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Intraspecific morphological variation and its implications in the taxonomic status of 'Bufo pisanoi,' a Pliocene anuran from eastern Argentina

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INTRASPECIFIC MORPHOLOGICAL VARIATION AND ITS IMPLICATIONS IN THE TAXONOMIC STATUS OF ‘*BUFO PISANOI*,’ A PLIOCENE ANURAN FROM EASTERN ARGENTINA

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ABSTRACT—The true toad family Bufonidae is one of the most speciose and widespread clades of neobatrachian anurans. Despite being well represented in South America at present, bufonids have a poor fossil record on this continent. ‘*Bufo pisanoi*’ from Pliocene outcrops of coastal Buenos Aires Province, Argentina, is the only extinct South American bufonid taxon recognized to date. In this study, we investigated the validity of the putative diagnostic characters of this extinct species to confirm its taxonomic status based on revision of the holotype and referred material. Comparisons with several extant bufonid species led us to include the taxon represented by this fossil material within the *Rhinella marina* clade, close to *R. arenarum*. Further comparisons and a geometric morphometric analysis based on a relatively large sample of the latter species demonstrated that the morphology of ‘*B. pisanoi*’ falls within the variation range of *R. arenarum*. Therefore, we consider that the names ‘*B. pisanoi*’ and *R. arenarum* represent the same taxon, with the latter having priority. This work emphasizes the importance of considering intraspecific variation whenever possible, because this leads to a more rigorous approach to the identification of fossil specimens and tests the taxonomic value of different osteological characters.

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

INTRODUCTION

The true toad family Bufonidae is one of the most speciose clades of neobatrachian anurans, with 558 extant species described to date (Frost, 2013). This large group has a nearly cosmopolitan distribution, being native to all areas except the Australopapuan Realm (excluding Sulawesi), Madagascar, Antarctica, and the Arctic. The diversification and intrarelationships of Bufonidae have been addressed recently based mostly on molecular data, as part of either broad-scaled (Frost et al., 2006; Pyron and Wiens, 2011) or more restricted (Pramuk, 2006; Pramuk et al., 2008; Maciel et al., 2010; Van Bocxlaer et al., 2010) studies. As a result of these investigations, several names of taxa within Bufonidae have been changed in accordance with the retrieved phylogenetic relationships. In this regard, the widely used generic name *Bufo* is now applied only to a small clade of Eurasian toads, whereas most of the South American species that had been referred to *Bufo* form a clade named *Rhinella* (Chaparro, 2007). *Rhinella* is recovered as the most derived clade among South American bufonids in recent phylogenetic analyses (e.g., Van Bocxlaer et al., 2010; Pyron and Wiens, 2011), although its relationships to the non-South American groups are still contentious. Despite being well represented in South America at present, bufonids have a poor fossil record in this continent consisting mostly of fragmentary, isolated bones that are generally difficult to identify at the species level. *Bufo* (now *Rhinella*) *pisanoi* from Pliocene outcrops of coastal Buenos Aires Province, Argentina (Fig. 1) (Casamiquela, 1967), is the only extinct South American bufonid taxon that has been

recognized to date, although Sanchiz (1998) placed this binomen into synonymy with the extant *Rhinella* (formerly *Bufo*) *schneideri* (Werner, 1894).

In this study, the holotype and the referred material of ‘*Bufo pisanoi*’ originally described by Casamiquela (1967) were examined with the aim to investigate whether the morphology of ‘*B. pisanoi*’ is actually different from other extant species and to test the validity of the putative diagnostic characters of this fossil species to confirm its taxonomic status. Preliminary observations revealed the similarity of ‘*B. pisanoi*’ to the extant *Rhinella* (formerly *Bufo*) *arenarum* (Hensel, 1867), a species that inhabits the region where the fossil sites that yielded this material are located. Therefore, our study focused on the skeletal morphology and intraspecific variability of this taxon, although we also expanded our comparative sample to avoid the problem of assumption of geographic or temporal stability as discussed by Bell et al. (2010).

Institutional Abbreviations—**FCEN**, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina; **MACN**, Museo Argentino de Ciencias Naturales Bernardino Rivadavia, Buenos Aires, Argentina; **MMP**, Museo Municipal de Ciencias Naturales Lorenzo Scaglia, Mar del Plata, Argentina; **PVL**, Paleontología de Vertebrados, Instituto Miguel Lillo, Tucumán, Argentina; **ZMB**, Zoologisches Museum, Berlin, Germany.

