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Description of a new species of *Eupsophus* (Amphibia: Neobatrachia) from the Valdivian Coastal range, Southern Chile: an integrative taxonomic approach

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Abstract

We describe a new species of *Eupsophus* from the Coastal Range of Southern Chile. The new taxon can be differentiated using an integrative taxonomic approach that includes advertisement call, reproductive mode, tadpoles, and mtDNA D-loop sequences. Based on karyological characters, the new species is assigned to the *roseus* Group (2N=30), and differs from all described species therein by having early winter breeding season, terrestrial tadpoles and its advertisement call with spectral elements reaching the 20 kHz. Phylogenetic analyses based on mitochondrial data place the new species as sister taxon of *E. migueli.* The discovery of this new species highlights the importance of the multisource approaches to discover cryptic diversity as well as to establish the basis for prioritizing policies and conservation efforts on Chilean batrachofauna.

Key words: Amphibia, Neobatrachia, *Eupsophus altor* **sp. nov**., integrative taxonomy, Chile

Introduction

The South American frogs of the genus *Eupsophus* Fitzinger 1843 are currently represented by ten species: *E. emiliopugini* Formas 1989, *E. vertebralis* Grandison 1961 (2n=28, *vertebralis* Group; Formas 1991), *E. roseus* (D & B 1841), *E. calcaratus* (Günther 1881), *E. insularis* (Philippi 1902), *E. migueli* Formas 1978, *E. contulmoensis* Ortiz, Ibarra-Vidal & Formas 1989, *E. nahuelbutensis* Ortiz & Ibarra-Vidal 1992, *E. septentrionalis* Ibarra-Vidal, Ortiz & Torres-Perez 2004 and *E. queulensis* Veloso, Celis-Diez, Guerrero, Méndez-Torres, Iturra-Constant & Simonetti 2005 (2n=30, *roseus* Group; Formas 1991) (Frost 2011). These species are restricted to the temperate *Nothofagus* forest of Southern Chile and Argentina (Formas 1979, Ibarra-Vidal *et al.* 2004).

During a series of herpetological surveys (2003–2011) in the vicinity of Valdivia city (Cerro Oncol, 39°41'S; 73°18'W, Coastal range, Valdivia Province, Southern Chile; Fig. 1) we collected frogs that make their specific determination problematic because they have external morphological characters that overlap with those of two geographically close species: *E. roseus* and *E. migueli*. Preliminary comparative observations of the external morphology of the specimens from Cerro Oncol (Valdivia province) suggested that those specimens were much more similar to *E. roseus* than *E. migueli* and for that reason those specimens were temporarily named as *Eupsophus* aff. *roseus*. However, despite its external resemblance, the specimens from Cerro Oncol have different reproductive patterns when is compared with *E. roseus*. For example, the tadpoles of *Eupsophus* aff. *roseus* are fully developed in terrestrial environments (particularly under fallen trunks), while the tadpoles of *E. roseus* developed in small water-filled cavities in the ground near to streams (Formas & Vera 1980). In addition the advertisement calls *Eupsophus* aff. *roseus* were registered during the austral winter (May to August), while those of *E. roseus* are detected between October to January (Formas & Vera 1980).

Integrative taxonomy is a conceptual multisource approach in which the nomination of a new species is considered as a hypothesis susceptible to be contrasted with different empirical evidences (Dayrat 2005, Padial *et al*. 2009, Schlick-Steiner *et al*. 2010). This proposition challenges subjective interpretations in the description and delimitation of new species, implicating that the resulting species hypothesis will be better supported as they are based on more comprehensive data from multiple sources.

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Taking into account these principles, and assuming that any kind of characters may be useful to propose species hypothesis, we proposed that the reproductive characteristics (reproductive season and terrestrial tadpoles) that present *Eupsophus* aff. *roseus* are enough to hypothesize that it belong to a independent lineage (*sensu* Baum & Donoghue 1995, De Queiroz 1998) of *E. roseus* and *E. migueli* and therefore constitutes a new species. To contrast our hypothesis we considered four lines of evidence: morphometry (nine continuous characters; see below), ethology (advertisement calls, reproductive mode) and molecular (mtDNA D-loop) data.

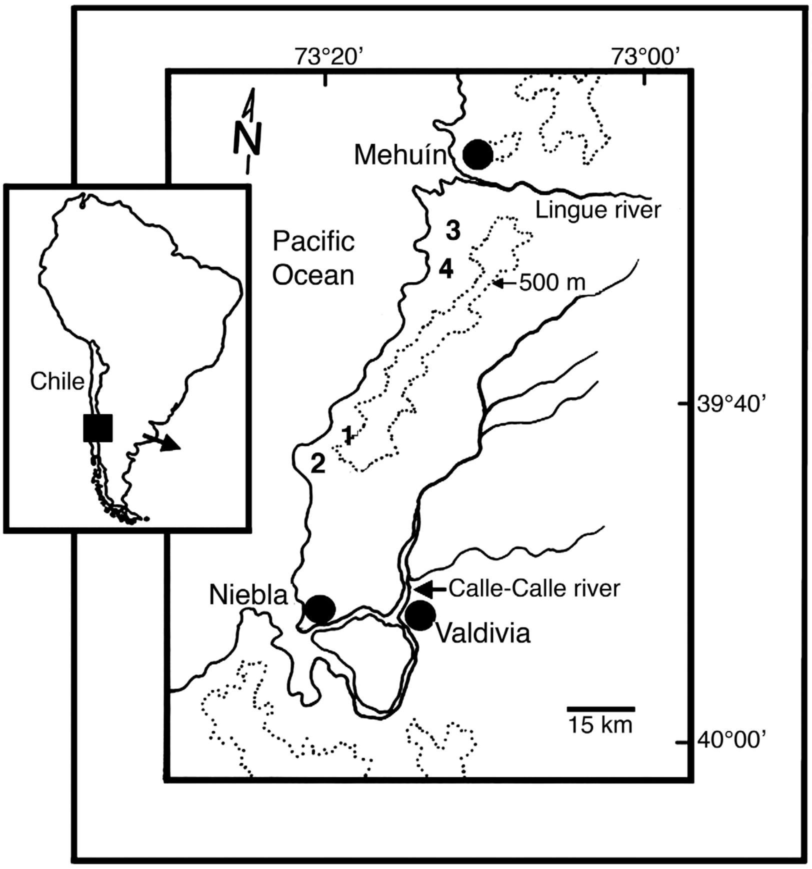

FIGURE 1. Distributional map of *Eupsophus altor* **sp. nov**. Numbers indicate the following localities: 1 Cerro Oncol (Type Locality), 2 Curiñanco, 3 Chan-Chán, 4 Alepue. (See coordenates in the text).

Material and Methods

Sampling. Because the geographical distribution of the genus *Eupsophus* extends from the Central Chile (*E. septentrionalis*; 35º49′S, Ibarra-Vidal *et al.* 2004) to the Southern Chile (*E. calcaratus*, 49º10′S, Nuñez *et al.* 2011) the sampling area was reduced to the western slopes of the Coastal Range of the Valdivia province, between Mehuín (39º25′S) and Cerro Oncol (39º41′S), where our target taxon (*Eupsophus* aff. *roseus*) and other species of the *roseus* Group species occur (*E. migueli* and *E. roseus*).

Morphometric characters. Morphological quantitative characters were analysed in 11 specimens of *Eupsophus* aff. *roseus* (6 males, 5 females; IZUA 3607-3618; Cerro Oncol; 39º41′S; 73º19′W, this paper), 10 specimens of *E. roseus* (3 males, 9 females, IZUA 3621-3633, La Saval; 39º48′S; 73º15′W; Nuñez *et al.* 1999) and 8 specimens of *E. migueli* (5 males, 3 females; IZUA 3634-3642; Colehual Alto; 39º22′S;73º04′W; this paper). The following variables were measured with a digital calliper (to the nearest 0.1 mm): snout-vent length (SVL), head length, head width, thigh length, tibia length, nostril-snout distance, nostril-eye distance, interocular distance and internarial distance. The Principal Component Analyses (PCAs) of morphometric characters was used to provide a representation of data useful to identify groups that may be related to previous assumptions about taxa without a priori subdivisions of the samples into discrete units (Wiley 1981). The PCA was performed with PAST v2.11 (PAleontological STatistics) (Hammer *et al*. 2001) on log10-transformed variables on variance-covariance matrices.

Osteology. Osteological observations were made on two adult male specimens of *Eupsophus* aff. *roseus* (IZUA 3619-3620), cleared and double-stained skeletons using the methodology of Song & Parenti (1995).

Tadpoles. Seventeen tadpoles of *Eupsophus* aff. *roseus* (IZUA 3643) stages 23–37 (Gosner 1960) were collected at Cerro Oncol. The following variables were measured with a digital calliper (to the nearest 0.1 mm): total length, body length, tail length, maximum tail height, tail muscle height, tail muscle width, internarial distance, and interocular distance.

Chromosomal analysis. The chromosomes were studied on one female of *Eupsophus* aff. *roseus* (IZUA 3544) according the methodology described by Formas (1991). We analyzed five well spread c-metaphases plates. Centromeric positions were determined according to Levan *et al*. (1964).

