchiz (1998). **1**, *Alytes* sp. from Moratilla 2 (MNCN Mor2-2001); **2**, *Bufo bufo*. Segment AB is drawn between the uppermost acetabular point in the iliac symphysis (A) and the dorsally lowermost point between tuber superius and pars ascendens (B). Segment CD is the parallel to AB drawn at a perpendicular distance of 47% AB length.

morphology present in small specimens of the genus *Bufo* (sensu stricto, the *Bufo* [*bufo*] species group of previous authors). However, one observable character is present in the Moratilla 2 fragment that allows its clear differentiation with respect to *Bufo*, and also from any other European Neogene anuran, except *Alytes* and the discoglossines. In relative terms, the pars ascendens of the fossil is much more developed than in the other taxa, a probable autapomorphic feature of Alytidae. The pars ascendens of the Moratilla 2 fossil is not completely preserved, but the observable zone is sufficient to obtain a numerical estimate, as indicated in Figure 6. The index (%) between the pars ascendens base (AB) and its parallel width (CD) at a perpendicular distance of 47% of AB is 68.03 for Moratilla 2, whereas *Bufo bufo* is significantly different with a mean index of 49.5% (n = 13, SD = 4.42). On the other hand, a combined *Alytes* sample (*Alytes cisternasii*, n = 12; *Alytes dickhilleni*, n = 9; *Alytes muletensis*, n = 5; and *Alytes obstetricans*, n = 12) has an index of 72.0% (SD = 4.97), which does not differ significantly from Moratilla 2. Within *Alytes* samples, the mean values range between 71.7% (*Alytes dickhilleni*) and 73.2% (*Alytes muletensis*). The detailed shape of the tuber superius in Moratilla 2 differs slightly from the most common shape observed in *Alytes* species; it is low and lacks abrupt anterior and posterior ends. However, variation in these features have never been properly studied nor quantified; thus, they could be phenotypically equivocal, as demonstrated in other anurans (Bever, 2005). In samples of all of the species of *Alytes*, we have observed specimens with morphotypes similar to Moratilla 2. In summary, based on the arguments presented here, we confidently identify this fossil as the ilium of an *Alytes* species.

Radioulna—As shown in Figure 3, the bone is complete, except at the distal end where it lacks most of the radial and ulnar epicondyles (capitulum radii and capitulum ulnae). However, the most distal point of fusion between the radius and ulna is preserved, thus providing a reliable position for landmark L1 (Fig. 4). Anuran radioulnae are considered elements with extremely low information content for taxonomic identification; therefore, they are rarely incorporated into phylogenetic studies of relationships. For this reason, as detailed below, we quantitatively identified the fossil fragment using geometric morphometrics. We followed two main steps. In the first one, within the different anuran samples studied, the shape most similar to the fossil one is detected. Only the information available from the fragment is used for this process. In the second step, using the shape affinities assessed during step 1, the complete morphology of the element is inferred using different quantitative reconstruction methods. These anatomical reconstructions are first used to find the shapes most phenetically similar to the fossil within the genus. Subsequently, this information is used to examine the morphological adaptive change within the known evolutionary framework of the group.

Theses analyses used representatives of the main European Neogene anuran groups: one species sample per included genus and all species of the genus *Alytes*. All of the *Alytes* species were considered because familiarity with their fossil record and previous classical biometric analyses indicated a probable a priori assignment to this group. In contrast, we were unable to include any representative from the family Palaeobatrachidae, as adequate samples of complete elements are unavailable. However, based on qualitative observations used for identification, the Moratilla 2 radioulna differs considerably in overall shape from the palaeobatrachid morphotype, as represented by *Palaeobatrachus hiri* (Fig. 7) from Mátraszőlős 2 (Hungary). For example, the relative position of radial diaphysis curvature (L7) with respect to ulnar diaphysis curvature (L3) is reversed compared with the position observed in Moratilla 2, and the orientation of the olecranon cavity edge in the facies caudalis (L5, L6) has a much lower angle with respect to the longitudinal axis. Furthermore, although negative evidence is never conclusive, it is worth mentioning that palaeobatrachids have never been found in the Iberian Neogene, a region with a relatively rich anuran fossil record (Martín and Sanchiz, 2013; Venczel et al., 2013; Wuttke et al., 2012).