MATERIALS AND METHODS

We examined all of the materials that have been assigned to ‘*Bufo pisanoi*,’ including the holotype, which was erected, described, and figured by Casamiquela (1967). These fossil

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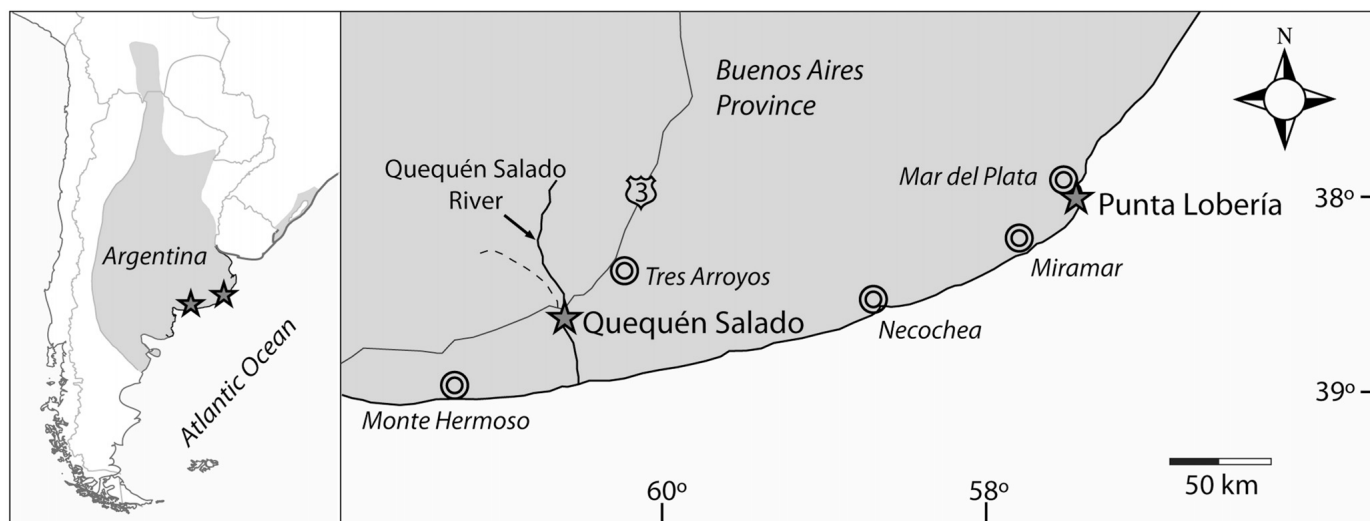


FIGURE 1. Map showing the provenance of the fossil materials of '*Bufo* (= *Rhinella*) *pisanoi*' in Buenos Aires Province (stars), Argentina, and the current distribution of *Rhinella arenarum* (highlighted in dark gray).

specimens, listed below, are three-dimensionally preserved and most belong to a single individual. Comparisons between this material and numerous bufonid species from South America were based on direct observation of skeletons whenever possible (Supplementary Data), as well as on published data. Given our preliminary observations, we focused on species of the *Rhinella marina* species group and, particularly, on the living *R. arenarum*. In this regard, the close resemblance of the fossil elements to the corresponding bones of this species led us to analyze the intraspecific variation of some discrete characters of cranial and postcranial elements in 34 specimens of *R. arenarum* and to perform a geometric morphometric analysis of the frontoparietals of the latter species and '*Bufo pisanoi*.' All of the examined skeletons of the living species belong to adult individuals, the snout-vent lengths of which range between 96.72 mm (male) and 147 mm (male). The majority of these specimens (27) were collected in a small area of northeastern Buenos Aires Province (Fig. 1) and presumably belong to the same population, although a few specimens of the same species (7) from other regions of its present distributional range were studied as well (Supplementary Data). Our total sample of *R. arenarum* included 22 males, five females, and seven specimens of undetermined sex.

The frontoparietal was selected for the morphometric analysis because it has frequently been used as a diagnostic element of bufonids in neontological studies (e.g., Martin, 1972) and also because it is well preserved enough to confidently place landmarks in one of the fossil specimens. The articulated condition of most of the skeletons in our sample of the living species prevented the use of the maxilla and nasal. The dorsal shape of the left frontoparietal in dorsal view was captured through five two-dimensional (2D) landmarks (Fig. 2). The image files (TPS) were created using tpsUTIL1.40 (Rohlf, 2008) and landmark digitization was carried out using tpsDIG 2.10 (Rohlf, 2006). Using TpsRelW 1.45 (Rohlf, 2007), landmarks were aligned by means of a generalized Procrustes analysis (Rohlf and Slice, 1990; Bookstein, 1991). Subsequently, the Euclidean distances between Procrustes coordinates were calculated and an unweighted pair group average (UPGMA) cluster analysis (Fig. 2) was carried out with Past 2.17 (Hammer et al., 2001) in order to summarize the phenetic relationships among the individuals of *R. arenarum* and '*Bufo pisanoi*.'