Call recording and analysis. During May and June 2009 the advertisement calls of five males of *Eupsophus* aff. *roseus* were recorded at Cerro Oncol with a digital recorder Marantz PMD 661 and an external unidirectional shotgun microphone (RØDE NTG2; frequency 20–20000 Hz). The spectral and temporal characteristics were analyzed using the software Audacity v1.2.6 at 44.1 kHz and 16 bit of resolution and a 512 FFT points. The Adobe Audition 3.0 was used to generate audiospectrograms and oscilograms. Calls were recorded at 30 cm from the emitter. Air and substrate temperature were measured immediately after each sound recording using a digital thermometer. The following quantitative parameters were analyzed: notes per call, call length (ms), fundamental and dominant frequencies (Hz), and maximum frequency (kHz).

Molecular data. For DNA extraction, 12 specimens of *Eupsophus* aff. *roseus* were collected from Cerro Oncol (IZUA 3607–3618), 12 specimens of *E. roseus* from La Saval (IZUA 3497–3509; 39°48′S; 73º15′W), three specimens from Lago Pellaifa (IZUA 3510–3512; 39º37′S; 71º59′W), nine specimens from Bosque San Martín (IZUA 3513–3521; 39º38′S; 73º12′W), six specimens of *E. migueli* from Mehuín (IZUA 3643–3648; 39º25′S; 73º12′W), three specimens of *E. calcaratus* from Reserva Valdivia (IZUA 3526–3528; 40º10′S; 73º29′W), and one specimen of *E. calcaratus* from Bahía Mansa (IZUA 3529; 40°33′S; 73°43′W). Whole genomic DNA for PCR was extracted from blood or liver samples using standard protocols of chemical digestion (0.1M Tris–HCl pH 8.0, 0.2M EDTA, 1% SDS, 100 µg/ml proteinase K), followed by phenol/chloroform extraction (Sambrook *et al*. 1989). For amplification and sequencing of a fragment of the D-loop region, we used primers ControlP-H and ControlJ2-L (Goebel *et al*. 1999). Sequences were deposited in GenBank under Accession Numbers JQ780164–JQ780170. Nucleotide sequences were aligned using MAFFT v5 (Katoh *et al*. 2005) under the iterative method of global pairwise alignment (G-INS-i). Default settings were chosen for all the parameters involved. Phylogenetic trees were constructed by the DNA maximum-likelihood method implemented in the GARLI v0.951 program (Zwickl 2006) using parameters for molecular evolution model as suggested by jMODELTEST v1.01 (Posada 2008), under the Akaike Information Criterion (Posada & Crandall, 1998; Posada & Buckley 2004). We also used a Bayesian phylogeny estimation with MrBAYES v3.0 program (Ronquist & Huelsenbeck 2003). Each Markov chain was started from a random tree and run for $1.0\text{x}10^7$ generations with every 1000th generation sampled from the chain. Stationarity was checked as suggested in Nylander *et al*. (2004). All sample points prior to reaching the plateau phase were discarded as ‘‘burn in’’ and the remaining trees combined to find the maximum a posteriori probability estimate of phylogeny.

Results

Morphometric data. The analysis of the morphometric characters of adult specimens based on Principal Component Analysis did not resolve discrete groups. In this analysis the Component 1 (eigenvalue = 24.06) explained 83.07% of the total variation and the Component 2 (eigenvalue = 1.98) explained 6.86%. Although the total variance explained is high (85.05%), the distribution of the individual values in the two factors did no conform discrete groups. A graphic representation of the eigenvalues of the first and second components is shown in Fig. 2. The measurements of males and females of *Eupsophus* aff. *roseus, E. roseus* and *E. migueli* are given in Table 1.

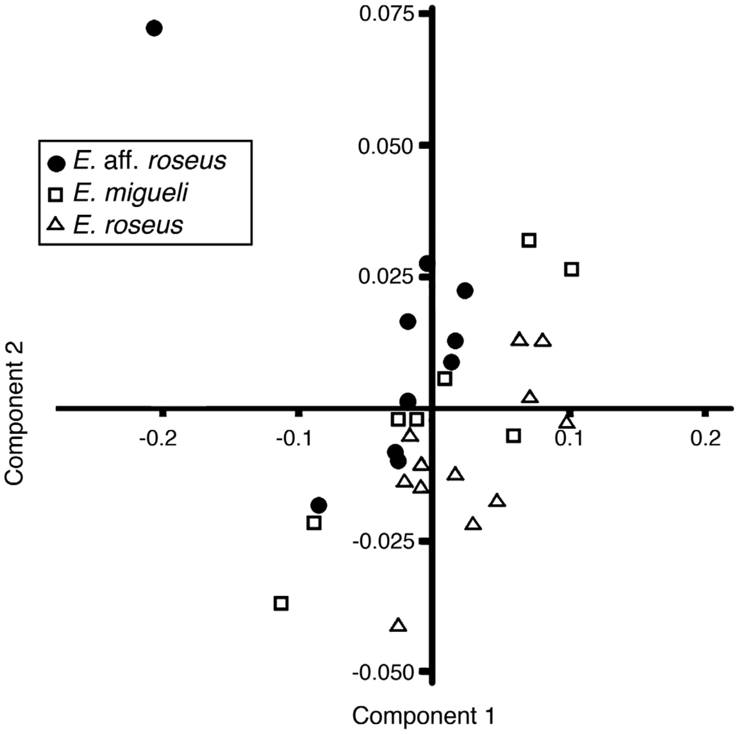

FIGURE 2. Principal component analysis (PCA) of morphometric characters of *Eupsophus* aff. *roseus*, *Eupsophus roseus* and *Eupsophus migueli*.

TABLE 1. Measurements (mm) of females and males of *Eupsophus* aff. *roseus*, *E. roseus,* and *E. migueli*. Means, standard deviation and ranges (parenthesis).

Character	*Eupsophus* aff. *roseus*	*E. roseus*	*E. migueli*
	females = 5, males = 6	females = 3, males = 9	females = 3, males = 5
Snout–vent length	38.2 ± 2.8 (33.5–42.0)	40.0 ± 3.2 (35.0–44.7)	39.4 ± 1.9 (31.2–47.2)
Head length	17.7 ± 0.8 (11.6–14.3)	12.5 ± 1.4 (10.2–14.6)	12.8 ± 0.6 (10.2–15.1)
Head width	14.2 ± 1.7 (10.9–15.1)	15.2 ± 0.9 (14.0–16.8)	14.4 ± 0.6 (11.9–16.9)
Thigh lenth	18.9 ± 1.5 (15.7–20.6)	20.8 ± 1.9 (18.2–25.2)	20.4 ± 0.7 (17.5–23.0)
Tibia length	19.5 ± 2.3 (12.9–21.0)	22.2 ± 1.4 (20.2–24.9)	20.9 ± 2.3 (17.8–24.1)
Nostril–snout length	2.9 ± 0.5 (2.5–3.8)	3.6 ± 0.7 (1.9–4.6)	3.3 ± 0.1 (2.7–3.8)
Nostril–eye distance	3.0 ± 0.5 (1.9–3.8)	3.5 ± 0.6 (2.6–4.7)	3.6 ± 0.2 (2.9–4.5)
Interocular distance	5.5 ± 0.6 (4.6–6.4)	6.1 ± 0.7 (5.0–7.5)	5.7 ± 0.3 (4.8–7.5)
Internarial distance	3.9 ± 0.3 (3.4–4.3)	4.0 ± 0.4 (3.5–4.5)	4.1 ± 0.1 (3.6–4.6)

Advertisement calls. The advertisements call of *Eupsophus* aff. *roseus* (air temperature 10.0–12.5°C) consists of a single note with the following characteristics: note duration 290–360 (336 ± ms), dominant frequency ranges between 1317–2098 (1882 ± 250 Hz) and the fundamental frequency ranges 304–1298 (756 ± 187 Hz). These vocalizations are rich in harmonics and show spectral elements which reach 20 kHz (Fig. 3). With the same method used to record the advertisement call of *Eupsophus* aff. *roseus*, one of us (FER) recorded the vocalizations of *E. migueli* and *E. roseus*. His results (not included herein) were in agreement to those previously obtained by Formas (1985) to *E. migueli* and Márquez *et al*. (2005) to *E. roseus* (Table 2).

TABLE 2. Advertisement calls characteristics (means and ranges) of *Eupsophus* aff. *roseus*, *Eupsophus roseus* and *Eupsophus migueli*. (n = number of males recorded).

Species	Notes per call	Call length (ms)	Fundamental frequency (Hz)	Dominant frequency (Hz)	Maximum frequency (kHz)	References
E. aff. *roseus* n = 15	1	336 (290–490)	756 (1317–2098)	1882 (1317–2098)	> 20	This paper
E. roseus n = 7	1	258 (124–235)	632 (346–1018)	1871 (1503–2166)	< 15[1]	Marquez *et al.* 2005
E. *migueli* n = 12	1	240 (200–350)	450 (390–987)	1835 (1500–2500)	< 15[1]	Formas 1985

[1] F. Rabanal; pers.obs.

