Internal larval characters in anuran systematic studies: a phylogenetic hypothesis for *Leptodactylus* (Anura, Leptodactylidae)

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**Abstract**

There are few systematic studies based on internal buccal and larval cranial morphology of anuran tadpoles. Here we hypothesized phylogenetic relationships of frogs of the *Leptodactylus* genus with 84 internal larval characters, where 63 of them were described for the first time. We recovered *Leptodactylus* as monophyletic with two major clades. A similar topological arrangement was found by combining the larval with 98 adult morphology, where 63 of them were described for the first time. We recovered *Leptodactylus* as monophyletic with two major clades. A similar topological arrangement was found by combining the larval with 98 adult morphology, where 63 of them were described for the first time. We recovered *Leptodactylus* as monophyletic with two major clades. A similar topological arrangement was found by combining the larval with 98 adult morphology, where 63 of them were described for the first time. We recovered *Leptodactylus* as monophyletic with two major clades. 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a synonym of *Leptodactylus*. Nevertheless, most authors recently rejected these conclusions (e.g. Ponssa 2008) because only few species had been sampled. Pyron and Wiens (2011) and Fouquet et al. (2013) considered *Adenomera* as a monophyletic valid genus. The phylogenetic hypothesis of Larson and de Sá (1998), based on characters of the larval cranium, neither supported the monophyly of the genus *Leptodactylus* nor the relationships among the groups proposed by Heyer (1969). Ponssa (2008) published a phylogenetic hypothesis for the *L. fuscus* group, based on external morphology, osteology, larval cranial morphology and behaviour. She corroborated the monophyly of the *L. fuscus* group and hypothesized that the *L. pentadactylus* and *L. fuscus* groups were sister groups.

We report on a comparative analysis of the larval internal buccal morphology and larval cranial of species of *Leptodactylus* to: (1) describe and investigate the use of new larval characters in phylogenetic studies of *Leptodactylus*, (2) propose a phylogenetic hypothesis for the genus, (3) assess the impact of larval characters on a combined tree, generated by larval (present work) and adult (previous analysis) characters and (4) evaluate congruence between these different datasets.

### Material and Methods

#### Sampling

Tadpoles and eggs of *Leptodactylus* species were collected in the field, and additional biological materials were obtained from scientific collections. Nineteen species of 30 different populations were sampled (Table S1). Three tadpoles, preferably at Stage 36 (Gosner 1960), of each species were dissected to examine internal buccal morphology, and three were also cleared and stained to examine the larval cranium.

#### Internal buccal morphology

The floor and roof of the oral cavity were exposed following the dissection procedure described by Wassersug (1976). Mouthparts were stained with methylene blue 5% and Sudan Black B + methylene blue, submerged in water and photographed with a stereomicroscope equipped with a digital photographic system. The identification and description of buccal structures are based on the terminology proposed by Wassersug (1976).

#### Larval cranial morphology

Tadpoles were cleared and stained following the protocols of Dingekus and Uhler (1977) and Song and Parenti (1995), with modifications. Observations, measurements and photographs of larval cranial structures were conducted with a stereomicroscope with eyepiece micrometre and digital photographic system. The identification and description of the structures are based on terminology employed by Larson and de Sá (1998), Cannatella (1999) and Haas (2003).

### Phylogenetic analyses

Characters coded based on previous studies – 12 characters of Larson and de Sá (1998) and nine characters of Prado (2006) – were used as described by the authors or adapted as needed (Tables S2 and S3). Character states of the internal buccal and larval cranial morphology for *Leptodactylus griculus* (Dunéril and Bibron, 1840), *L. notoaktites* Heyer 1978 and *L. pastulatus* (Peters, 1970) are based on published descriptions (Wassersug and Heyer 1988; Larson and de Sá 1998; de Sá et al. 2007a, b) and accounts of the buccal microanatomy of *L. labyrinthicus* (Spix, 1824), *L. petesi* (Steindachner, 1864), *L. podicipinus* (Cope, 1862) and *L. rufus* Lutz, 1930 by Vieira et al. (2007) and Miranda and Ferreira (2008, 2009).

Among the 76 species of the genus *Leptodactylus*, 19 were sampled in this study (Table S1). Five species of four genera that have been suggested to be close relatives of *Leptodactylus* (Larson and de Sá 1998; Frost et al. 2006; Grant et al. 2006; Ponssa 2008) were used as outgroups: *Aloides vanzolinii* (Domingo-Barros, 1974); *A. verrucosus* (Philipps, 1902) (Alsidodidae); *Ceratophrys cranwelli* Barrio, 1980 (Ceratophryidae); *Telmatobius scrobiclit* Laurent and Laville, 1986 (*Telmatobiidae*); and *Crossodactylus gauchichaudii* Duméril and Bibron, 1841 (*Hylodidae*). Character states of outgroups were obtained from the literature (Laville and Fabrezi 1992; Larson and de Sá 1998; Ramón Formas and Brieva 2004; Vera Candioti 2005; Weber and Caraschi 2006; Vera Candioti 2008). Characters varying between populations were treated as polymorphisms and were included in the analysis. Maximum parsimony analyses using heuristic searches were implemented in PAUP 4.0b1 (Swofford 1998) and TNT 1.1 (Goloboff et al. 2003). Treelength, indices (consistency and retention) and character changes in the clad of clades were analysed with MacClade 4.0 ( Maddison and Maddison 1992) and Mesquite 2.71 ( Maddison and Maddison 2009). In heuristic searches, the most parsimonious trees were found by 2000 multiple random addition sequences, and the method of branch swapping was tree bisection and reconnection (TBR), retaining replicate trees. Support for clades was obtained by bootstrap analysis of 10 000 pseudoreplications (Felsenstein 1985; Hillis and Bull 1993; Müller 2005) and by Bremer support (Bremer 1994). Bootstrap values were calculated by PAUP 4.0b1 and Bremer support by TNT 1.1. All series of transformations were considered as unordered and unweighted.