Osteological terminology mainly follows that of Trueb (1973), although we also have used terms exclusively applied to bufonids (Martin, 1972).

SYSTEMATIC PALEONTOLOGY

ANURA Fischer von Waldheim, 1813

NEOBATRACHIA Reig, 1958

BUFONIDAE Gray, 1825

RHINELLA Fitzinger, 1826

RHINELLA ARENARUM (Hensel, 1867)
(Figs. 3, 4A)

Bufo pisanoi Casamiquela, 1967:162–163.

Holotype—Not designated. Seven syntypes noted, although location of depository not designated. Kwet et al. (2006) located three of the seven syntypes in the Zoologisches Museum of Berlin and designated ZMB 6779 as lectotype (Frost, 2013).

Type Locality—Rio Grande, Rio Grande do Sul, Brazil.

Emended Diagnosis—A large toad of the *R. marina* group that differs from other species of the group in having enlarged paratoid glands, which are posteriorly followed by prominent round or granular warts, and tubercular cranial ornamentation.

Referred Material of '*B. pisanoi*'—(1) PVL 2197, an individual represented by three-dimensionally preserved cranial and postcranial elements, which consist of a left, almost complete frontoparietal fused to the very incomplete otoccipital, complete right maxilla, presacral vertebrae VII and VIII with poorly preserved transverse processes, sacrum with incomplete left diapophysis, urostyle lacking its posterior part, and fragmentary diaphysis of right femur (Fig. 3). This material was designated as the holotype of '*B. pisanoi*' by Casamiquela (1967). (2) MMP 779, three-dimensionally preserved right nasal and anterior portion of left maxilla (Fig. 3). The nasal is proportionally larger than the maxilla; therefore, these elements represent two different individuals. These bones were referred to '*B. pisanoi*' by Casamiquela (1967), who listed the nasal as a left element.

Localities and Horizons of '*B. pisanoi*'—(1) Outcrops of the Irene 'formation' sensu Reig (1955), about 2 km south of the bridge of National Route 3, right margin of the