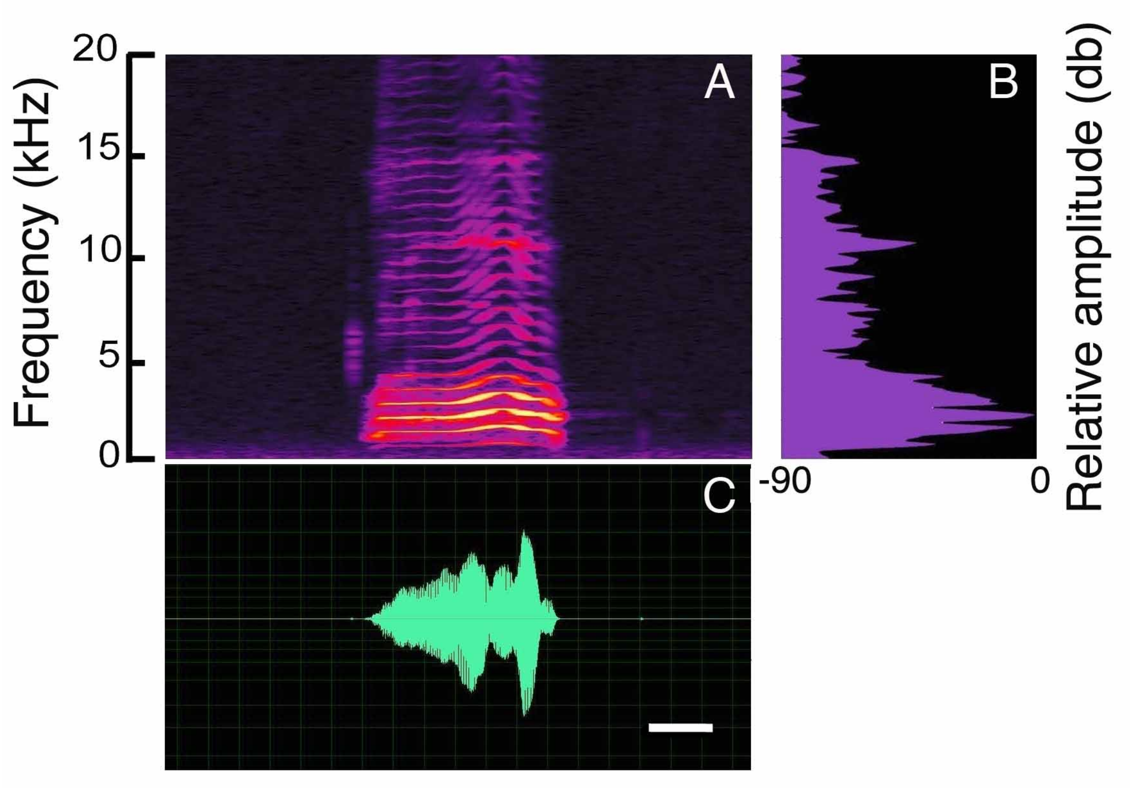

FIGURE 3. Sonogram (A), oscillogram (B) and power spectrum (C) of the advertisement call of *Eupsophus* aff. *roseus* (air tempature 10.0–12.5°C). Bar indicates 1 ms.

Molecular relationships. Hierarchical likelihood test implemented in jModeltest (Posada 2008) selected a TRN+I+Γ substitution model as best fitting the data (A = 0.2917, C = 0.2279, G = 0.1373 and T = 0.3431; gamma shape parameter = 0.1480). The single phylogram obtained from Maximum Likelihood analysis (Likelihood score -lnL =1200.5051) is shown in Fig. 4. Bayesian searches resulted in a topology that completely agreed with Maximum Likelihood analysis. This analysis shows two results: first the specimens assigned to *Eupsophus* aff. *roseus* are clearly differentiated from *E. roseus* and second that *Eupsophus* aff. *roseus* share in this analysis a common ancestor with *E. migueli*.

Taxonomic Conclusions. Two independent lines of evidence (bioacoustics and molecular) allow to us to conclude that *Eupsophus* aff. *roseus* is a new species. This conclusion, together our initial observations (winter breeding season and terrestrial oviposition/clutches and tadpoles) supports the hypothesis that *Eupsophus* aff. *roseus* belongs to a lineage distinct from *E. roseus* and *E. migueli*.

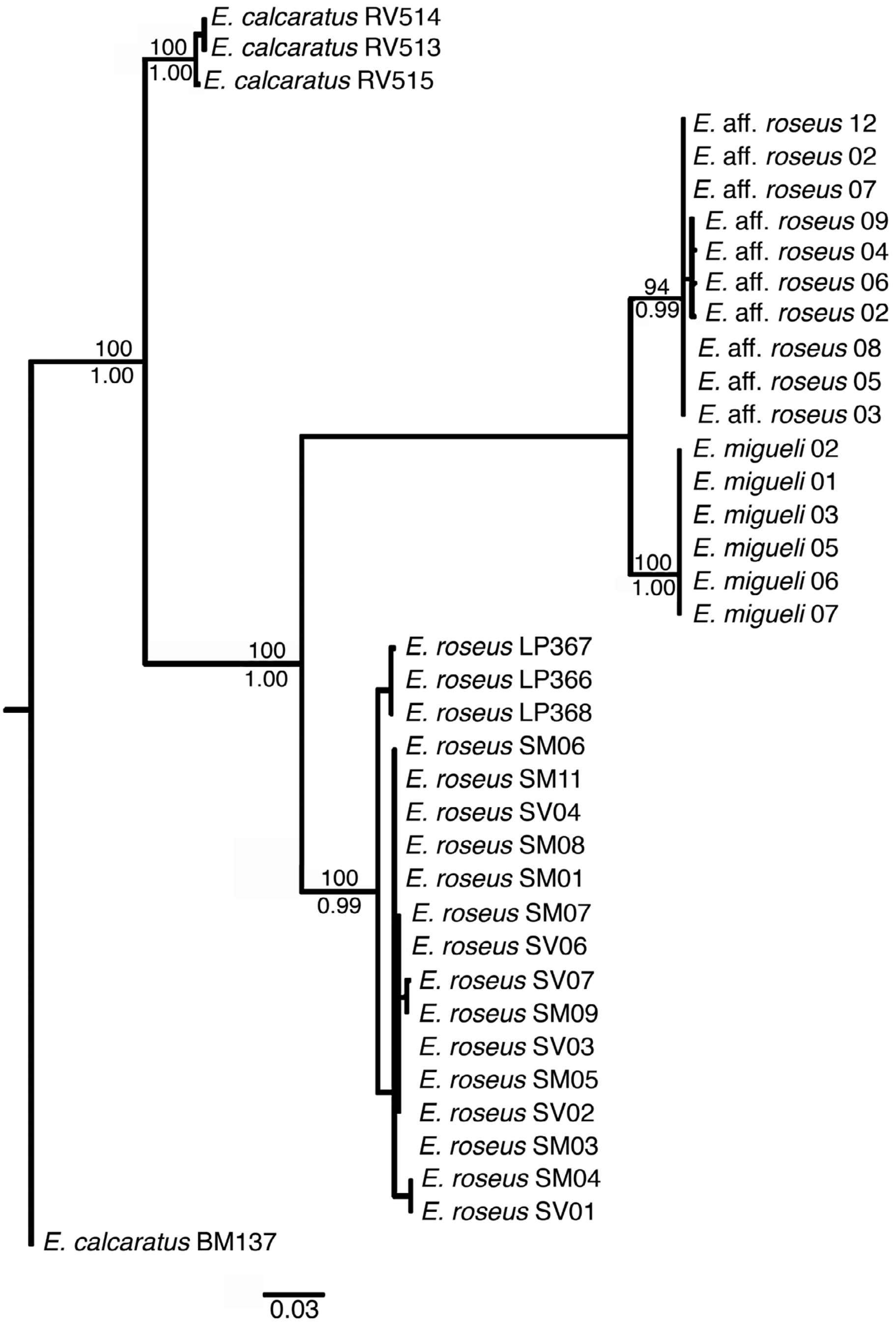

FIGURE 4. Single tree recovered in the Maximum Likelihood analysis; Bayesian analyses recovered a consensus tree with identical topology. The support indexes for each of the nodes are Maximum Likelihood and Bayesian posterior probabilities. Labels correspond to species and sampling localities (RV =Reserva Valdivia, LP =Lago Pellaifa, SM = Bosque San Martín, SV =La Saval, BM = Bahía Mansa).

***Eupsophus altor* sp. nov.** Nuñez, Rabanal & Formas (Fig. 5)
Oncol's ground frog (English) Rana de hojarasca de Oncol (Spanish)

Type Material. Holotype. IZUA 3607, adult male collected by Felipe Rabanal and José Nuñez on 11 June 2008 at the Cerro Oncol (39º41'S; 73º19'W, 650 m a.s.l.), Coastal range, Valdivia Province, 40 km W (by road) of Valdivia city, Chile (Fig. 1). **Paratypes.** IZUA 3608–3620, collected at the type locality, same data as holotype.

Diagnosis. The species is assigned to the genus *Eupsophus* because it has the following osteological cranial pattern (cervical cotylar arrangement type II; palatal shelf of premaxilla relatively deep; palatal shelf of maxilla of moderate width; pterygoid process moderately large; nasals small, widely separated medially; nasals in broad contact with maxillae, not in contact with pterygoids; epiotic eminences prominent; zygomatic ramus of squamosal of moderate length, widely separated from maxilla; otic ramus of squamosal as long as zygomatic ramus, expanded medially into small otic plate; squamosal-maxillary angle 50–55°; palatines broad, widely separated medially, bearing odontoid ridges; sphenethmoid entire, extending anteriorly to anterior edge of nasals parasphenoid alae oriented at right angles to anterior ramus of parasphenoid, broadly overlapped laterally by median rami of pterygoid) as has been described by Lynch (1971) and endotrophic tadpoles (Formas 1985). *Eupsophus altor* is assigned to the *E. roseus* Group species by having 2n = 30 chromosomes. *Eupsophus altor* differs from all species described of the *E. roseus* group species by having early winter breeding season, terrestrial tadpoles and its advertisement call with spectral elements reaching the 20 kHz, and nine mitochondrial D-loop nucleotide site substitutions from its congeneric species phylogenetically closest.