### Impact of larval characters to infer *Leptodactylus* phylogeny and homogeneity test

We also assessed the impact of larval characters to infer the relationships of species of the *Leptodactylus* genus. To evaluate this impact, we conducted the following steps: (1) we combined the larval partition generated in the present work with the data set of Ponssa (2008) (see Fig. 1 representing the schematic diagram of partitions used in the combined analysis). Note that we excluded Ponssa’s larval characters because these originated from Larson and de Sá’s (1998) work and were replaced by our own data. In our data matrix, characters 1–98 are derived from Ponssa (2008) and characters 99–182 were from this work (Data S1). Only species sampled in both studies were included in the combined analysis resulting in 18 terminal taxa, and (2) a phylogenetic analysis was performed (under maximum parsimony) with these two partitions combined. The most parsimonious trees were obtained by 2000 multiple random addition sequences using the method of branch swapping (TBR) (TNT 1.1), and (3) by performing a Partitioned Bremer Support (PBS) analysis (Gatesy et al. 1999). PBS examines the impact of each partition (among multiple partitions) to the overall tree, estimating support values to each node produced by each data set. PBS analysis was conducted in TNT 1.1 by using the script published by Peña et al. (2006). A test of homogeneity (incongruence length difference ILD) (Farris et al. 1995; Dolphin et al. 2000) to verify congruence between the two partitions was implemented in PAUP, with 1000 replications, random additions of taxa and heuristic search.

### Results

#### Overview of *Leptodactylus* internal larval morphology

Overall, the buccal morphology among *Leptodactylus* tadpoles presented two remarkable features: the diversity of structures and reduction (in size and quantity) of these structures (Fig. 2). The infralabial papillae are small and simple in most species studied, except in *L. riveroi* where those are more complex. The number of papillae delimiting of the buccal floor area showed a large variation. Some tadpoles as *L. chaquensis* and *L. latrans* have more papillae (Fig. 2A). Also, the presence of a glandular zone is clearly noticed in the species of aquatic habits such as *L. natalensis*, *L. latrans*, *L. petesi*, *L. podicipinus* and *L. pastulatus* (Fig. 2B–D). These species are known to deposit the spawn directly in water bodies and tadpoles developed entirely in the aquatic environment (Heyer 1969). Conversely in tadpoles of *L. riveroi*, *L. rhodomystax*, *L. vastus*, *L. knudseni* and *L. labyrinthicus* (Fig. 2E–F), buccal structures are particularly reduced.
Only species sampled in both studies were included in the analysis. The total data set examined in this study therefore represented 18 taxa and 182 characters. The second was obtained from this work, eliminating 16 species (diagonal stripes; b). The resulting matrix is then shown in c: characters 1–98 are derived from Ponsse (2008) and characters 99–182 from this work (diagonal stripes; 84 characters). Only species sampled in both studies were included in the analysis. The total data set examined in this study therefore represented 18 taxa and 182 characters.

The reduction in the number of papillae in the buccal roof arena is a widespread feature in *Leptodactylus*, reaching the full absence in *L. riveroi* (Fig. 2G).

Among larval cranium, we found nine features common to all *Leptodactylus* species sampled: processus posterior dorsalis in ala of suprarostral; crista parotica; commissura quadratoorbitalis; processus dorsomedialis, p. ventromedialis and p. retroarticularis present in Meckel’s cartilage; ceratobranchials fused to the hypobranchial plates; lateral projections present in ceratobranchials I, II and III; p. branchialis anterior; palatoquadrate narrow anteriorly and broad and slightly rounded posteriorly and otic capsules representing about 30% of the total cranial length (Fig. 3).

To illustrate the structures of floor and roof of buccal cavity, larval cranium was analysed in the present work (see Figs 2 and 3). These figures help to understand how characters were delimited and coded. Full morphological descriptions of buccal anatomy and larval cranium of *Leptodactylus* species are in preparation for further publication.

**Phylogeny reconstruction using larval characters**

Our anatomical analysis resulted in a matrix of 42 characters of internal oral features and 42 larval cranium characters (Data S2). Characters are listed in Table S2 (internal oral morphology) and Table S3 (larval cranium morphology), where 63 of them were described for the first time (35 from internal oral and 28 of larval cranial morphology). In the 24 taxa examined, three of the 84 characters were uninformative. Heuristic searches resulted in four most parsimonious trees (Data S3) with an optimal parsimony score of 417 steps. The strict consensus cladogram (Fig. 4) has 423 steps, a consistency index of 0.371, a consistency index excluding uninformative characters of 0.366 and a retention index of 0.484.