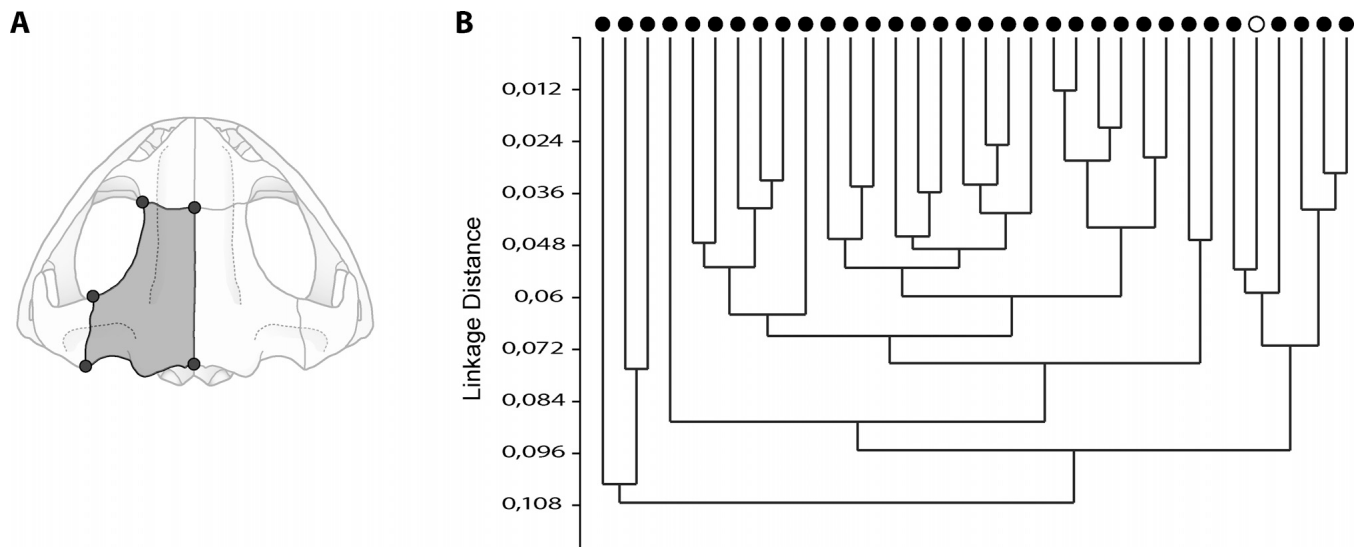


FIGURE 2. **A**, skull of *Rhinella arenarum* in dorsal view showing the position of landmarks of the frontoparietal. **B**, UPGMA cluster analysis of Procrustes distances between specimens of *R. arenarum* (black dots) and PVL 2197 (white dot).

Quequén Salado River valley, Buenos Aires Province, Argentina. The Irene ‘formation’ has been traditionally considered to be correlated with the Chapadmalalan Age/Stage (3.9–3.2 Ma), at least in part (Goin et al., 1994; Tambussi, 1998). However, recently it has been proposed that this informal stratigraphic unit spans the Huayquerian-Montermosan-lower Chapadmalalan ages (late Miocene–late Pliocene) based on study of the caviomorph rodent remains from pertinent sequences of Buenos Aires Province (Deschamps et al., 2012). The lack of precise stratigraphic data on the PVL 2197-bearing levels prevents ascription to a more specific age. (2) Punta Lobería, Buenos Aires Province, Argentina. Chapadmalal Formation, ‘upper Chapadmalalan’ Stage, Pliocene (Deschamps et al., 2012).

Comments—The dermal cranial elements formerly assigned to ‘*Bufo pisanoi*’ bear heavy ornamentation consisting of clusters of tubercles (Fig. 3A–D). The tubercles are present on the entire dorsal surface of both the frontoparietal (PVL 2197) and the nasal (MMP 779), but clusters are particularly well developed on the crests (Fig. 3A, C). The ornamentation of the maxillae is best developed on the most dorsal part of the pars facialis and posterior to the preorbital process (Fig. 3B).

The anterior margin of the frontoparietal (PVL 2197) is perpendicular to the skull midline (Fig. 3A), indicating the presence of a transverse suture between this element and the nasal, whereas the referred nasal (MMP 779) presents an oblique posterior margin (Fig. 3C).

RESULTS AND DISCUSSION

The holotype of ‘*Bufo pisanoi*’ (PVL 2197) clearly belongs to the *Rhinella* (formerly *Bufo*) *marina* species group, as concluded by Casamiquela (1967). The *R. marina* group is one of the phenetic groups of extant bufonids recognized by different authors (e.g., Martin, 1972) based mainly on skeletal, mostly cranial, features. In recent molecular phylogenetic analyses (e.g., Van Bocxlaer et al., 2010), this bufonid group was recovered as monophyletic. With the exception of *Rhinella marina*, the range of which reaches as far north as Texas, species of the *R. marina* group are endemic to South America (Vallinoto et al., 2010). It is noteworthy that

members of this clade of well-ossified, relatively large toads are characterized by a similar osteology (Tihen, 1962a), making it problematic to identify fossil material at the species level. Only one of the skeletal synapomorphies proposed for this group (the anterior edge of the sacral diapophyses are angled posteriorly with respect to the longitudinal axis of the vertebrae; Maciel et al., 2010) can be assessed in the available material of ‘*B. pisanoi*’ due to its incompleteness. However, this material has a combination of features that only occurs in members of the *R. marina* group amongst South American bufonids. This combination consists of ‘broad’ skull (sensu Martin, 1972), heavy cranial ornamentation, defined supraorbital crests, nearly transverse anterior margins of frontoparietals, and the absence of parietal crests.

With regard to the 10 species that currently compose the *R. marina* group (Maciel et al., 2010), a survey of their cranial ornamentation patterns shows that the tubercular type of ornamentation of ‘*B. pisanoi*’ is only present in *R. arenarum*. In contrast, a striated or wrinkled pattern characterizes the remaining living species of the clade (Fig. 4), including *R. rubescens*, *R. achavali*, and *R. icterica*, which form the sister clade of *R. arenarum* (Maciel et al., 2010; Vallinoto et al., 2010), and *R. schneideri*, the taxon to which Sanchiz (1998) synonymized ‘*B. pisanoi*.’ Further detailed osteological comparisons among members of the ‘south-central clade’ (*R. arenarum*, *R. rubescens*, *R. achavali*, and *R. icterica* sensu Maciel et al., 2010) were limited by the limited sample available to us.