FIGURE 5. A. Holotype of *Eupsophus altor* **sp. nov.** (IZUA 3607) from Cerro Oncol (Valdivia Province). B: Terrestrial embryos of *Eupsophus altor* **sp. nov**. C: Nest with tadpoles of *Eupsophus altor* **sp. nov.** Stage 30. D: Tadpoles at stages 37–41. Stages according to Gosner (1960). Bar indicates 10 mm.

Description of the holotype. Adult male 36.6 mm in SVL. Head 0.93 times narrow than body; head length 31.7% of SVL; head width 1.2 times broader than long. Snout rounded dorsal view and obtuse in lateral view; loreal region flat, nostrils slightly prominent, oriented laterally; internarial distance 0.31 times the head width, internarial region slightly convex; nostril slightly closer to the anterior border of the eye than the terminus of snout; canthus rostralis marked. Eyes prominent, laterally oriented, 0.44 times the head length; tympanum round, 0.67 times the eye diameter; dorsolateral fold well developed, extending from the posterior corner of eyelid, terminating dorsal to forelimb. Maxillary and premaxillary teeth present; seven prevomerine teeth (4 on the left and 3 on the right), obliquely located between the choanae, small sized (0.26 times internarial distance) and subcircular in shape; tongue rounded, posterior the border slightly notched, attached approximately 66.4% of its length interiorly. Forelimbs slender; dorsal and ventral surfaces smooth. Relative length of the fingers: III>IV>II>I; tips of the fingers rounded and slightly protuberant; subarticular tubercles rounded distributed on fingers as follows: I-II-IV

(1), III (2); inner palmar tubercle ovoid; outer almost rectangular, as long as the inner; one subarticular tubercle on fingers I–IV; supernumerary palmar tubercles absent; nuptial pads on fingers I–II with tiny spines unpigmented. Hind limbs long and slender (162.8% of SVL); tibiotarsal articulation reaching the posterior border of eye when hind limb is adpressed along the body; toes long and thin, their relative length are: IV>V=III>II>I; a small delicate web between toes III–IV; tips rounded; inner metatarsal tubercle ovoid and developed, external tubercle conical and small, one-fourth of length of inner metatarsal tubercle; subarticular tubercles rounded, distributed on toes as follows: I(1), II(1), III(2), IV(3), V(2). Skin of head, dorsal surface, flanks and limbs smooth; ventral surfaces of limbs smooth; cloacal opening directed posteriorly at ventral level of thighs; cloacal opening unornamentated, covered by a fold of skin; Ventral surface of thighs smooth.

Colour in life. The dorsum is reddish-pink with light gray spots. Extremities with light gray bracelets. The flanks are whitish but with yellow in axillary and inguinal areas. Throat, chest and belly with minute melanophores regularly distributed. The ventral surface is white or creamy (Fig. 5). Upper part of the iris yellowish with black reticulations. Color in preservative (70% ethanol plus NaCl) similar to the live specimens.

Osteology. Neurocranial braincase: The neurocranial braincase is made up of three bones: sphenethmoid, prootics and exoccipitals. It is partially covered by the frontoparietals dorsally and the parasphenoid ventrally (Fig. 6A). The sphenethmoid forms the floor, edges of the roof and the anterolateral wall of each side of the braincase. Dorsally occupies a large area (its width is equal to the length of the nasals) between the nasals, frontoparietals and frontoparietal fontanella. Its ventral face overlaps with the inner half of the neopalatines, vomers and anterior end of the cultriform process of the parasphenoid. The prootics are fused with the exoccipitals, forming the posterior region of the braincase and the otic capsules. The exoccipital, which are covered ventrally by the parasphenoid forming the posteromedial walls of the otic capsules, the margin of the foramen magnum, and the occipital condyles.

Dermatocranium: The frontoparietals are paired and their anterior extremes are slightly divergent. The frontoparietals are expanded posteriorly and they overlap the prootics but do not reach the foramen magnum. The frontoparietal fenestra extends forward from the anterior third of the skull. The fontanella is 0.24 times the skull length. The nasals are paired subrectangular bones, transversally oriented and superimposed on the sphenethmoid (Fig. 6B). A space separates the nasals from the oblique cartilage of the nasal capsule. The parasphenoid is T-shaped, not fused with the subjacent bones. The cultriform process, which rests on the sphenethmoid, is long not keeled and anteriorly serrated, their tips do not reach the level of the neopalatines. The posteromedial process of the parasphenoid is acuminate and near the foramen magnum margin. The alae deflect posteriorly and they gradually expand to the cartilaginous extreme. The neopalatines are curved bones, concave posteriorly. One-third of it inner length overlaps with the sphenethmoid. The outer edge reaches the pars palatine of the maxilla. The vomers are paired bones that overlap the sphenethmoid. Each vomer comprises a dentigerous process with a transverse and concave row that bears 4 to 5 teeth. The posterior margin forms the posterior margin of choanae. The maxillary arcade is complete. The upper jaw is composed of the premaxillae, maxillae and quadratojugal bones. Each premaxilla bears 4–5 teeth. The alary process is subrectangular, oriented dorsally curved backwards and not reach the nasals. The pars palatina is subrectangular and the palatine process is pointed. The maxilla is well-developed, pars fascialis is wide and pars palatina narrow. The teeth (19–24) are conical.

Suspensorium: Each pterygoid has well-developed the anterior, medial and posterior rami. The anterior ramus is expanded anteriorly, articulating with the inner side of the maxilla. The medial ramus does not reach the allae of parasphenoid, but it contacts on the prootic. The posterior ramus invests the cartilaginous quadrate process medially and terminates posteriorly at the angle of the jaw. The zygomatic ramus of the squamosal is slightly curved (Fig. 6C). The otic ramus is expanded and shorter than zygomatic ramus. The ventral ramus is straight and the angle with the maxilla is about 45º. The annulus tympanic is oned dorsally, cartilaginous and joined to the zygomatic ramus. The coronoid process of the mandible is trapezoidal and normally developed. Columella presents.

Hyoid apparatus: The hyoid plate is cartilaginous and mineralized at posteromedial processes (Fig. 6E). At midline the hyoid corpus is 1.2 times wider than long; the hypoglossal sinus is broad and U-shaped, approximately as deep as wide and its margins are parallel. The allary processes are thin, perpendicular to the axial axis of the hyoid plate, slightly oriented forward and with a distal expansion. The hyales are thin and curved; the anterior lateral processes are developed and slightly curved laterally. The postero-lateral processes are thin and oriented postero-laterally, their tips are sharp. The posterior ends of the posteromedial processes are cartilaginous and slightly expanded.

Pectoral girdle: The pectoral girdle is arciferous, the omosternum is cartilaginous with an expanded distal end

(Fig. 6D). The anterior expansion is shorter than the cartilaginous sternum. The omosternum slightly expanded anteriorly and stick-like. The sternum is wide and its distal end is rounded. The procoracoid is present and extends to the level of the internal extreme of the clavicle, the clavicles do not touch each other. The prolongation of the procoracoid extends between the clavicle and the scapula. In ventral view, the right cartilaginous epicoracoid overlaps the left. The pectoral fenestra, whose inner margin is concave is 1.5 times wider than long. Each of these apertures is anteriorly bordered by the procoracoid cartilage, medially by the epicoraciod cartilage, and posteriolly by the coracoid. Each clavicule is concave anteriorly. The glenoid end of the clavicle is expanded dorsolaterally into a wedge-shaped process that articulates with the pars acromialis of the scapula. The clavicles do not reach the glenoid fossa. The scapula is rectangular in shape and 1.3 times the coracoid. The scapula is composed of two planes: pars acromialis concave and the pars glenoid concave posteriorly. The coracoid is subrectangular and the distal ends are distally expanded. The glenoid cavity is limited by the pars glenoidalis and coracoid. The outer edge of the supra scapular cartilage is cartilaginous. The cleitrum consists of an ossified thin and bifid lamina, the posterior ramus is shorter of the anterior ramus slightly expanded, as long as the scapula; the anterior border ossified as cleitrum; the posterior and lateral margins unmineralized.