The genus *Leptodactylus* is recovered as monophyletic, supported by the following buccal morphological characters (Character number: State; Data S2): triangular buccal floor arena (10:1); slightly prominent projections on the posterior margin of ventral velum (17:1); postnarial arena small (27:0); lateral ridge papillae small (30:1); and glandular zone of the dorsal velum occupying half the surface (41:1). Larval cranium characters supporting the monophyly of *Leptodactylus* are as follows (Character number: State; Data S2): ventromedial fusion of the corpus of the suprarostral narrower than the body (43:1); presence of ventrolateral projections in the corpus of suprarostral (44:1); posterolateral extension of the palatoquadrate extends beyond the anterior margin of otic capsules (63:3); angle between the posterior margin of the processus ascendens and the braincase between 70° and 80° (65:1); presence of the commissura quadranoroortalis (70:1); Meckel’s cartilage long and curved (71:1); pars reuniens and Copula II with the same length (74:0); processus urobranchialis wide (77:1); lateral process of the ceratobranchial triangular (78:1); and presence of projections just in the posterior portion of ceratobranchials (84:2).

Our phylogenetic analysis of larval characters produced two major clades within *Leptodactylus* (Fig. 4). One (clade 1) is composed of species traditionally assigned to the *L. fuscus* group (*L. bufonis* Boulenger, 1894; *L. camauara* Sazima and Bokermann, 1978; *L. latinasus* Jimenez de la Espada, 1875; *L. troglodytes* Lutz, 1926; *L. notoaktites*; *L. mystacinus* Burmeister, 1861). *L. latrans* (Burmeister, 1861); *L. tapiti* Sazima and Bokermann, 1978; *L. furcatus* Sazima and Bokermann, 1978; *L. gracilis*; *L. spixi* Heyer, 1983; and *L. fuscus*), the *L. latrans* group (*L. chaquensis* Cei, 1950 and *L. latrans*) and the *L. melanomorus* group (*L. natalensis* Lutz, 1930; *L. petersii*, *L. podicipinus* and *L. pustulatus* Peters, 1870). *Leptodactylus fufonis* and *L. camauara* are successive basal branches in this clade, which is supported by the following characters of internal oral anatomy: infralabial posterolateral papillae conical or triangular (5:1); triangular median ridge (28:3); and absence of pustules and/or papillae on the posterior and/or dorsal of the dorsal velum (42:0). Four larval cranium characters also support the clade 1: otic capsules representing 30% or more of the length of larval cranium (54:1); cornua trabecularia representing over 20% of the length of larval cranium (60:0); posterolateral extension of the palatoquadrate reaching the level of attachment of processus ascendens to the braincase (63:1); and hypobranchial plates separated (80:0).

Figure 1. Schematic diagram representing how different matrices were combined to this study. One data set was taken from Ponsse (2008), originally containing 43 taxa and 113 characters (complete square at top left). We deleted Ponsse’s larval matrix characters (99–113) (cross-hatched; a), eliminating 25 species and 15 characters. The second was obtained from this work, eliminating 16 species (diagonal stripes; b). The resulting matrix is then shown in c: characters 1–98 are derived from Ponsse (2008) (horizontal stripes) and characters 99–182 from this work (diagonal stripes; 84 characters). Only species sampled in both studies were included in the analysis. The total data set examined in this study therefore represented 18 taxa and 182 characters.

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The other clade (clade 2; Fig. 4) comprises the species *Leptodactylus labyrinthicus*, *L. knudseni* Heyer, 1972 and *L. vastus*, which traditionally have been assigned to the *L. pentadactylus* group. This clade is defined by five character states of internal buccal anatomy: central papillae anteriorly positioned in relation to lateral papillae (9:1); buccal floor arena rectangular or trapezoidal (10:3); more than 30 pustules on the buccal floor arena (11:2); five or less papillae limiting the buccal roof arena (13:1); and posterior wall of the nostrils low (24:0). Among the larval cranium characters are suprararostral corpus and alis of the same width (47:0); a distinct processus posterolateralis at the crista parotica (62:0); and Meckel’s cartilage short and curved (71:0). Among the larval cranial characters are suprararostral corpus and alis of the same width (47:0); a distinct processus posterolateralis at the crista parotica (62:0); and Meckel’s cartilage short and curved (71:0).

Bootstrap and Bremer supports indicate that *Leptodactylus* is a monophyletic clade well supported in the analyses with 82% and decay index of 5, respectively. The clade (*L. vastus + (L. knudseni + L. labyrinthicus)*) has a bootstrap support of 79% and Bremer support of 5. The clade composed of species of groups *L. fuscus*, *L. melanotus* and *L. latrans* has a Bremer value of 3. The clade ((*L. chaquensis + L. latrans*) + (*L. pustulatus + (L. podicipinus + (L. natalensis + L. petersi))*) has a bootstrap support of 60% and a Bremer of 5.

**Impact of larval characters to infer Leptodactylus phylogeny and homogeneity test**

The analysis of 182 characters and 18 taxa (Data S1) reveals 18 constant characters, and 34 uninformative and 130 informative characters. We recovered 25 most parsimonious trees (Data S4).
with 487 steps each. The strict consensus cladogram (Fig. 5a) has 557 steps, a consistency index of 0.472, a consistency index excluding uninformative characters of 0.424 and a retention index of 0.309.

The monophyly of the genus *Leptodactylus* is supported, but there are some polytomies (Fig. 5a). However, close relationships among the species allocated to the *L. fuscus*, *L. latrans*, *L. melanonotus* and the *L. pentadactylus* groups are maintained.