Geometric morphometric procedures were used for size and shape comparisons. ANOVA of centroid size analysis showed

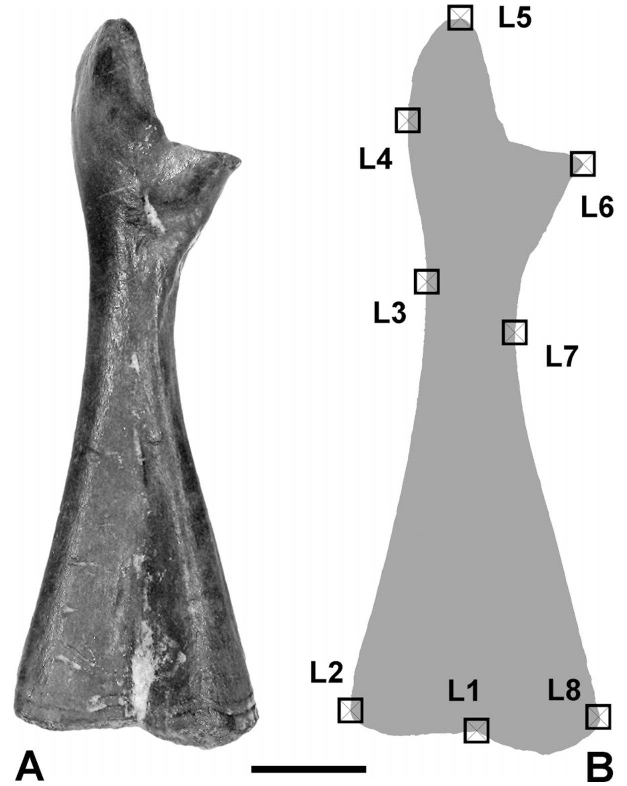

FIGURE 7. Left radioulna of *Palaeobatrachus hiri* (MMP Pásztó, uncataloged) from Mátraszőlős 2 (Hungary). **A**, complete bone in posterior view; **B**, position of landmarks. Note that the relative position of landmarks L3 and L7 is the reverse to the Moratilla 2 one. Scale bar equals 2 mm.

TABLE 2. Descriptive statistics of centroid size (CS) on anuran radioulnar samples.

Species	Mean	SE	CI −95%	CI +95%	N
Moratilla 2	4.51				1
Alytes cisternasii	5.96	0.16	5.64	6.28	16
Alytes dickhilleni	7.00	0.20	6.59	7.40	10
Alytes maurus	7.12				1
Alytes muletensis	7.49	0.17	7.17	7.82	15
Alytes obstetricans	6.92	0.16	6.60	7.23	16
Bombina variegata	7.92	0.20	7.52	8.32	10
Bufo bufo	7.90	0.16	7.58	8.22	16
Discoglossus jeanneae	6.95	0.19	6.57	7.34	11
Epidalea calamita	7.37	0.16	7.05	7.69	16
Hyla meridionalis	8.34	0.16	8.02	8.66	16
Latonia gigantea	7.45	0.19	7.08	7.82	12
Pelobates cultripes	7.08	0.16	6.77	7.40	16
Pelodytes ibericus	9.04	0.21	8.62	9.46	9
Pelophylax perezi	7.13	0.16	6.82	7.45	16
Rana iberica	8.23	0.16	7.91	8.55	16

Abbreviations: **SE**, standard error; **CI**, confidence interval; **N**, sample size.

significant variation in overall size (F(15, 181) = 17.333, P = 0.000) and also that all species were significantly larger than M2 (Table 2). A principal components analysis (not shown) of all specimens (N = 197) was applied to extract the main patterns of variation. However, the PC scatter plots showed large and overlapping distributions, with M2 in the upper central part of the distributions. Thus, because no clear pattern emerged from these distributions, we compared the shape of the M2 fossil with the mean shapes of the comparative species by evaluating the Procrustes distance of the fossil to the closest species. This analysis showed that three of the four closest neighbors in Procrustes distance belonged to the genus *Alytes* (i.e., *Alytes dickhilleni*, d = 0.050; *Alytes maurus*, d = 0.039; *Alytes muletensis*, d = 0.044; *Pelophylax perezi*, d = 0.058), which fits also with morphological diagnostics based on iliac morphology.

Therefore, M2 was analyzed again by PCA but in the closer phylogenetic framework of the genus *Alytes* only (Fig. 8). This analysis suggested that M2 was likely relatively narrow and long (gracile) and more similar in shape to *Alytes dickhilleni*, *Alytes maurus*, and *Alytes muletensis* than to the robust forms of *Alytes obstetricans* and *Alytes cisternasii*.

However, because of the significant size differences between M2 and the remaining groups, and because of the well-known allometric growth throughout life in anurans, allometric effects on the shape affinities of M2 to the remaining species could not be ruled out. To control for these allometric sources of shape variation and affinities, further analyses were carried out.