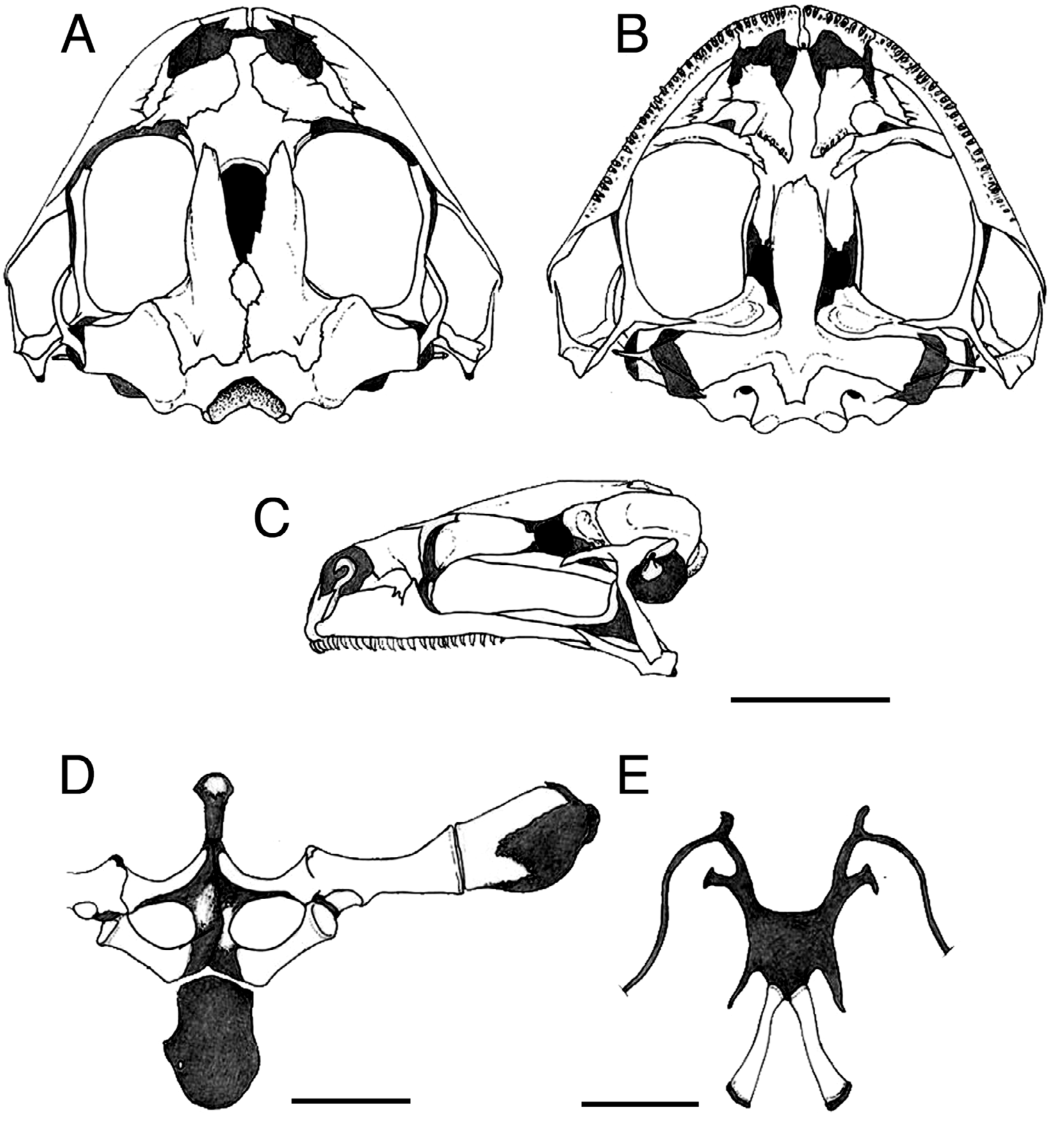

FIGURE 6. Skull of *Eupsophus altor* **sp. nov.** (IZUA 3619); in dorsal (A),ventral (B), and lateral (C) views. Pectoral girdle (D) and hyoid plate (E) of *Eupsophus altor* **sp. nov**. Bar indicates 5 mm. Gray areas indicate cartilaginous tissue.

Axial osteology: The vertebral columnae is composed of eight procoelus, non-imbricate, independent presacral vertebrae (Fig.7A, B). Presacral I (atlas) wide, shallow cervical cotyles widely separated. The presacrals II–IV with low neural spines, presacrals V–VIII with neural spine absent. Relative lengths of transverse processes and sacrum: III = sacrum < IV< II< V–VII<VIII. Distal ends of presacral III slightly expanded; transverse processes of

presacrals V–VIII acuminated. Presacral II oriented anteriorly, III–VII oriented posteriorly and VIII oriented perpendicularly to the longitudinal axis. Sacral diapophysis rounded, end slightly expanded, oriented posterolateraly; sacrum with bicondylar articulation with urostyle. Urostyle robust bearing dorsal crest that is more developed anteriorly, approximately 1.2 times larger than sacrum plus presacral vertebral column. Overall length of pelvic girdle 1.6 times the length of sacrum plus presacral vertebral column. Ilial shaft poorly developed, interilial profile U-shaped, width of the U at the anterior ends of the ilia approximately 2.5 times its base. Ilium forming anterior margin of round acetabulum; preacetabulum forming approximately a 45º angle to the ilial shaft; ilia articulating with one another medially forming the anterior margin of acetabulum; ventral margin of acetabulum formed by cartilaginous pubis. The ischium is prominent, articulating with the ilium and fused with the pubis (Fig. 7C).

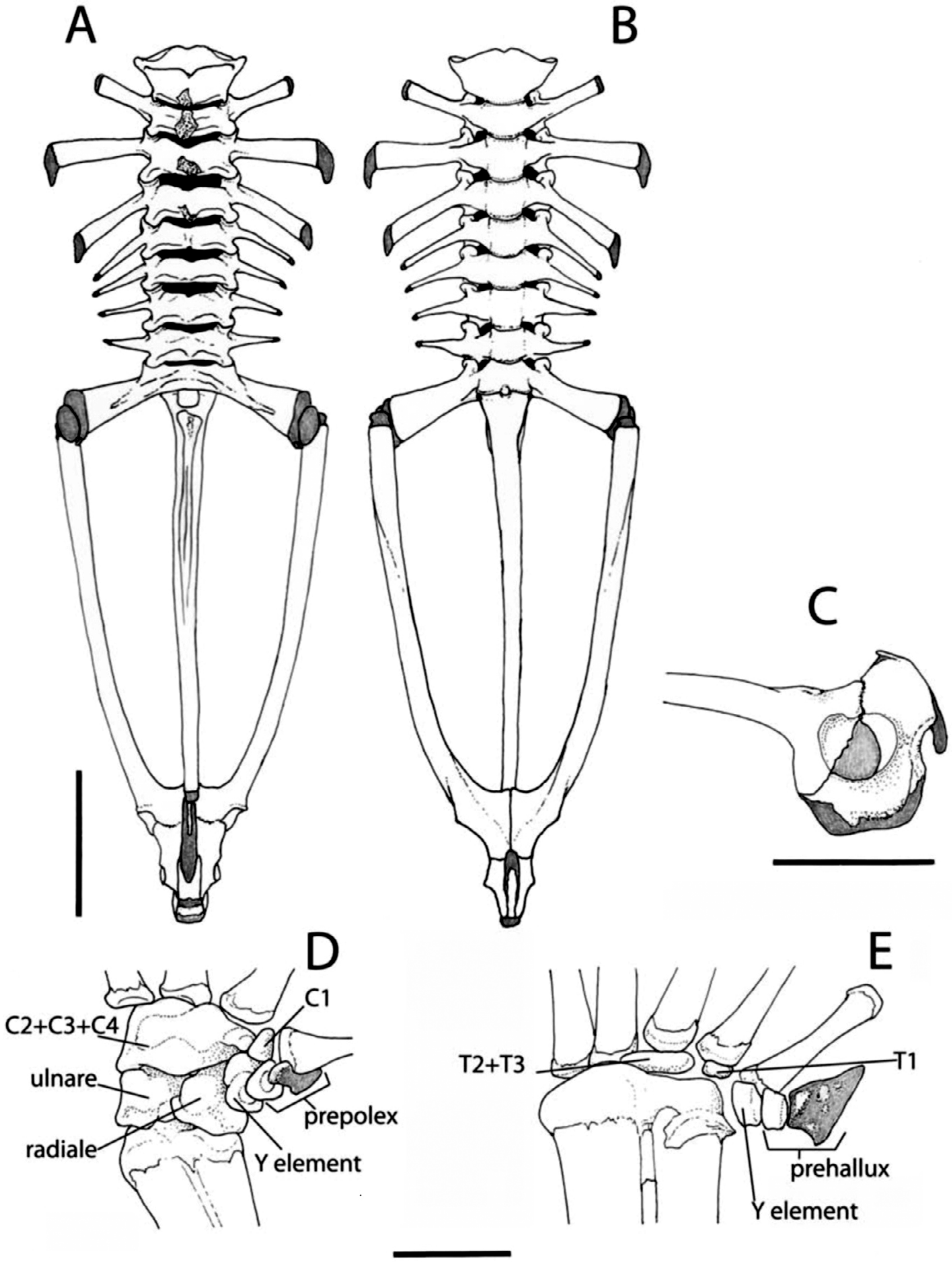

FIGURE 7. Vertebral column of *Eupsophus altor* **sp. nov.** (IZUA 3619) in dorsal (A), and ventral (B) views. Left acetabulum of *Eupsophus altor* **sp. nov.** (C). Dorsal views of the hand (D) and foot (E) of *Eupsophus altor* **sp. nov**. Bar indicates 5 mm. (C1–C4= carpal bones; T1–T3 = tarsal bones).