Thirty-five characters with unambiguous changes support the *Leptodactylus* clade; among these, 12 characters were provided by Ponssa (2008).

It is expected that if the PBS results’ partitions (in this case, larval and adult characters; see Fig. 1) support a relationship represented by a node in the combined tree, then the PBS value will be positive. If, conversely, a partition supports an alternative relationship, the PBS value will be negative. The magnitude of PBS values indicates the level of support for or disagreement with a node. Our results of PBS test revealed that each data set (larval and adult characters) was congruent and supports monophyly of the *Leptodactylus* clade (Fig. 5a; both positive values). Although, this contribution shows to differentially support this arrangement (2.8 for adult characters and 6.2 for larval characters). The clade formed by *Leptodactylus chaquensis* + *L. podicipinus* and the other by *L. knudseni* + *L. labyrinthicus* (Fig. 5a) contributed differentially for this arrangement (both ~5.2 for adult characters and 8.2 for larval characters). Adult and larval

**Figure 3.** Representations of larval cranium morphologies of *Leptodactylus* tadpoles: (a–b) dorsal and ventral overviews of *L. fuscus*, stage 38 (Gosner 1960); (c) the arrows represent the two states of the Character 56 (angle between *cornua trabeculae* in relation to the width of larval cranium) of *L. trosodytes*, stage 35 (state 0, left figure) and *L. chaquensis*, stage 35 (state 1, right figure); (e–f) robustness of infrarostral elements and states of Meckel’s cartilage, *L. knudseni*, stage 33 (more robust, state 0) and *L. tapiti*, stage 39 (less robust, state 1); (g–h) represent two states of the Character 61 (*processus anterolateralis* of crista parotica), *L. fuscus*, stage 32 (state 0) and *L. vastus*, stage 34 (state 1). AS, alas of suprarostral; CB, ceratobranchials; CH, ceratohyal; COP, copula; CP, crista parotica; CQ, commisura quadratoorbitalis; CS, corpus of suprarostral; CT, *cornua trabeculae*; FJ, foramen jugulare; IR, infrarostral; HP, hypobranchial plates; LP, lateral projections; MC, Meckel’s cartilage; OC, otic capsule; PAH, *p. anterior hyalis*; PALH, *p. anterolateralis hyalis*; PAL, *processus anterolateralis*; PAQ, *pars articularis quadrati*; PAS, *p. ascendens*; PB, *p. branchialis*; PDP, *p. dorsalis posterior*; PM, *p. muscularis quadrati*; PPL, *p. posterolateralis*; PPH, *p. posterior hyalis*; PQ, palatoquadrate; PQE, *p. quadratoethmoidalis*; PR, *pars reuniens*; PRA, *p. retroarticularis*; PU, *p. urobranchialis*; TS, *tectum synoticum*; TTM, *taenia tecti marginalis*. Scale bars = 1.0 mm.
characters support the clade formed by *L. knudseni* + *L. labyrinthicus* + *L. rhodomystax*; however, both contributed with small values to this representation (0.8 for adult characters and 0.2 for larval characters; Fig. 5a).

The homogeneity test showed that the compared partitions (present work and Ponssa’s) are incongruous (*p* < 0.001).

The 50% majority rule consensus tree of the combined analyses (Fig. 5b) is more similar to the tree based only on larval characters. We recovered a large clade composed by species of the *L. fuscus*, *L. latrans* and *L. melanomystax* groups, and another composed by species of the *L. pentadactylus* group. The bootstrap analyses of the strict consensus tree support the *Leptodactylus* clade to a level of 96%.

**Discussion**

**Polymorphisms found in internal larval morphologies of Leptodactylus**

We recorded intraspecific and populational morphology variation among tadpoles sampled. Intraspecific and intrapopulational variations in buccal morphology were equally observed between the left and right sides of the same individual. The features with variations were the amount of pustules throughout arenas of floor and buccal roof and the amount of papillae rounding it, as well as the presence of pustules or papillae in the region anterior to buccal pocket. These kinds of variation (quantity) are expected and therefore should not be used for systematic of tadpoles (Wassersug and Heyer 1988). But the disproportion among variations (extensions) observed could be used for phylogenetic reconstruction purpose.

Interpopulational variations in buccal morphology could be evaluated only in cases of species sampled for more than one locality: *L. chaquensis*, *L. fuscus*, *L. mystacinus*, *L. latrans*, *L. podicipinus* and *L. troglodytes*. Leptodactylus chaquensis and *L. latrans* presented variation in infralabial papillae. In the first species mentioned, we found four infralabial papillae in Argentinian populations and three in the population from Corumbá municipality, Mato Grosso do Sul state, Brazil. Populations of *L. latrans* from Brazilian states of Paraná, São Paulo and Roraima presented three infralabial papillae, while population of
Seropédica municipality, Rio de Janeiro state, presented five. Populations of *L. latrans* also presented variations in lingual papillae. The populations from Brazilian states of Paraná, São Paulo and Roraima presented four lingual papillae, and population of Rio de Janeiro state presented three lingual papillae. The amount of dispersed pustules on the arena of buccal floor was a quite variable character, being all the species already mentioned presenting such variation. The number of papillae limiting the arena buccal floor varied in populations of *Leptodactylus fuscus*. Population from Chapada dos Guimarães National Park in Mato Grosso Brazilian state showed a greater amount of papillae limiting the arena (Miranda and Ferreira 2009). Differences in height of the nostrils posterior wall were observed in tadpoles of *L. mystacinus* and *L. podicipinus* (Miranda and Ferreira 2009). Tadpoles of *L. mystacinus* from Argentina presented four postm- nal papillae per side and the individuals collected in Brasilia, Federal District, Brazil, two papillae per side. The median ridge was relatively small in species examined as already demonstrated by Wassersug and Heyer (1988), although the shape gradually varied between semicircular to triangular in *L. fuscus*, *L. latrans* and *L. troglodytes*. The shape of lateral ridge papillae varied in tadpole populations of *L. latrans*. Individuals from Paraná, São Paulo and Rio de Janeiro Brazilian states presented ramified and complex papillae, while tadpoles from Roraima Brazilian state presented chela-shaped lateral ridge papillae. The shape of the buccal roof arena presented by *L. fuscus* and *L. podicipinus* varied between triangular and trapezoidal. Differences in amount of pustules in the buccal roof arena were observed in tadpoles of *L. podicipinus* as already verified by Miranda and Ferreira (2009).