Regression analysis of shape on size on the full sample confirmed the hypothesis of allometry and accounted for approximately 16% of total variance ($P < 0.001$), suggesting a need for controlling size variation. Consequently, we used species-specific regression models to standardize all specimens of *Alytes* species to the shapes they would have at centroid size 4.5, the size of M2 (Table 2). In doing so, we controlled quantitatively for size, and thus allometric variation at the most appropriate level of morphometric comparisons, namely, with all specimens standardized in shape by downscaling them to smaller (CS = 4.5) size (Bastir and Rosas, 2004). As expected, after removal of the allometric fraction of variation, the distribution patterns became much clearer (and narrower), although the principal affinities of M2 to *Alytes dickhilleni*, *Alytes maurus*, and *Alytes muletensis* remained similar (Fig. 8).

Statistical and Geometric Reconstructions

Once the phenetic affinities had been identified, we used four different multiple multivariate regressions for statistical reconstructions of M2, using as reference *Alytes dickhilleni*, *Alytes cisternasii*, *Alytes obstetricans*, or *Alytes muletensis*, and the TPS

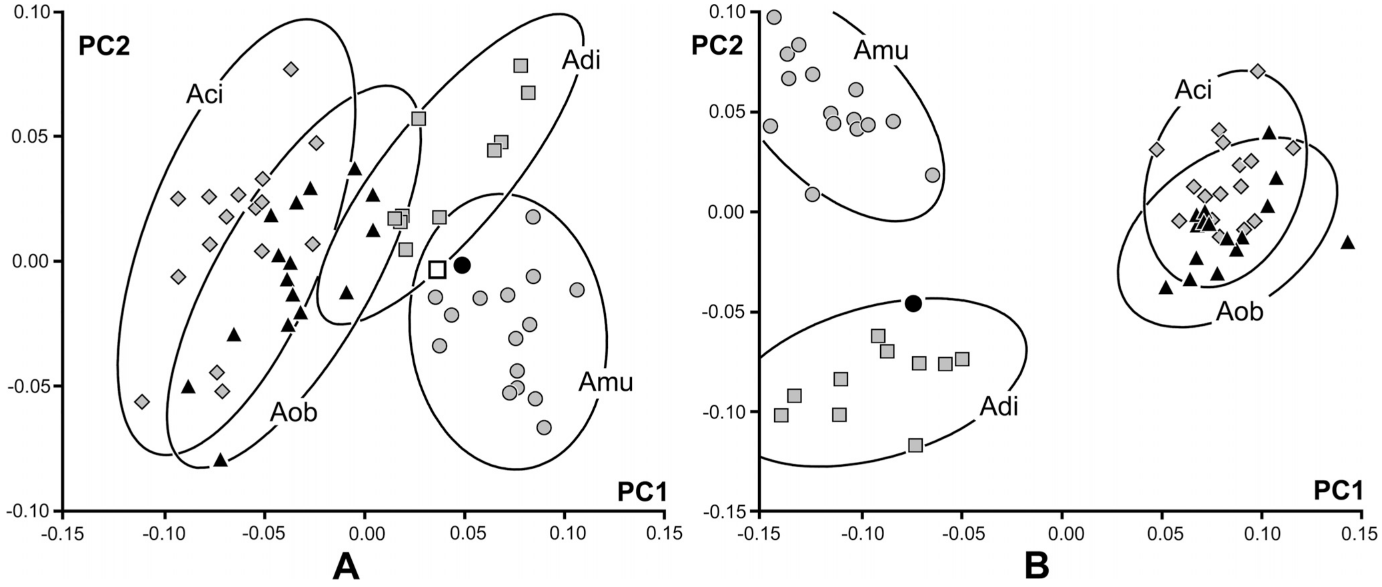

FIGURE 8. *Alytes* radioulnae. **A**, principal components analysis (six landmarks) plus 95% confidence ellipses showing scores along PC1 and PC2. PC1 accounts for 55% of total variance, PC2 for 18.6%. Note that in this projection Moratilla 2 (M2) plots along PC1 within 95% confidence intervals of the *Alytes muletensis* distribution but also very close to 95% range of *Alytes dickhilleni*; **B**, PC1 (65.3% of total variance) and PC2 (17.9% of total variance) plot of the same data, but after species-specific control for allometric variation, and downscaled to the shapes predicted for the size of Moratilla 2 (centroid size = 4.5). Note that Moratilla 2 is still on the PC1 scores of *Alytes muletensis* and *Alytes dickhilleni*, but now within the 95% range of the latter.