Forelimb: Humerus as long and robust as the radioulna; phalangeal formula for manus 2-2-3-3; terminal phalanges pointed, distal carpals 2–4 fused; distal carpal 1, element Y, radiale and ulnare independent; prepollex with two elements (pattern C; Fabrezi 2001), the proximal triangular in shape and ossified; the distal element cartilaginous (Fig.7D).

Hind limb: Femur and tibiofibula similar in length; tibiale and fibulare fused at the ends. Tarsal elements ossified; metatarsal IV and V articulate with the end of fibulare; metatarsal III articulates with tarsal 2–3, and metatarsal II articulates with the element Y; the metatarsal I articulate with tarsal 1. Phalangeal formula of the foot: 2-2-3-4-3, terminal phalanges pointed. The prehallux has two elements; proximal one ossified and smaller than the cartilaginous distal element (Fig. 7E).

Tadpole. The tadpoles of *E. altor* are endotrophic and its description is based on five specimens collected at the type locality (one in stage 33 and four in stage 34). The total length ranges between 18.4–19.6 mm. Larva type IV (Orton 1953) with elliptical body in dorsal view, slightly depressed (Fig. 8A, B). Snout slightly truncated in lateral and dorsal profiles. Pupils are circular. The small nostrils are oval in shape, not protruding. The narial opening situated anterolaterally nearer to the snout than the anterior border of the eyes. The internarial distance 0.6 times the interorbital distance. The oral disc is ventral (*sensu* Mijares-Urrutia 1998), nearly circular in shape (Fig. 8C). The margin of the oral disc slightly emarginated with a single row of the marginal papillae. Rostral gap 0.75 times the oral disc; mental gap absent. Intramarginal papillae absent. Upper and lower jaw sheaths well keratinized and with serrations. Labial tooth formula 2(2)/2. Tube spiracular short, reduced and sinistrally disposed; its aperture corresponds 0.5 times the eye diameter. Intestinal convolutions poorly defined and with abundant vitellum. Vent medial, large (0.22 times the body length), tubular in shape with its posterior extreme slightly sharpened, the aperture is longitudinal (Fig. 8D). The caudal fins are low. Tail length 0.70 times the total length. Tail height low (0.85 times the body height). Tail width, 1.5 times the internarial distance. Tail axis curved dorsoventrally with myomeres well developed. Tip of the tail rounded. Body and tail unpigmented. The measurements of the tadpoles are shown in Table 3.

TABLE 3. Measurements (Mean and standard deviation in mm) of the tadpoles *Eupsophus altor.* Stages *sensu* Gosner (1960).

Character	Stages							
	23	24	26	28	33	34	36	37
	n = 1	n =1	n = 2	n = 2	n = 1	n = 3	n = 3	n = 4
Total length	11.2	13.0	13.1–13.3	13.1–13.3	19.6	18.4 ± 1.6	21.2 ± 1.1	20 ± 1.2
Body length	4.2	5.5	6.1–6.2	6.0–6.3	6.4	6.8 ± 0.4	7.1 ± 0.3	6.8 ± 0.3
Tail length	6.3	7.5	7.2–7.4	7.1–8.2	13.2	11.6 ± 1.4	14.1 ± 0.8	13.2 ± 1.1
Maximum tail height	2.3	2.7	2.1–2.2	2.3–2.5	3.5	3.1 ± 0.9	4.0 ± 0.4	3.2 ± 0.4
Tail muscle height	1.3	1.6	1.5–1.7	1.5–1.7	1.8	1.8 ± 0.4	2.1 ± 0.2	2.2 ± 0.1
Tail muscle width	0.9	1.0	1.0–1.2	1.1–1.3	1.0	1.4 ± 0.5	1.6 ± 0.3	1.6 ± 0.1
Internarial distance	0.6	0.6	0.9 –1.0	0.9–1.2	1.2	1.3 ± 0.1	1.4 ± 0.1	1.3 ± 0.1
Interocular distance	1.2	1.5	1.4 –1.5	1.5–1.6	2.1	2.1 ± 0.4	2.5 ± 2.8	2.8 ± 0.1

Chromosomes. The diploid number is 2n=30 and the Fundamental Number (NF) is 44. Calculation of arm ratios identifies pairs 1, 5, 9, 10 and 11 as metacentric (m), pairs 2 and 3 submetacentric (sm), and pairs 4, 6–8, 12–15 as telocentric (Table 4; Fig. 9).

TABLE 4. Relative length, arm ratio and type of chromosomes of *Eupsophus altor.* * Chromosome with secondary constriction.

	Pair														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Relative length[1]	165	109	93	66	64	63	61	59	58	56	48	46	37	35	35
r [2]	1.2	2.7	3.4	∞	∞	1.3	∞	1.1	∞	1.1	∞	∞	1.1	∞	∞
Type[3]	m	sm*	st	t	m	t	t	t	m	m	m	t	t	t	t

[1] According to Bogart 1970
[2] *r* = large arm/short arm
[3] m = metacentric, sm = submetacentric, st = subtelocentric, t = telocentric

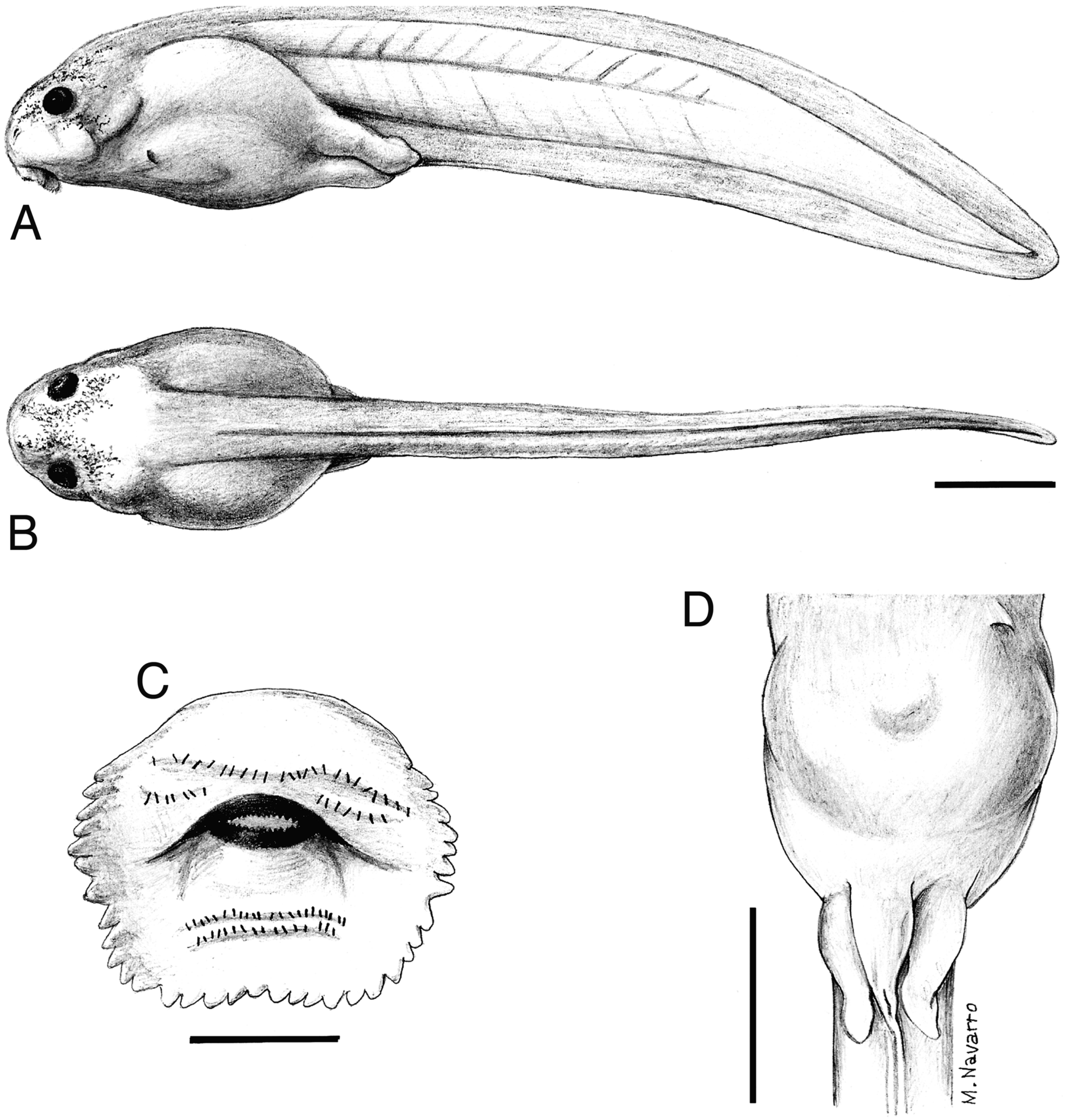

FIGURE 8. Tadpole of *Eupsophus altor* **sp. nov.** (IZUA 3621) (Stage 33, Gosner 1960). Lateral (A), dorsal (B) and ventral (D) views; oral disc (C). Bar indicates 5 mm (A, B, D), and 1.5 mm (C).

Reproductive mode. Nests (*N*=30) and tadpoles were observed between May and August between the forest and meadowlands. The terrestrial eggs were easy to separate as in *E. roseus* (Formas &Vera 1980). Eggs (17–30, 5.5–5.7 mm in diameter) and tadpoles were grouped in moist burrows in the ground covered by logs or stones. In all cases the tadpoles were found away from any aquatic environment. A male was always observed near the tadpoles.