Differences in the buccal morphology and larval cranial presented less intraspecific and populational variations. Features presented intraspecific variation were ceratobranchial process, ornamentations of etmoidal plate and fusion of infrarostral ele-

Figure 5. Combined maximum parsimony analysis of 98 characters of external morphology, osteology, ethology and morphometric data of adult specimens (Ponssa 2008) and 84 larval characters. (a) strict consensus cladogram (length = 557 steps, CI = 0.472 and RI = 0.309). Numbers above nodes are bootstrap values, and PBS values are found below nodes inside squares (adult characters left, larval characters right). (b) 50% majority rule consensus cladogram (length = 11 steps, CI = 0.580, RI = 0.550). Numbers above nodes are bootstrap values and below the frequencies of clades.

Performance of internal buccal larval morphology characters

This is an innovative work that accessed internal morphological variations in tadpoles as well as adults and molecular data (Wynn and Heyer 2001).
strongly correlated with the larval ecology, and homoplasy may render buccal characters phylogenetically uninformative. Nonetheless, these authors also argued that there was a phylogenetic pattern in buccal characteristics. This set of data was not exploited in previously published phylogenetic analyses that used larval characters (Larson and de Sá 1998; Maglia et al. 2001; Haas 2003; Pugener et al. 2003).

We have identified four features of buccal larval anatomy that are synapomorphies of Leptodactylus, as follows:

(1) Buccal floor area triangular (Character 10: State 1). This character state also occurs in Physalaemus Fitzinger, 1826 (Wassersug and Heyer 1988; Miranda and Ferreira 2009), suggesting that this characteristic may have arisen earlier or more than once in the evolution of anurans.

(2) Slightly prominent projections on the posterior margin of ventral velum (17:1). This character occurs in species that are not closely related. The convergence is present in all Leptodactylus sampled in this study. The codification of this character needs to be refined in future studies.

(3) Small lateral ridge papillae (30:1). Absence of the lateral ridge papillae is an attribute found in basal families of Anura (Pipidae, Rhinophrynidae, Alytidae, Leiopelmatidae and Bombinatoridae) (Wassersug 1980). The absence of these structures was also reported in Ceratophrys cranwelli (Ceratophryidae; Vera Candioti 2005). These observations suggest that this character may have arisen more than once in the evolutionary history of Anura, and their presence or absence may be a result of multiple evolutionary events (Maglia et al. 2001). The presence of a pair of lateral ridge papillae was observed in all previously examined Leptodactylus tadpoles.

None of the larval characters analysed are synapomorphic for the phenetic species groups of Leptodactylus. Among the 42 buccal characters, 16 are homoplastic.

(1) Presence of spherical protuberances on the anterior portion of the infrarostral region (Character 1: State 1). This character occurs in Leptodactylus chaquensis, L. latrans, L. podicipinus and L. labyrinthicus. We expected such anatomical similarity among L. chaquensis, L. latrans and L. podicipinus; though, L. labyrinthicus is not closely related. The convergence is surprising because these species do not occupy the same type of environment, having disparate dietary habits (Agostinho et al. 2002; Prado et al. 2005; Silva et al. 2005).

(2) A pair of narrowly separated medial infralabial papillae (3:2). This feature is present in Leptodactylus natandensis, L. notoaktites, L. spixi and L. vastus and shows no apparent distributional pattern.

(3) Lateral pair of infralabial papillae quadrangular (5:2). This character occurs in species that are not closely related.

(4) Lingual papillae in number of three (7:2). Lingual papillae occur in the Leptodactylus fuscus, L. latrans and L. melanotus groups, and the L. pentadactylus clade. Associations between the larval ecology and the amount of lingual papillae are unknown.

(5) Arrangement of lingual papillae (9:1). Central papillae anteriorly positioned in relation to lateral papillae were observed in tadpoles of the Leptodactylus pentadactylus group and in L. spixi, which traditionally is placed in the L. fuscus group.

(6) More than 30 pustules on the buccal floor area (11:2). This feature is found in many species of Leptodactylus, including those not closely related; it also occurs in the Crossodactylus gaudichaudii.