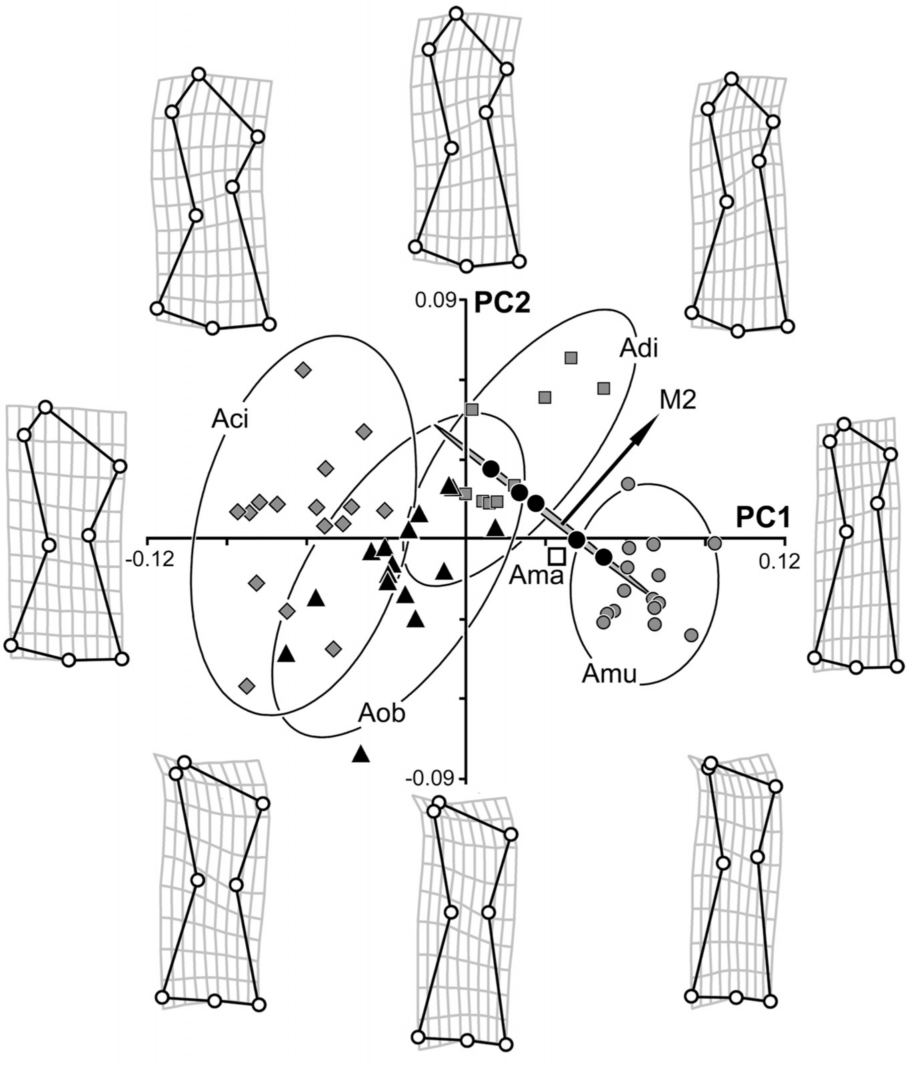

FIGURE 9. Principal components analysis of genus *Alytes* radioulnae with all five quantitative reconstructions of Moratilla 2 (M2) after each of the living species (eight landmarks), showing scores along PC1 and PC2 plus 95% confidence ellipses. PC1 accounts for 54.4% of total variance, PC2 for 16.7%. TPS grids illustrate the corresponding shape variations in this subspace, at axes and mid-quadrant regions. Note the separation of the gracile group (*Alytes muletensis*) from the different robust groups (*Alytes dickhilleni*, *Alytes obstetricans*, *Alytes cisternasii*). Note also how the different Moratilla 2 reconstructions run from intermediate to gracile positions. Left quadrants, with PC1 negative scores, show the range of robust specimens (proximal and distal ends wider relative to length), whereas right quadrants, with positive PC1 scores, contain more gracile specimens. The upper right quadrant (positive PC1 and PC2 scores) points to an increased robustness of the distal bone end, and the olecranon cotyle, interlandmark line D(5–6), is oriented at a closer angle to the main axis (D1–5), about 46.2°, whereas the lower right quadrant shows morphologies in which the olecranon cotyle is more orthogonal (angle about 63.7°) and the distal bone end relatively more gracile.

method for geometric reconstruction of M2 using *Alytes maurus*, for which only one specimen was available. As a result, we obtained five different quantitative M2 reconstructions, the morphometric affinities of which were reassessed and compared with previous size, shape, and allometric analyses using PCA and CVA on the full landmark set (LM = 8). The PCA plots (Fig. 9) showed a clear range of M2 reconstructions from the center of the *Alytes dickhilleni* distribution towards *Alytes muletensis*, i.e., always within the gracile realm, and away from the robust *Alytes obstetricans* and *Alytes cisternasii* groups. A final CVA was carried out that revealed three highly significantly different axes (Axis 1: Wilks lambda = 0.0039, $\chi^2 = 269.0060$, df = 48, $P < 0.001$; Axis 2: Wilks Lambda = 0.1048, $\chi^2 = 109.3861$, df = 33, $P < 0.001$; Axis 3: Wilks Lambda = 0.3249, $\chi^2 = 54.5236$, df = 20, $P < 0.001$). Jackknife assignment tests (N = 100), leaving out 10% of known data, identified correctly and significantly 81.3% of 600 ‘unknown’ specimens. The CVA model suggested a phenetic assignment of the five different M2 reconstructions to *Alytes dickhilleni* on four occasions and to *Alytes muletensis* on one.