The tubercular pattern of cranial ornamentation is invariably present in the studied sample of *R. arenarum*. Despite this similarity, the supraorbital crests show a range of development, varying from low to relatively high (Fig. 5), although the crests are less developed than in other species of the *R. marina* group (e.g., *R. schneideri*) (Fig. 4). The extent and degree of ornamentation of the frontoparietals are also variable in *R. arenarum*. In some individuals, the ornamentation is limited to the supraorbital crests, whereas the medial surface of the frontoparietals is rather smooth (Fig. 4D, G); conversely, other specimens have the ornamentation more evenly distributed (Fig. 4F). Likewise, the tubercles may be sparse or densely distributed. This variation is not linked to sexual dimorphism and occurs in specimens from the same

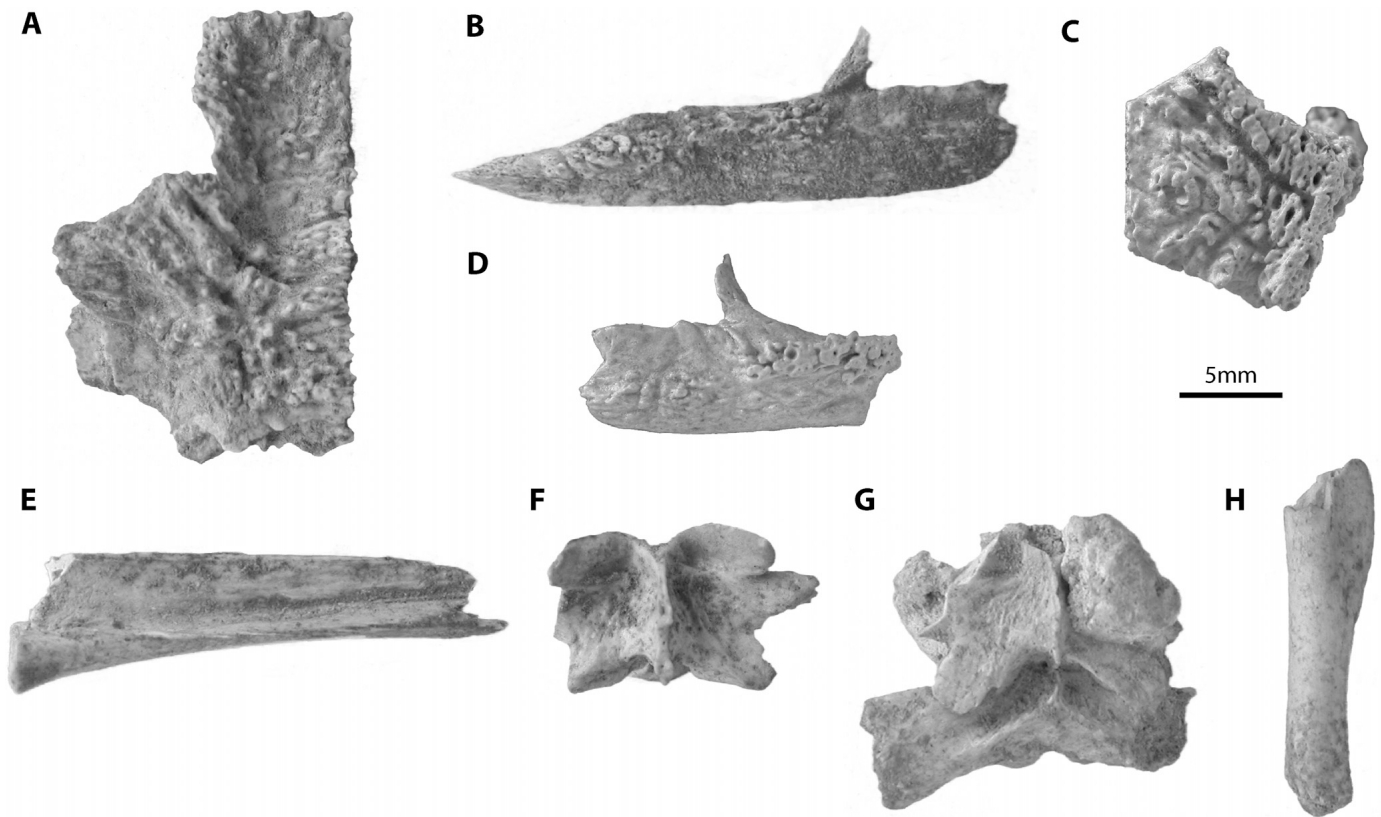


FIGURE 3. '*Bufo* (= *Rhinella*) *pisanoi*.' PVL 2197: **A**, left frontoparietal, dorsal view; **B**, right maxilla, lateral view; **E**, urostyle, left lateral view; **F**, presacral vertebra, dorsal view; **G**, last presacral and sacral vertebrae, dorsal view; **H**, right femur, medial view. MMP 779: **C**, right nasal, dorsal view; **D**, left maxilla, lateral view.