(7) Projections grouped laterally within the anterior limits of ventral velum, absent or with pustules (15:0 and 15:2). The absence and reduction in number and size of selection structures (infralabial papillae, lingual papillae and arena papillae) is related to macropagous and carnivorous habits. Thus, the absence of these projections and their presence with reduced size (pustules) in the Leptodactylus pentadactylus group is expected. These features also occur in L. bufonius, L. camaquara, L. furnarius and L. spixi, for which there are no data on foraging habits.

(8) Prominent projections on the posterior margin of ventral velum (17:2). This character state is present in species of the Leptodactylus fuscs + L. latrans + L. melanotus clade and also occurs in L. knudseni.

(9) More than five projections on each side of the posterior margin of ventral velum (18:1). This character is present in out-groups, especially in Crossodactylus Duméril and Bibron, 1841 and Telmatobius Wiegmann, 1834 (Weber and Caramschi 2006; Vera Candioti 2008), and also occurs in Leptodactylus bufonius and L. vastus.

(10) Orientation of choanae in relation to the longitudinal axis of the body (21:0 and 21:1). This character is highly homoplastic and variable and probably has slight systematic value.

(11) Posterior wall of the nostril, low (24:0). All tadpoles in the Leptodactylus pentadactylus clade, L. tapiti and L. spixi share this character, which has no known ecological significance.

(12) Median ridge shape (28:0 and 28:2). The median ridge is relatively small in all species of Leptodactylus previously analysed (Wassersug and Heyer 1988), but its shape is convergent in nine of 22 species examined.

(13) Border of median ridge (29:1 and 29:2). The shape of the median ridge margin also showed to be convergence in the species analysed. This character together with Character 28 indicates that this anatomical feature (median ridge) should be used with prudence in future studies.

(14) Buccal roof arena quadrangular or trapezoidal (33:3). This character state occurs in seven species of the Leptodactylus fuscus group, L. labyrinthicus and in one out-group (Telmatobius scrochii).

(15) Dorsal velum narrow in relation to buccal roof (37:1). This feature was found in all species traditionally assigned to the Leptodactylus latrans and L. melanotus groups, and also L. notoaktites and L. labyrinthicus. A narrow velum and the presence of a poorly developed glandular zone are associated with macrophagous larvae (Wassersug 1980), such as L. latrans and L. labyrinthicus (Agostinho et al. 2002; Prado et al. 2005; Silva et al. 2005). The dietary habits of the other species are unknown.


Our results corroborate Wassersug and Heyer’s (1988) observation that many features of larval internal buccal morphology are homoplastic. However, this does not refute the importance of including these characters in phylogenetic studies. Maglia et al. (2001) claimed that it is useful to know whether certain morphologies have evolved in parallel, because this information enables identification of potential functional or ecological pressures (in convergences), as well as the complexity of development processes. Recognition of parallelisms in developmental patterns in the evolutionary histories of taxa may yield useful insights into the homologies of the adult morphologies (Maglia et al. 2001).
More studies should be performed to assess the utility of such characters in phylogenetic inference in anurans.

**Performance of larval cranium morphology characters**

Larval cranium characters have been widely used in phylogenetic studies (Larson and de Sá 1998; Maglia et al. 2001; Haas 2003; Pugener et al. 2003) and are considered to present less homoplasy than features of internal buccal morphology. We found four larval cranial characters to be synapomorphic for *Leptodactylus*:

1. Ventromedial fusion of the corpus of the suprarostral narrower (Character 43: State 1). Larson and de Sá (1998) also observed this trait in *Leptodactylus* species.

2. Ventrolateral projections in the corpus of suprarostal (44:1). Variously sized projections are present in all species of *Leptodactylus* examined. In *L. fuscus* + *L. latrans* + *L. melanotus* (except *L. bufonius*) clade, the projections are small. The projections are well developed and fused in the *L. pentadactylus* clade and *L. bufonius*.

3. Angle of the posterior margin of the processus ascendens in relation to the braincase at an angle between 70° and 80° (65:1). This feature was found in most species of the genus, except members of the *Leptodactylus fuscus* group, in which the posterior margin of processus ascendens is nearly perpendicular to the braincase (Larson and de Sá 1998).

4. Lateral process of ceratobranchials triangular (78:1). This feature is absent in *Leptodactylus riveroi* and in the *L. pentadactylus* clade. Few are known about its distribution, because the character has not been used before.

The clade composed by the species traditionally assigned to the *Leptodactylus fuscus*, *L. latrans* and *L. melanotus* groups is supported by one synapomorphy (Character 63: State 1). The *L. pentadactylus* clade also is supported by one synapomorphy (47:0).

Among 42 larval cranium characters, 22 showed some degree of convergence, suggesting that this data partition has fewer homoplasy characteristics than that of internal buccal anatomy, at least for *Leptodactylus*.

1. Presence of ventrolateral projections in the corpus of the suprarostal (Character 44: State 2). Members of the *Leptodactylus pentadactylus* clade and *L. bufonius* share this feature. Larson and de Sá (1998) also observed the same structure in the *Leptodactylus pustulatus* clade and *L. bufonius* during the process of clearing and staining.

2. Presence of adastral (51:1). Both presence and absence of adastral seem not to be related to common heritage because are observed in species that are not phylogenetically closely related (i.e. in-group and out-groups).

3. Presence of adastral more than 90% of its length (52:2). Wide chondrocranium occurs in *Alsodes* Bell, 1843 and *Ceratophrys cranwelli*, in some species of the *Leptodactylus fuscus* + *L. latrans* + *L. melanotus* clade and in *L. knudseni*.