The eggs develop into endotrophic tadpoles that completing their total development within the nest. This particular reproductive mode, including terrestrial tadpoles, is an additional argument that support the hypothesis that *E. altor* is an independent lineage of *E. roseus*.

Distribution and natural history. At present, specimens of *E. altor* are known from the type locality and three additional sites [Alepue (39º36'S; 73º14'W), Chan-Chán (39º33'S; 73º12'W) and Curiñanco (39º39'S; 73º18'W)] located the western slopes of the Coastal range, between the mouth of the rivers Lingue (39º26'S;

73º12’W) and Valdivia (39º52’S; 73º23’W), Valdivia province. This area is covered by the typical vegetation of Valdivian rain forest (Veblen 2007) where the following plants were identified: *Drymis winteri*, *Laurelia phyllipiana*, *Weinmannia trichosperma*, *Dendroligotrichum dendroides*, and *Cladonia* spp. There, the most common frogs are: *Alsodes norae*, *Batrachyla antartandica*, *Batrachyla leptopus*, *Eupsophus vertebralis*, *Pleurodema thaul*, and *Rhinoderma darwinii*. During January and February (austral summer) specimens of the small lizards *Liolaemus pictus* and *L. cyanogaster* were observed. Throughout the year, adult specimens of *E. altor* were found under rotten trees and at night walking through the vegetation ground. Mating calls (maximum activity at night), males with nuptial pads and gravid females were observed throughout May and August (austral winter). The clutches and tadpoles were always observed during May and June, and tadpoles between May and October in terrestrial nests, under logs or tree holes until 1 m above the ground (Fig 5B, C, D).

Etymology. The specific name *altor* is derived from the Latin meaning “one who looks after or brings up its offspring”. This epithet is given in the masculine form of the word (the female form is *altex*) because we observed that in this species males are the care givers for offspring.

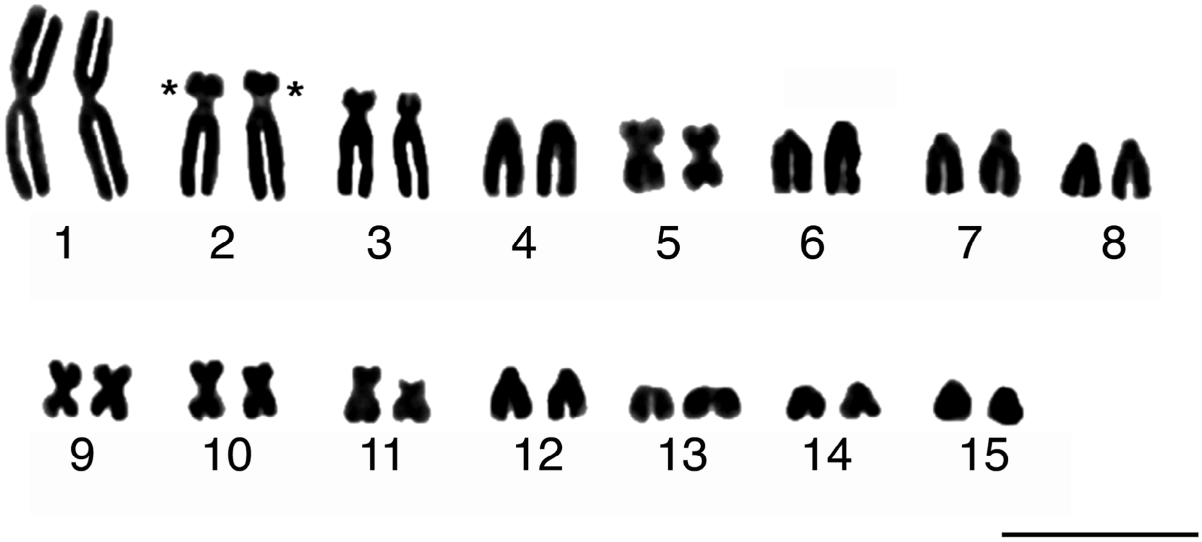

FIGURE 9. Chromosomes of *Eupsophus altor* **sp. nov. (**IZUA 3620). Bar indicates 10µm. Asterisk indicates chromosome with secondary constriction.

Discussion

Application of integrative taxonomy in *Eupsophus* species. Frogs of the genus *Eupsophus* has been considered a taxonomic collective supported by a unique synapomorphy, endotrophic tadpoles with reduced mouthparts and pigmentation (Formas 1989). Although this hypothesis is suggestive it should be considered with caution because the tadpoles of *E. migueli*, *E. contulmoensis*, *E. insularis* and *E. septentrionalis* are unknown. From the karyological standpoint the genus has been divided into two phenetic group species: the *E. vertebralis* Group and the *E. roseus* Group (Formas 1991). The members of the former (*E. vertebralis* and *E. emiliopugini*) are almost unrecognizable externally; however their advertisement calls are notoriously different and this feature is the best way to distinguish both taxa (Formas 1989). Conversely, the members of the *E. roseus* Group species have similar advertisement calls (Formas 1985); but in contrast to this ethological uniformity they show remarkable morphological polymorphism (colour patterns and colouration). As a result of this phenomenon the specific determination based only on a single character set is often uncertain. Our problem arose from the difficulty of assigning the specimens of Cerro Oncol to some of the known species of the *E. roseus* Group species. Faced with this difficulty we decided to apply the theoretical principles and methodologies used by the integrative taxonomy. This approach is conceptual and methodologically robust and has proved to be useful in resolving taxonomic problems, and determination and discovery of cryptic species (Padial *et al*. 2009, Padial & De la Riva 2009). As a result of the application of the integrative approach we conclude that the specimens from Cerro Oncol belong to a new species, *Eupsophus altor*, distinct from *E. roseus* and *E. migueli*.

The resolution of our taxonomic problem suggests that this approach could be also used in the elucidation of conflictive taxonomic cases detected in three species of *E. roseus* Group species. First, the specific taxonomic clarification of the northern populations of *E. calcaratus* (Nuñez *et al*. 2011); second, the elucidation on the probable conspecificy of *E. queulensis* and *E. septentrionalis*, two sympatric species restricted to a small area of Central Chile (Reserva Nacional Los Queules) (Ibarra-Vidal *et al*. 2004, Veloso *et al*. 2005) and third, the taxonomic status of the populations of *E. roseus*, a species widely distributed in southern Chile (Cei 1962), that show low allozymic variation (Formas *et al*. 1991) and high levels of inter and intrapopulational morphological variation (Nuñez *et al*. 1999).

The singularities of *Eupsophus altor*. Two singularities distinguish *E. altor* from the other members of *E. roseus* Group species: advertisements calls with spectral elements that reach the 20 kHz, terrestrial eggs, and terrestrial endotrophic tadpoles. If the advertisements calls of the *E. roseus* Group species are compared, it is observed that they have a similar call patterns consisting of a single note lasting 0.15–0.35 sec, concentrating the energy between 1100–2900 Hz and the harmonics have frequencies ranging from 2020 to 3900 Hz (Formas 1985). In general, the advertisement call of *E. altor* agrees to those of the members of *E. roseus* Group species; however differs by containing spectral elements above 20 kHz. Among the Neotropical frogs this character look like to be unique and only has been reported in two South Asian frogs *Odorrana tormota* and *Huia cavitympanum* (Feng *et al*. 2005, Arch *et al*. 2008). The calling males of these allopatric frogs were found in similar habitats, fast-moving streams, which contrast with the males of *E. altor* that are calling at the border of the forest. These results encourage further comparative insights into the mechanisms underlying high-frequency communication in the anuran of the temperate forests of South America.

Among the frogs of the temperate *Nothofagus* forests, members of the *Eupsophus* genus are unusual because after hatching, they remain in dark conditions in small water-filled holes or galleries near small streams or flooded holes ground (*E. roseus*, *E. calcaratus*, *E. nahuelbutensis*, *E. vertebralis* and *E. emiliopugini*) (Ubeda & Núñez 2006). For these features they are assigned to the endotrophic nidicolous guild as defined by Altig & Johnson (1989). The reproductive mode of *E. altor* diverges from that described from the *Eupsophus* species, because eggs and endotrophic tadpoles of this species hatch only in terrestrial environments. This unique reproductive pattern, that including terrestrial tadpoles, has only been reported in some species of the genera *Adenomera* and *Cycloramphus* (see Verdade 2005). We do not draw any large conclusions from the developmental pattern of *E. altor*, but we wish to point out that the developmental pattern of this frog is an appropiate model that can be used to studying the adaptations of the tadpoles to the terrestrial environment in the temperate forests of Southern Chile.

Distribution and conservation. Our results show that *E. altor* occupies a restricted distributional area (four sites up the moment) on the western slopes of the Coastal Range, between the mouths of the Valdivia and Lingue rivers. Other species of the *E. roseus* Group (*E. migueli*, *E. contulmoensis*, *E. nahuelbutensis*, *E. insularis*, *E. septentrionalis* and *E. queulensis*) also present small distributional areas in the Coastal Range and suggest a distributional pattern that contrasts with the broad distributional ranges of *E. roseus* and *E. calcaratus* (Nuñez *et al*. 1999, Nuñez *et al*. 2011). The origin of both distributional patterns is unknown, however, before proposing any hypothesis, the specific delimitation of each taxon and its exact spatial distribution should be defined in advance. Frogs of the temperate forests of South America has been studied extensively during the last 20 years, but our data suggest that current species account is yet an underestimate of the real diversity of frogs. In such sense, this paper shows that the integrative taxonomic approach could be an useful tool to discover cryptic diversity as well as a basis for prioritizing policies and conservation efforts.