area. This demonstrates that the frontoparietal of '*B. pisanoi*' falls within the variation range of that of *R. arenarum*. It is evident that Casamiquela (1967), in his discussion of '*B. pisanoi*,' was not aware of this variation when he stated that '*B. pisanoi*' can be distinguished from *R. arenarum* by a unique combination of poorly developed cranial crests, a character that he misinterpreted as a juvenile feature, together with a heavy cranial ornamentation, an adult feature. The observed variation also contrasts with the characterization of *R. arenarum* as having very high cranial crests and a relatively smooth cranial surface between the crests of previous studies (Estes and Wassersug, 1963).

In addition to the features commented on above, the cluster analysis based on the morphometric data of the frontoparietal resulted in the element of '*B. pisanoi*' being recovered nested among the *R. arenarum* specimens (Fig. 2). This indicates that the overall shape of the frontoparietal of '*B. pisanoi*' agrees with that of the extant species.

The nasal (MMP 779) ascribed to '*B. pisanoi*' by Casamiquela (1967) has distinctive canthal and preorbital crests (sensu Martin, 1972), and contacted the maxilla only by means of the maxillary process, as in members of the *R. marina* group among South American bufonids. It also bears the tubercular ornamentation characteristic of *R. arenarum*, as stated previously. It is also worth noting that although the posterior margins of the nasals when in articulation with the frontoparietals frequently seem perpendicular to the midline in *R. arenarum*, disarticulated nasals actually have oblique posterior margins, as in MMP 779.

As with the frontoparietal, other features that Casamiquela (1967) considered unique for '*B. pisanoi*' (e.g., obtuse angle formed between the anterior margin of the crest of the urostyle and the horizontal plane of the element) also occur in some individuals of our sample of *R. arenarum*. Although the fossil specimens represent individuals larger (130–140 mm in snout-vent length; Casamiquela, 1967) than most *R. arenarum* (88–112 mm; Ceí, 1980), the inferred body size is similar to those of aged toads of the extant species that we examined.

In summary, after examining a relatively large sample of *R. arenarum*, we were unable to find features differentiating the holotype and referred specimens of '*B. pisanoi*' from this extant species. Furthermore, the putative distinguishing morphological traits and shape of the frontoparietal of the fossil species occur within the range of intraspecific variation of the living species. Therefore, we consider that the names '*B. pisanoi*' and *R. arenarum* represent the same taxon, with the latter having priority.

Anuran remains from the Oligocene of Bolivia (Báez and Nicoli, 2004) and Mio-Pliocene of Argentina (Tihen, 1962b; Tauber, 1989) have been considered to be close to the extant *R. arenarum*, but their fragmentary condition casts doubt on their species-level assignment. Thus, the fossil remains studied herein represent the oldest confident fossil record of *R. arenarum*. The presence of this species in ?upper Miocene–Pliocene strata at the Quequén Salado and Punta Lobería fossil localities of Buenos Aires Province is in accordance with its present geographic distribution (Fig. 1). Our results are also consistent with the late