Based on these results, both the iliac and radioulnar fragments from Moratilla 2 can be identified with confidence as belonging to a new, currently unnamed species of the genus *Alytes* (Alytidae). A formal proposal of a new species will be made when other elements of the skeleton become available, and a more complete diagnosis is possible.

Among living representatives of *Alytes* (Fig. 10), the overall morphology most similar to the fossil is found in Balearic midwife toads (*Alytes muletensis*) and perhaps also to *Alytes maurus*, although the variation of the latter is unknown. Using a combined sample of *Alytes*, a RMA (reduced major axis) regression model analysis inferring size (SVL) was performed on the most accurate interlandmark distance estimators for the radioulna D(1–5), and for the ilium (dorsal extreme of tuber superius to anterior-most point of acetabulum in the main axis). The results indicated that Moratilla 2 fossils are within the size range of *Alytes*, with a minimum SVL of approximately 36 mm as predicted from the radioulna. The ilium corresponds to a younger and smaller individual (SVL of approximately 28 mm).

MORPHOLOGICAL EVOLUTION IN *ALYTES*

The Fossil Record of *Alytes*

The knowledge management system Lisanfos KMS (Martín and Sanchiz, 2013) indicates that, excluding historical

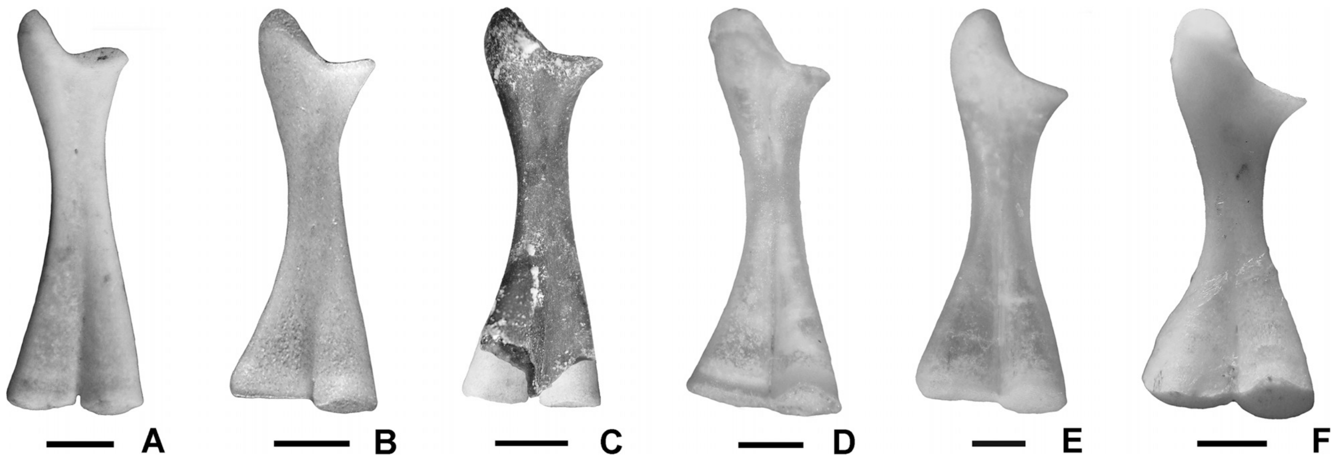

FIGURE 10. Left radioulnae from species of *Alytes* in facies caudalis view. **A**, *Alytes muletensis* (Cova de la Barxa 02); **B**, *Alytes maurus* (MNCN 40768); **C**, Moratilla 2 fragment (MNCN Mor2-2000) reconstructed; **D**, *Alytes dickhilleni* (MNCN 16781); **E**, *Alytes obstetricans* (MNCN 15102); **F**, *Alytes cisternasii* (MNCN 15504). Scale bars equal 1 mm.

misidentifications, fossil remains of *Alytes* have been recovered at least from 61 localities. With the exception of a Holocene German site (*Alytes obstetricans*), a Russian vertebral fragment (cf. *Alytes* sp.) from the middle Pleistocene (Ratnikov, 1997) that requires confirmation, and a lower Pleistocene record from Morocco (cf. *Alytes maurus*), all of the Holocene (18 sites) and Pleistocene (37 sites) records come from France and Spain. Excluding one Spanish Holocene site with a probable *Alytes cisternasii* assignment, all of the Quaternary fossil records in continental Europe, for which species identification is possible, are considered *Alytes obstetricans*, and have been found within this species contemporary range.

