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Phylogenetic relationships of a Patagonian frog radiation, the *Alsodes* + *Eupsophus* clade (Anura: Alsodidae), with comments on the supposed paraphyly of *Eupsophus*

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Abstract

The frog clade composed of the alsodid genera *Alsodes* + *Eupsophus* is the most species-rich of the Patagonian endemic frog clades, including nearly 31 of the slightly more than 50 species of that region. The biology of this group of frogs is poorly known, its taxonomy quite complex (particularly *Alsodes*), and its diversity in chromosome number striking when compared with other frogs (collectively, there are species having $2n = 22$, $2n = 26$, $2n = 28$, $2n = 30$ or $2n = 34$). We present a phylogenetic analysis of this Patagonian frog clade based on mitochondrial and nuclear gene sequences. We sequenced five mitochondrial genes (*cytochrome b*, *cytochrome oxidase I*, *12S*, *16S*, *NADH dehydrogenase subunit 1*) with three intervening tRNAs, and fragments of three nuclear genes (*seven in absentia homolog 1*, *rhodopsin* exon 1, *RAG-1*), for a maximum of 6510 bp for multiple specimens from 26 of the 31 species. We recovered *Eupsophus* as polyphyletic, with *E. antartandicus*, *E. sylvaticus*, and *E. taeniatus* in Batrachylidae, in accordance with most previous hypotheses. Based on this result, we transfer *E. antartandicus* and *E. taeniatus* back to *Batrachyla*, and *E. sylvaticus* to *Hylorina* (resurrected from the synonymy of *Eupsophus*), remediating the paraphyly of *Eupsophus*. Our results strongly corroborate the monophyly of *Alsodes* + *Eupsophus* (sensu stricto), the individual monophyly of these genera, and the monophyly of the species groups of *Eupsophus*. They also show the non-monophyly of all non-monotypic species groups of *Alsodes* proposed in the past. Our results expose several taxonomic problems particularly in *Alsodes*, and to a lesser extent in *Eupsophus*. This phylogenetic context suggests a rich evolutionary history of karyotypic diversification in the clade, in part corroborating previous hypotheses. In *Alsodes*, we predict three independent transformations of chromosome number from the plesiomorphic $2n = 26$. All these, strikingly, involve increments or reductions of pairs of haploid chromosomes. Finally, the phylogenetic pattern recovered for *Alsodes* and *Eupsophus* suggests a trans-Andean origin and diversification of the group, with multiple, independent ingressions over *cis*-Andean regions.

The vast surface of Patagonia includes a striking diversity of environments, from very wet forest (> 3000 mm annual precipitation) in the west to an extreme dry steppe in the east, and landscapes that include the Andes and adjacent mountain systems up to 4700 m a.s.l. With nearly 60 species (Frost, 2011), the Patagonian amphibian fauna is not particularly diverse, but includes 10 endemic genera, some having one or a few species (*Calyptocephalella*, *Chaltenobatrachus*, *Insuetophrynus*, *Rhinoderma*, *Telmatobufo*), to as many as 18 (*Alsodes*). Knowledge of phylogenetic relationships of

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Patagonian frogs is still very sparse. Previous studies have focused on the relationships of some genera using a few exemplars, but resolution within them remains elusive.

Alsodes (18 species) and *Eupsophus* (13 species) are the two most species-rich frog genera of Patagonia. While species of *Eupsophus* are restricted to forested areas at southern latitudes, some species of *Alsodes* also reach the arid Andean slopes in central Chile and Argentina. Some interesting aspects of both genera are the extreme karyotypic variation in terms of chromosome numbers (there are species having diploid complements of 22, 26, 28, 30 or 34 chromosomes), fundamental numbers (FN), C-band patterns, and heteromorphic sex chromosomes (Veloso et al., 1981, 2005; Iturra and Veloso, 1989; Cuevas and Formas, 1996, 2003, 2005a,b). Additionally, *Eupsophus* has endotrophic larvae (with the exception of three species recently transferred from *Batrachyla* and *Hylorina* to *Eupsophus*; see Pyron and Wiens, 2011) that develop in water housed in small hollows in the ground of the forest or holes at the end of flooded tunnels (Formas and Vera, 1980; Úbeda and Nuñez, 2006).