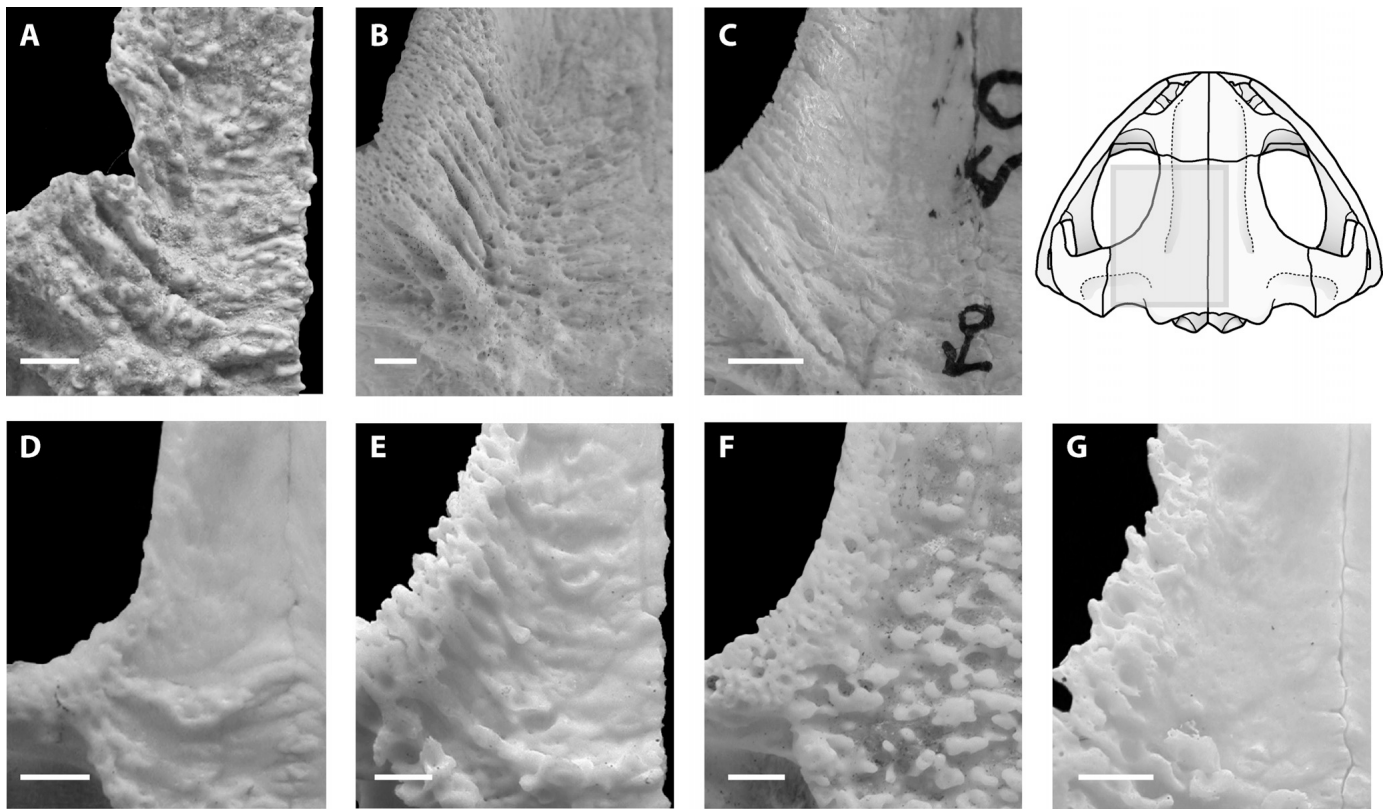


FIGURE 4. Detail of left frontoparietals in dorsal view. **A**, ‘*Bufo* (= *Rhinella*) *pisanoi*,’ PVL 2197, showing the tubercular pattern of ornamentation; **B**, *Rhinella schneideri*, FCEN 664, and **C**, *R. icterica*, FCEN 20, showing the striated pattern of ornamentation; **D–G**, *R. arenarum* (all adult males from northeastern Buenos Aires Province), MACN-He 45380 (**D**), MACN-He 45381 (**E**), FCEN uncataloged (**F**), and MACN-He 45388 (**G**), respectively, showing the variation in the development of tubercular ornamentation. All scale bars equal 2 mm.

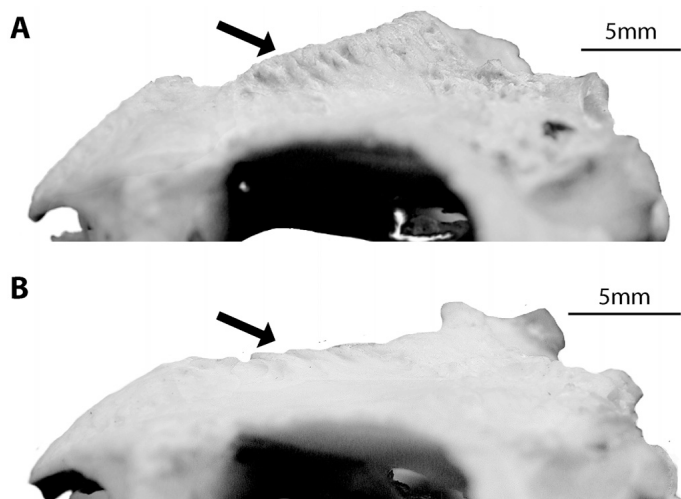


FIGURE 5. Detail of the roof of the skull of *Rhinella arenarum* in lateral view showing the variation of development of supraorbital crests. **A**, MACN-He 45395, well developed; **B**, MACN-He 45380, poorly developed.