The records of *Alytes* in non-Continental Europe, all of them Quaternary, are based on the former genus *Baleaphryne* from the Balearic Islands, initially discovered as an extinct insular form (Sanchiz and Adrover, 1977), but later found living in relict mountain areas of Majorca. A comprehensive analysis of the biological peculiarities of *Baleaphryne* (Hemmer and Alcover, 1984) suggested that it should be considered a synonym of *Alytes*. However, recent molecular studies indicate that subgeneric status for *Baleaphryne* may be maintained (Martínez-Solano et al., 2004; Gonçalves et al., 2007).

Besides Moratilla 2, two localities older than the Plio-Pleistocene, Willershausen (lower Pliocene; Germany) and Salobreña (uppermost Miocene; Spain), might contain *Alytes* remains; however, these remains have not been fully described, and their identification needs to be confirmed (Sanchiz, 1998). Two extinct species have been proposed, one of them (*Alytes grandis*), from the German middle Pleistocene, is a synonym of *Rana temporaria* (Ranidae), as indicated by Rage (1984) and analyzed by Sanchiz and Schleich (1986). The other species, *Alytes talaioticus* (Sanchiz and Alcover, 1982) from the Holocene of Minorca (Balearic Islands), is currently considered a synonym of the extant *Alytes muletensis* from the near island of Majorca.

Phylogenetic Relationships within *Alytes*

The genus *Alytes* is probably the only representative of a very old anuran lineage (Alytinae) that separated from its nearest living relatives (Discoglossinae and Bombinatoridae) during the late Lower Cretaceous (Blackburn et al., 2010). This is likely because the Upper Jurassic and early Lower Cretaceous alytids (e.g., *Eodiscoglossus*) do not belong to any of the living clades (contra Sanchiz, 1998), and this is in congruence with the latest sequence-based molecular clock data (Blackburn et al., 2010; Pyron and Wiens, 2011). The relationships among living species of *Alytes* are indicated in Figure 11, based upon the molecular phylogenetic results obtained by Martínez-Solano et al. (2004) and

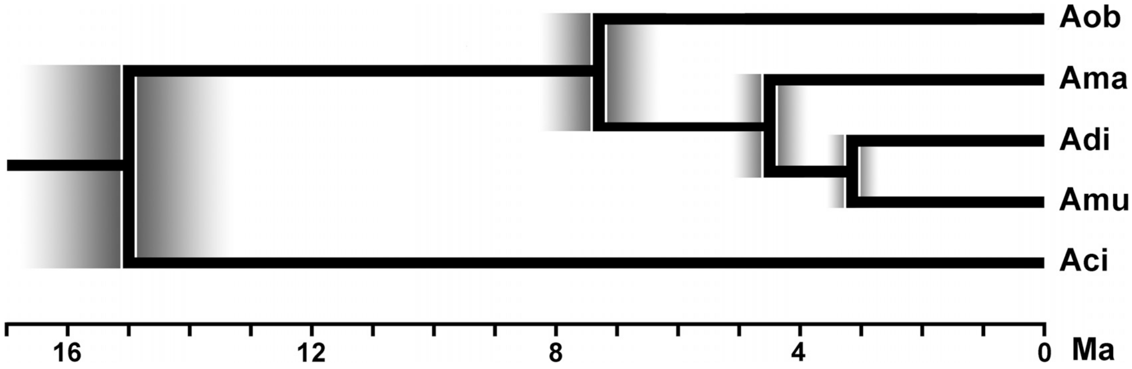

FIGURE 11. Phylogenetic species tree of the living genus *Alytes* based on DNA sequences, simplified from Martínez-Solano et al. (2004) and Gonçalves et al. (2007). Gradations at the nodes indicate the confidence intervals of the molecular clock. **Abbreviations**: **Aci**, *Alytes cisternasii*; **Adi**, *Alytes dickhilleni*; **Ama**, *Alytes maurus*; **Amu**, *Alytes muletensis*; **Aob**; *Alytes obstetricans*.

Gonçalves et al. (2007). Because we considered the Moratilla 2 fossil as the outgroup of the living *Alytes* clade, its similarity with any of these species would be only based on primitive character states; therefore, the fossil could not be taxonomically assigned to any of the living species.