4. Length of the otic capsule 30% or more of the length of larval cranial (54:1). This condition is widely distributed in *Leptodactylus* and is unrelated to the size of the tadpoles. It was found in small *Leptodactylus riveroi*, *L. bufonius*, *L. camaquara*, *L. petesi*, *L. pustulatus*, *L. notaokites* and *L. gracilis* and large tadpoles (*L. knudseni*).

5. Ornamentation in the ethmoid plate (Character 55). This character is polymorphic and convergent among the species analysed.

6. Narrower angle (<10%) between *cornua trabeculae* in relation to larval cranial (56:0). This character occurs in outgroups, in the *Leptodactylus fuscus* + *L. latrans* + *L. melanotus* clade and also in *L. labyrinthus*.

7. Presence of a *taenia tecti transversalis* and *taenia tecti medialis* subdividing the fontanelle into a frontal and two parietal fontanelles (57:1). This character was observed in the outgroup (tadpoles of *Alsdotes verrucosus*) in the *Leptodactylus fuscus* + *L. latrans* + *L. melanotus* clade and tadpoles of *L. labyrinthus* and *L. rhodomystax*. It seems to be related to larval ontogeny and its heritage pattern is difficult to access.

8. Foramen prooticum visible in lateral view (more than 1/3 of its opening) (58:1). This character state was observed in *Leptodactylus chaquensis*, *L. natalensis*, *L. podicipinus*, *L. spixii*, *L. knudseni* and in *Alsdotes*. All other species (except *L. rivieri*) had a small *f. prooticum* in lateral view. This character seems to be unrelated to the size of the tadpole, but as the knowledge of this structure is limited, it is necessary to examine different species to understand their distribution patterns.

9. Foramen oppticum greater than the *f. oculomotorius* (59:0). This state character is present in some species of the *Leptodactylus fuscus* + *L. latrans* + *L. melanotus* clade and also in the tadpole of *L. labyrinthus*. It seems that this character is not related to the body size or other ecological similarity between species.

10. Length of *cornua trabeculae* relative to larval cranial length (60:1). Feature observed in tadpoles of *Alsdotes* and in both clades of the genus resurrected in this phylogenetic analyses. For studies involving a greater diversity of species, a new coding could be required to elucidate the evolutionary pattern of this character.

11. Processus anterolateralis of crista parotica small and triangular (61:0). This character occurs in *Leptodactylus podicipinus* and *L. petesi*. Species with similar morphologies. The character state was also observed in *L. rhodomystax*. No morphological or ecological similarities are known between *L. rhodomystax* and (*L. petesi* + *L. podicipinus*). The convergence in this character could correspond to multiple evolutionary events.

12. Processus posterolateralis of crista parotica distinct (62:0). As this process is slender when compared to the previously mentioned, its use must be viewed with some caution as is hard to analyse, and also there is risks of loss or damage of this structure during the process of clearing and staining.

13. Processus posterolateralis extension of the palatoquadrate (63:2 and 63:3). We followed the coding of Larson and de Sá (1998), Characters states 0, 1 and 4 are informative and non-convergent. States 2 and 3 are homoplasic, both in the in-group and out-groups, suggesting that this character requires better delineation.

14. Triangular projection at anterolateral margin of the *cornua trabecula* reduced or absent (68:1). This polymorphic character is homoplasic and, thus, not phylogenetically informative.

15. Width of *processus muscularis* (in dorsal view) two-thirds that of *pars articularis quadrati* (69:1). *Leptodactylus rhodomystax* shares this character with some species of the *L. fuscus* + *L. latrans* + *L. melanotus* clade and members of out-groups (*Alsdotes* and *Crossodactylus*). The shape of this structure is similar in all species examined.

16. Presence of a commissura quadratoorbitalis (70:1). This character occurs in all species of *Leptodactylus* examined, as well as *Telmatobius*. This suggests that this attribute may have arisen before and/or more than once in the evolution of *Leptodactylidae*.

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L. pentadactylus

(18) Copula II larger than the pars reuniens (74:2). Sizes are variable in both in-group and out-group.

(19) Large processus urobranchialis (77:1). The size of p. urobranchialis proved to be slightly informative and homoplastic.

(20) Processus anterior hyalis and p. posterior hyalis equal in size (79:1). Leptodactylus knudseni shares this character with a few other species of the L. fuscus + L. latrans + L. melanonyx clade.

(21) Processus branchialis closed (82:1). According to Larson and de Sá (1998), all species of Leptodactylus belonging to the L. fuscus and L. pentadactylus groups have an open p. branchialis, whereas in species of L. latrans and L. melanonyx groups and in L. riveroi and L. silvanimbus, the p. branchialis is closed – data corroborated by our analysis. Although in the clade formed by the L. latrans, L. melanonyx and the L. fuscus groups, the character is homoplastic.

(22) Ceratobranchials presenting lateral projections only in the posterior portion (84:2). This character may be plesiomorphic for Leptodactylus, which is corroborated by presence of this character state in tadpoles of L. riveroi and L. rhodomystax. But it may also have arisen more than once during the evolutionary history of the group, as it is present in L. petersii (L. fuscus + L. latrans + L. melanonyx clade) and in L. labrosus (L. pentadactylus clade).