Miocene and early Pliocene divergence of the lineage represented by this species proposed by Maciel et al. (2010) based on a calibrated phylogenetic analysis of molecular data.

The interspecific and intraspecific comparisons performed in this study show that the tubercular cranial ornamentation is a consistent and distinctive feature of *R. arenarum* among members of the *R. marina* group; thus, we included this trait in the diagnosis of this species.

Species Recognition and Intraspecific Variation in Paleontology

Species are the fundamental units of evolution; thus, the nature of these entities, as well as how they are recognized, has long been the object of thoughtful discussions (e.g., Mayr, 1982; Templeton, 1989). Species delimitation is essential to carry out biogeographic and ecological studies, as well as to deduce macroevolutionary patterns and processes (Smith, 1994). Although different and apparently incompatible species concepts have been erected (see Mayden, 1997, for an account), it has been proposed that a conceptual unity about what species are underlies all of these definitions, and what differentiates them is how these entities can be delimited (e.g., De Queiroz, 2007). According to De Queiroz (2007), the differences between species delimitation criteria rely on which properties acquired by lineages during the course of divergence are taken into account. In this regard, systematists typically delimit species based on the presence of ‘fixed’ (i.e., intraspecifically invariant) morphological characters, or combination of characters, that don’t overlap those of other species (Wiens and Servedio,

2000); although it is not always made explicit, the notion that non-overlapping, fixed characters (if genetically based) provide evidence that gene flow with other similar entities has been interrupted underlies these studies. However, the decoupling of morphological and taxic changes during the evolutionary process suggests that there can be no absolute criteria for recognizing species based on morphology (Tattersall, 1986). Even so, fossil species are distinguished as separate entities only if they are morphologically distinct.

When fixed morphological characters are used as species delimitation criteria, two main problems may occur (Wiens and Seredidio, 2000): (1) the number of species present in a sample of individuals could be underestimated if the characters taken into account are insufficient to differentiate the real units; and (2) the number of species could be overestimated by considering characters as fixed when they are actually intraspecific variants. Studies of fossil vertebrates are particularly prone to these problems because in general, the only available information is based on the hard parts, and, in addition, these remains are seldom preserved well enough to retain the full suite of skeletal characters observable in living specimens. Moreover, the acceptance of certain characters as fixed is a sensitive issue when dealing with single fossil individuals from different localities/horizons, where the properties of their respective populations are inferred by extrapolating the morphology of these unique specimens.

Not surprisingly, the identification at the species level of the fossil material studied herein raised these theoretical and methodological issues concerning the delimitation and recognition of species in paleontology. It is true that the range of morphological variation between closely related species may partially, or even substantially, overlap, but in his discussion on '*Bufo pisanoi*,' Casamiquela (1967) considered some skeletal characters of *Rhinella arenarum* as fixed, probably as an artifact of sample size, when actually they are not, as revealed by our sample of this extant species. The putative diagnostic (fixed) characters of '*B. pisanoi*' overlap the range of variation of those of *R. arenarum* and, thus, fail to discriminate the two species with the available evidence. We were unable to find any other discrete character or suite of characters that might support the distinction of these two entities. However, aware of the above-stated problem number 1, we cannot discard that the discovery of new and more complete remains might provide new data suggesting the presence of an extinct species of *Rhinella* in the Neogene of Buenos Aires Province.

Although from an instrumentalist point of view species can be recognized as the smallest cluster of individuals that might be distinguished from other clusters by unique traits or combination of traits, substantial variation may occur within a cluster (e.g., Bever, 2005). In this regard, it has been demonstrated that polymorphic (excluding ontogenetic and between-gender variation) morphological characters may contain significant phylogenetic information (Campbell and Frost, 1993; Wiens, 1995; Wiens and Seredidio, 1997); therefore, this variation should be evaluated carefully whenever possible, as recommended by Trueb (1977) in one of the few studies dealing with intrapopulational variation in anurans. This information, however, is not usually discussed explicitly (Bell et al., 2010), and, frequently, features that vary in the terminal taxa are excluded from phylogenetic analyses.

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