Morphological Differentiation in *Alytes*

Concerning locomotion, the genus *Alytes* is one of the few anuran groups that includes semifossorial species that are forelimb diggers. As detected in an early comparative osteometrical analysis (Sanchiz, 1984), generic differentiation fluctuates between robust forearms in the more fossorial species (*Alytes cisternasii* and *Alytes obstetricans*) and gracile forearms associated with 'climbing' movements and fissure hiding in extreme vertical ravines (*Alytes muletensis*). In the first cladistic analysis made on this topic (Sanchiz, 1984), the robust forearm morphology, common to all continental species known at that time, was considered primitive, and the gracile morphology was considered an autapomorphic adaptation, detected only in the newly discovered insular sample. Other species discovered or recognized afterwards (*Alytes dickhilleni* and *Alytes maurus*) showed intermediate morphotypes.

Geometric morphometric analysis of the whole group confirms that a robust and a gracile pattern can be morphologically distinguished (Fig. 9). The robust pattern is characterized by relatively increased proximal and distal breadth compared with its length, whereas gracility is indicated by increased relative length. This pattern is strongly reflected in the results of the PCA (Fig. 9), where PC1 (54.8% of total variance) tends to polarize gracile (PC1 positive) and robust (PC1 negative) morphologies. Shape variations along PC2 (16.7% of total variance; Fig. 9) also show features of robusticity but at the distal bone end only, along with variations at the fossa olecrani (L5, L6). At negative loadings on PC2, this fossa is oriented more orthogonally relative to the main axis and the distal end of the bone is relatively narrower and thinner. Towards the positive PC2 loadings, the fossa olecrani is less orthogonal to the main axis, and the distal radioulnar part is relatively wider and thicker, as exemplified by *Alytes cisternasii* and *Alytes dickhilleni* (PC2 positive).

Radioulnae of *Alytes dickhilleni* show intermediate morphological features, combining robustness at the distal part with gracility at the proximal one (see also images in Fig. 10). The results of this PCA suggest thus that robust radioulnae are achieved in different configurations. As a consequence, when mapped on the evolutionary tree based on molecular data (Fig. 11), this morphological difference indicates that robustness was achieved independently three times, in *Alytes cisternasii* and *Alytes obstetricans* to a convergent morphotype, and by *Alytes dickhilleni* to another robust design. Our results support the hypothesis that Moratilla 2 likely represents a generalized, intermediate morphology, certainly not as robust as *Alytes cisternasii* or *Alytes obstetricans*, and neither as gracile as *Alytes muletensis*.

The radioulnar analysis suggests that the last common ancestor for the living species of *Alytes* (Moratilla 2 record) was phenetically more similar to the clade *Alytes* (*Baleaphryne*), and that at least four different morphological evolutionary trends occurred: one towards increased gracility and three towards increased robustness (Fig. 12). Starting from the Moratilla 2 morphotype, two parallel trends for increased robustness are detected, one directly leading to *Alytes cisternasii* and the other to the common ancestor of all of the other *Alytes* species (node A), which continues on to the living *A. obstetricans*. The only gracility trend starts in node A and leads to node B, the common ancestor of the living clade *Alytes* (*Baleaphryne*), then continues very strongly to *Alytes muletensis*, most probably by insular evolution. From node B, the trend towards the African *Alytes maurus* cannot be reliably inferred at present because only one specimen was available for analysis, and is very similar to the node B morphotype. However, also from node B, a clear trend towards robustness, leading to *Alytes dickhilleni*, is inferred as a third semifossorial convergent adaptation.