Additional phylogenetic remarks

The most remarkable difference between the phylogenetic hypotheses presented here and earlier ones is the fact that the monophyly of the Leptodactylus fuscus group could not be corroborated. Larson and de Sá (1998) identified two major clades in Leptodactylus (Leptodactylus – fuscus and latrans – melanonyx) based on 26 characters of larval cranial morphology and suggested that the species of the L. pentadactylus group might be paraphyletic once two species placed in the L. fuscus group (L. albilabris and L. labrosus) were more closely related to the species of the L. pentadactylus group than any other species. We could not verify this relationship because L. albilabris ( Günther 1859) and L. labrosus Jiménez de la Espada, 1875 were not sampled in this study. Ponssa (2008) corroborated the results of Larson and de Sá (1998) by showing a close relationship between those species and the L. fuscus group. Ponssa (2008) and Pyburn and Wiens (2011) recovered L. fuscus group as monophyletic and most closely related to the L. pentadactylus group. These relationships were not corroborated in the present study. We identified two major clades – a clade formed by the L. fuscus + L. latrans + L. melanonyx groups and the other formed by the L. pentadactylus group. The L. latrans and L. melanonyx groups are closely related and embedded within the L. fuscus group. This major clade is closely related to the L. pentadactylus clade. We did not recover L. rhodomystax (currently considered a member of the L. pentadactylus group) in the L. pentadactylus clade; instead, L. rhodomystax is basal in relation to the two major clades – that is the L. fuscus + L. latrans + L. melanonyx clade and L. pentadactylus clade. This relationship was also recovered by Pyburn and Wiens (2011). Leptodactylus riveroi is sister of all other species in the genus, suggesting that this species does not represent an evolutionary transition between the L. latrans and L. melanonyx groups (Heyer and Pyburn 1983; Larson and de Sá 1998).

Heyer (1969) suggested a close relationship between the Leptodactylus latrans and L. melanonyx groups, with the L. pentadactylus group being the sister group of the former two, based on behaviour, morphology and ecology of adults. The L. fuscus group formed the sister group of the large clade composed of species of the other three species groups – L. latrans, L. melanonyx and L. pentadactylus. The L. marmoratus group (now Adenomera) is not closely related to any of the aforementioned groups previously. In recent phylogenetic hypotheses, Pyburn and Wiens (2011) and Fouquet et al. (2013) resurrected Adenomera as sister group of Leptodactylus species. Because we were unable to sample species of Adenomera, we have no insights on the issue. There are a few discrepancies of data processing by the present analysis and the hypothesis presented by Ponssa (2008). Ponssa (2008) ordered some characters in her analysis and also implemented the method of character successive weighting (Goloboff 1993, 1995). This methodology has been criticized (Turner and Zandee 1995; Kluge 1997; Grant and Kluge 2003), and we prefer not to implement it in the present work.

Most amphibian systematic studies include data from adult specimens and/or molecular data. However, as mentioned by Maglia et al. (2001), the morphology of an organism, including those with a bimodal life cycle, is not restricted solely to adult; it is part of a continuous ontogenetic process that includes different forms with different attributes that can be assessed. Our phylogenetic analyses based on larval and adult morphological data sets yielded the same two clades (fuscus – latrans – melanonyx and pentadactylus) for larval characters alone and combined larval and adult datasets. The unique difference was the topological position of Leptodactylus rhodomystax, which was included in the L. pentadactylus clade in the combined analysis. This confirms that different classes of characters are important in the resolution of relationships among species of Leptodactylus. Unfortunately, only 15 species of Leptodactylus were included in the combined analysis. The great amount of missing data in Ponssa’s (2008) data set may be a source of inconsistencies, affecting the results. It is necessary to sample a larger number of species in the genus Leptodactylus to clarify both the relationships and the behaviour of larval and adult characters. In the present work, when different partitions were combined (i.e. Ponssa 2008 and the larval characters analysed by the present work), the resulting phylogeny proved to be mostly congruent to the one obtained by using larval characters only. Hillis and Wiens (2000) suggested that subsampling characters and/or taxa may produce phylogenetic inconsistencies.

Some of the characters used in the phylogenetic analysis, both from this work and that coded by Ponssa (2008), were polymorphic. The inclusion of polymorphic characters in phylogenetic analysis does not necessarily make the matrix more informative (Wiens 2000). Some authors code polymorphic characters as missing data or choose to exclude them from analysis (Farris 1966; Kluge and Farris 1969). Although the polymorphic characters are less informative than the characters with fixed states (Wiens 1995), their inclusion is more informative than their exclusion (Wiens 2000).

PBS results suggest an incongruence produced by larval and adult characters found in many topological areas of the strict consensus tree (Fig. 4a). ILD also supports this incongruence. The considerable disagreement between two partitions on the combined trees is probably related to the nature of characters, which are subject to different evolutionary processes.

The monophyly of the Leptodactylus is corroborated by both larval and adult morphological characters. Nevertheless, the infragenic relationships remain to be resolved as additional species, and different data sets (e.g. molecular characters) can be sampled.

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Additional Supporting Information may be found in the online version of this article:

Table S1. Species, locality, acronym and collection number, and tadpoles analysed.

Table S2. List of characters and character states of the internal buccal morphology used in this study.

Table S3. List of characters and character states of the larval cranial morphology used in this study.

Data S1. Combined matrix used this study.

Data S2. Matrix representing 84 larval characters and 27 taxa.

Data S3. Additional Supporting Information may be found in the online version of this article.