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References

Altig, R. & Johnston, G.F. (1989) Guilds of anuran larvae: relationships among developmental modes, morphologies, and habitats. *Herpetological Monographs*, 3, 81–109.

Arch, V.S., Grafe T.U. & Narins, P.M. (2008) Ultrasonic signalling by a Bornean frog. *Biology Letters*, 4, 19–22.

Baum, D. & Donohue, M. (1995) Chosing among alternative “Phylogenetic” species concepts. *Systematic Botany*, 20, 560–573.

Bogart, J.P. (1970) Systematic problems in the amphibian family Leptodactylidae (Anura) as indicated by karyotypic analysis. *Cytogenetics*, 9, 369–383.

Cei, J.M. (1962) *Batracios de Chile*. Ediciones de la Universidad de Chile, Santiago, Chile, 128p.

Dayrat, B. (2005) Toward integrative taxonomy. *Biological Journal of the Linnean Society*, 85, 407–415.

De Queiroz, K. (1998) The general lineage concept of species, species criteria, and the process of speciation. A conceptual unification and terminological recommendations. *In*: Howard, D.J. & Berlocher, S.H. (Eds.), *Endless Forms: Species and Speciation*. Oxford University Press, England, pp. 57–75.

Di Castri, F. & Hajek, E. (1976) *Bioclimatología de Chile***.** Vicerrectoría Académica de la Universidad Católica de Chile, Santiago de Chile, Santiago de Chile, 128p.

Fabrezi, M. (2001) Patterns of carpal development among anuran amphibians. *Journal of Morphology*, 249, 210–220.

Feng, A., Naris, P., Chun-He, X., Wen-Yu, L., Zu-Lin, Y., Qiu, Q., Zhi-Min. X. & Jun-Xian S. (2005) Ultrasonic communication in frogs. *Nature*, 440, 333–336.

Formas, J.R. (1979) La herpetofauna de los bosques temperados de Sudamérica. *In*: Duellman, W. (ed.), *The South America Herpetofauna: its origin, evolution and dispersal*. Monograph, 7. Lawrence, Kansas, pp 341–369.

Formas, J.R. (1985) The voices and relationships of the Chilean frogs *Eupsophus migueli* and *E. calcaratus* (Amphibia: Anura: Leptodactylidae*). Proceedings of the Biological Society of Washington*, 98, 411–415.

Formas, J.R. (1989) A new species of *Eupsophus* (Amphibia: Anura: Leptodactylidae). *Proceedings of the Biological Society of Washington*, 98, 568–576.

Formas, J.R. (1991) The karyotypes of the Chilean frogs *Eupsophus emiliopugini* and *E. vertebralis* (Amphibia: Anura: Leptodactylidae). *Proceedings of the Biological Society of Washington*, 104, 7–11.

Formas, J.R. & Vera, M.A. (1980) Reproductive patterns of *Eupsophus roseus* and *E. vittatus*. *Journal of Herpetology*, 14, 11–14.

Formas, J.R., Lacrampe, S. & Brieva, S. (1991) Allozymic and morphological differentiation among three South American frogs, genus *Eupsophus* (*E. roseus*, *E. insularis* and *E. contulmoensis*). *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, 102, 57–60.

Frost, D.R. (2011) Amphibian Species of the World: an Online Reference. Version 5.5 (31 January, 2011). Electronic Database accessible at http://research.amnh.org/vz/ herpetology/ amphibia/American Museum of Natural History, New York, USA.

Goebel, A.M., Donnelly, J.M. & Atz, M.E. (1999) PCR primers and amplification methods for 12S ribosomal DNA, the control region, cytochrome oxidase I, and cytochrome *b* in bufonids and other frogs, and an overview of PCR primers which have amplified DNA in amphibians successfully. *Molecular Phylogenetics and Evolution*, 11, 163–199.

Gosner, K.L. (1960) A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica*, 16, 183–190.

Hammer, Ø., Harper, D. & Ryan, P. (2001) PAST: PAleontological STatistical software package for education and data analysis. v2.11 *Palaentological Electronica*, 4, 1–4.

Ibarra-Vidal, H., Ortiz, J.C. & Torres-Pérez, F. (2004) *Eupsophus septentrionalis* n. sp., nueva especie de Leptodactylidae (Amphibia) de Chile Central. *Boletín de la Sociedad de Biología de Concepción*, 75, 91–102.

Katoh, K., Kuma, K., Toh. H. & Miyata, T. (2005) MAFFT version 5: improvement in accuracy of multiple sequence alignment. *Nucleic Acids Research*, 33, 511–518.

Levan, A., Fredga, K & Sandberg, A. (1964) Nomenclature for centromeric position on chromosomes. *Hereditas*, 52, 201–220.

Lynch. J.P. (1971) Evolutionary relationships, osteology, and biogeography of leptodactyloid frogs. *University of Kansas, Museum of Natural History History, Miscellaneus Publication*, 53, 1–238.

Márquez, R., Penna, M., Marques, P. & do Amaral, J.P. (2005) Diverse types of advertisement calls in the frogs *Eupsophus calcaratus* and *E. roseus* (Leptodactylidae): a quantitative comparison. *Herpetological Journal*, 15, 257–263.

Mijares-Urrutia, A. (1998) Los renacuajos de los anuros altoandinos de Venezuela: morfología externa y claves. *Revista de Biología Tropical*, 46, 119–143.

Nuñez, J.J., Wood, N., Rabanal, F.E., Fontanella, F & Sites J.W. (2011) Amphibian phylogeography in the antipodes: refugia and postglacial colonization explain mitochondrial haplotype distribution in the Patagonian frog *Eupsophus calcaratus* (Cycloramphidae). *Molecular Phylogenetics and Evolution*, 58, 343–352.

Nuñez, J.J., Zárraga, A.M. & Formas, J.R. (1999) New molecular and morphometric evidence for the validation of *Eupsophus calcaratus* and *E. roseus* (Anura: Leptodactylidae) in Chile. *Studies on Neotropical Fauna and Environment*, 34, 50–155.

Nylander, J.A., Ronquist, F., Huelsenbeck, J.P. & Nieves-Aldrey, J.L. (2004) Bayesian phylogenetic analysis of combined data. *Systematic Biology*, 53, 47–67.

Orton, G.L. (1953) The systematics of vertebrate larvae. *Systematic Zoology*, 2, 63–75.

Padial, J.M. & De la Riva, I. (2009) Integrative taxonomy reveals cryptic Amazonian species of *Pristimantis* (Anura:

Strabomantidae). *Zoological Journal of Linnean Society*, 155, 97–122.

Padial, J.M., Castroviejo-Fisher, S., Köhler, J., Vilà, C., Chaparro, J.C. & De la Riva, I. (2009) Deciphering the products of evolution at the species level: the need for an integrative taxonomy. *Zoologica Scripta*, 38, 431–447

Posada, D. (2008) jModelTest: Phylogenetic model averaging. *Molecular Biology and Evolution*, 25, 1253–1256.

Posada, D. & Crandall, K.A. (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics*, 1, 817–818.

Posada, D. & Buckley, T.R. (2004) Model selection and model averaging in phylogenetics: advantages of the AIC and Bayesian approaches over likelihood ratio tests. *Systematic Biology*, 53, 793–808.

Ronquist, F. & Huelsenbeck, J.P. (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, 19, 1572–1574.

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Second Edition. Cold Spring Harbour Laboratory: Cold Spring Harbor Press.

Schlick-Steiner, B.C., Steiner, F. M., Seifert, B., Stauffer,C., Christian, E. & Crozier R.H. (2010) Integrative taxonomy: a multisource approach to exploring biodiversity. *Annual Review of Entomology*, 55, 421–438.

Song, J. & Parenti, L.R. (1995) Clearing and staining whole fish specimens for simultaneous demonstration of bone, cartilage and nerves.*Copeia*, 1995, 114–118.

Veblen, T.T. (2007) Temperate forests of Southern Andean Region. *In* Veblen, T.T., Orme, A & Young, K. (Eds.), *Physical Geography of South America*. Oxford University Press, UK, pp. 217–231.

Veloso, A., Celis-Diez, J., Guerrero, P.C., Méndez, M.A., Iturra, P., Simonetti, J.A. (2005) Description of a new *Eupsophus* species (Amphibia, Leptodactylidae) from the remnants of Maulino forest, central Chile. *Herpetological Journal*, 15, 159–165.

Verdade, V. (2005) Relações filogenéticas entre as espécies dos gêneros *Cyclorhamphus* Tschudi 1838 e *Zachenus* Cope 1866 (Anura, Leptodactylidae). PhD Thesis, Universidade de São Paulo, São Paulo, Brazil.

Wiley, E.O. (1981) *Phylogenetics. The Theory and Practice of Phylogenetic Systematics.* John Wiley & Sons, New York,USA, 439 p.

Zwickl, D.J. (2006) Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the Maximum-Likelihood criterion. PhD Dissertation. The Graduate School of the University of Texas, Austin, USA.