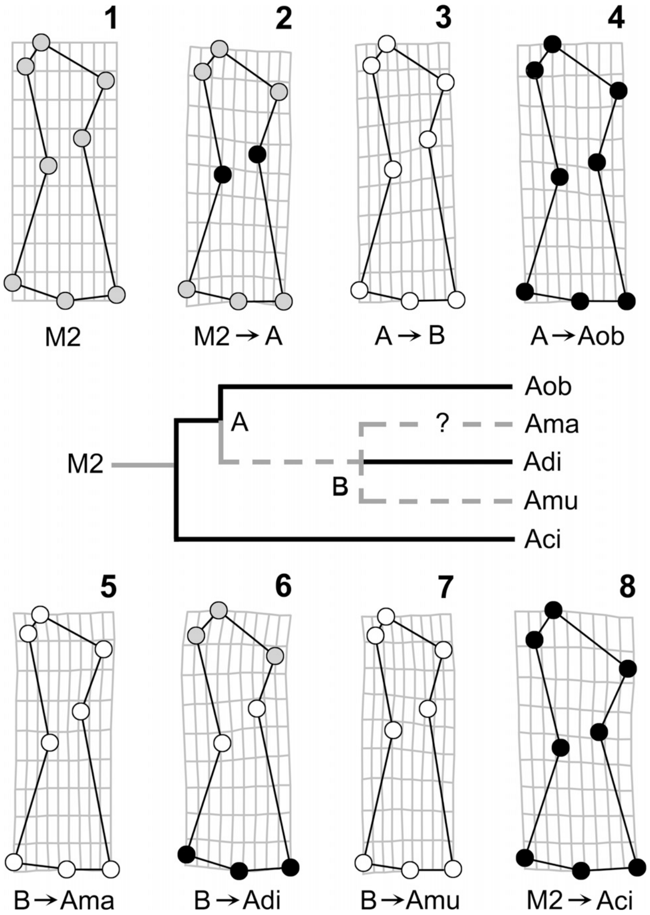

FIGURE 12. Model of morphological evolution in *Alytes* radioulnae. Morphometric analysis mapped on the phylogenetic tree indicates that robustness has been acquired independently in *Alytes dickhilleni*, *Alytes obstetricans*, and *Alytes cisternasii*. The color of each landmark indicates that in the corresponding branch it has undergone a trend towards more robust (black), more gracile (white), or stable (gray points) morphologies. The branches are indicated bt arrows. **1**, *Alytes* sp. from Moratilla 2 (M2); **2**, Node A (A). Hypothetical ancestral morphology estimated as mean shape of descending species *A. obstetricans*, *A. dickilleni*, *A. maurus*, and *A. muletensis*; **3**, Node B (B), subgenus *Alytes* (*Baleaphryne*) clade. Hypothetical ancestral morphology estimated as mean shape of descending species *A. dickhilleni*, *A. maurus*, and *A. muletensis*; **4**, *Alytes obstetricans*; **5**, *Alytes maurus*; **6**, *Alytes dickhilleni*; **7**, *Alytes muletensis*; **8**, *Alytes cisternasii*.

Discovery of fossil *Alytes* in Moratilla 2 is fortunate because the locality can be dated to a time slightly prior to the initial diversification of the living species of the genus. The morphological locomotor adaptations that resulted in the diversification of *Alytes* since the middle Miocene can only be deduced as general trends because the current lack of other fossils of *Alytes* prevents correlating them with the increasingly detailed models of environmental time changes available (e.g., Böhme et al., 2011).

CONCLUSIONS

The conclusions drawn from the study of the middle Miocene Moratilla 2 anuran remains can be grouped into several levels of generalization:

1. Paleontological history of midwife toads (*Alytes*). We have shown that the Moratilla 2 remains likely belong to a new unnamed species of the genus *Alytes*. This extinct species lived slightly prior in time to the initial and early splits that resulted in the current biodiversity pattern of *Alytes*. As the only potential ancestral species known, parsimony suggests that its morphology is the primitive condition for the genus.
2. Evolutionary history of midwife toads (*Alytes*) as a case example of the usefulness of fossil fragments. We selected the Moratilla 2 fossil sample and analyzed the least informative element available to demonstrate how fragmentary material, even elements normally considered taxonomically uninformative and frequently overlooked, can (and should) be studied using numerical quantitative approaches, because they might provide a relevant scientific value that cannot be obtained otherwise. In our case, we were able to infer different convergent adaptive trends on forearm locomotor performance. On the one hand, the common increased relative widths of the proximal and distal bone ends in *Alytes cisternasii* and *Alytes obstetricans*, but having a fossa olecrani differently orientated, thus not being parallelism, but convergence. A different robust trend is found in *Alytes dickhilleni*, increasing the distal relative width but not the proximal one. The insular lineage of *Alytes muletensis* shows instead a reverse trend towards extreme gracilization, presumably an adaptation to a ravine environment requiring a 'climbing,' and not fossorial, locomotory performance.

   The important relevance of this result is to point out that this inference would have remained hidden, because neither molecular nor comparative osteological approaches of living forms would have detected it in the absence of the Miocene fossil fragment.
3. Evolutionary history of midwife toads (*Alytes*) as a case example of how divergence times and molecular evolutionary models can be combined with paleontological data at lower taxonomic levels. We present another example of how paleontology can be used as a potential refutation test for time estimations derived from molecular clocks and, more importantly, demonstrate that much informative data can be extracted using the synergistic combination of evolutionary frameworks, imported from molecular data, with the polarities, change rates, and adaptive trends inferred from morphological data from fossil and living forms. A scientific history of adaptation now seems feasible, even at low taxonomic levels and using few anatomical elements. Moreover, in the future, these historical models of adaptation can potentially be compared with independently derived data based on the environmental history of Earth.

As a final technical observation, it is important to recognize that in order to biometrically analyze fossil fragments, such as the ones presented here, access to appropriate osteological comparative material in museum collections is critical. For example, it is fundamental to have samples of dry skeletons, representing natural variation, which also have isolated elements from which measurements can be taken. We have verified that these types of collections are not presently available in Europe for almost all anuran genera.

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