The monophyly of *Alsodes* was partially tested by Correa et al. (2006), Formas et al. (2008), and Pyron and Wiens (2011). Correa et al. (2006) included five species (*A. nodosus*, *A. monticola*, *A. barrioi*, *A. tumultuosus*, and *Alsodes* sp.) in the context of a phylogenetic analysis using the mitochondrial genes *12S* and *16S*. That study was designed to examine the phylogenetic relationships of Chilean "leptodactylids" (*sensu lato*) and their position within Neobatrachia; it recovered *Alsodes* as paraphyletic with respect to *Eupsophus*. Formas et al. (2008) presented a small analysis to evaluate the relationships and identity of the controversial *Eupsophus coppingeri*, including six species (*A. australis*, *A. barrioi*, *A. coppingeri*, *A. kaweshkari*, *A. vanzolinii* and *A. verrucosus*). They analysed a 304 bp fragment of *cytochrome b*, recovered a monophyletic *Alsodes* (with *E. coppingeri* nested within it), and transferred *E. coppingeri* to *Alsodes*. Recently, Pyron and Wiens (2011) included eight species (*A. vanzolinii*, *A. nodosus*, *A. australis*, *A. tumultuosus*, *A. gargola*, *A. kaweshkari*, *A. monticola* and *A. barrioi*) in the context of a broad phylogenetic analysis across amphibians using sequences from GenBank. There is also morphological evidence that apparently supports the monophyly of *Alsodes*, such as the presence of pectoral round spiny patches in males (Barrio, 1970; Lynch, 1978) and the enlarged *cristae medialis* and *lateralis* of the humerus, also in males (Lynch, 1978). The taxonomy of *Alsodes* is quite complex and confusing, in particular the type species of the genus, *A. monticola* Bell, 1843, a taxon with uncertain identity and known only from the poorly preserved holotype (see Formas et al., 2008). Another problematic issue is the lack of diagnostic morphological characters, as some species are distinguished mainly on the basis of chromosome number (Formas et al., 2002; Cuevas and Formas, 2003, 2005b).

The monophyly of *Eupsophus* has been partially tested by Veloso et al. (2005, three species included), Correa et al. (2006, five species), and Formas et al. (2008, two species). Correa et al. (2006) found a monophyletic *Eupsophus* in the context of a reasonably broad taxon sampling of Hyloides. Formas et al. (2008) did not recover a monophyletic *Eupsophus*; however, the few exemplars and limited sequence evidence did not allow a rigorous test. On the other hand, there is evidence from reproductive biology and morphology that suggests the monophyly of *Eupsophus*. The genus has endotrophic-nidicolous tadpoles that develop in water housed in small hollows in the ground or holes at the end of flooded tunnels (Formas and Vera, 1980; Formas, 1992; Úbeda and Nuñez, 2006). Furthermore, *Eupsophus* has a peculiar character state in the hand muscles, a wide origin of muscles transversi metacarporum 1 and 2 in Metacarpus III and IV, respectively (Burton, 1998a). Finally, Vera Candioti et al. (2011) proposed additional synapomorphies from tadpoles: the connection of the processus muscularis to neurocranium through a ligament, and the absence or reduction of the third lower labial tooth row.

The studies that examined the phylogenetic relationships of the genus *Eupsophus* are those of Veloso et al. (2005), Correa et al. (2006) and Formas et al. (2008). The first of these was designed to study relationships of *E. queulensis* (*E. vertebralis*, *E. calcaratus*, and *E. queulensis* included) using a fragment of 319 bp of the mitochondrial gene *12S*. The analysis of Correa et al. (2006) was focused on exploring the relationships of Chilean "leptodactylids" among hyloids, including *E. emiliopugini*, *E. calcaratus*, *E. migueli*, *E. queulensis*, and *E. roseus*, using the mitochondrial genes *12S* and *16S*. Formas et al. (2008), in a study designed to place *A. coppingeri* using a fragment of the mitochondrial gene *cytochrome b*, included one species per group of *Eupsophus* (*E. emiliopugini* and *E. calcaratus*); the genus was not recovered as monophyletic. Of these analyses, only the taxon samplings of Veloso et al. (2005) and Correa et al. (2006) allowed a test of the monophyly of the *E. roseus* group. On the other hand, the monophyly of the *E. vertebralis* group (*E. emiliopugini* and *E. vertebralis*) was tested by Pyron and Wiens (2011), and was not recovered as monophyletic (see below).

Despite the above-mentioned references to the monophyly and phylogenetics of *Eupsophus*, a recent paper by Pyron and Wiens (2011) did not find *Eupsophus* monophyletic. Instead, *Eupsophus* was recovered as paraphyletic with respect to *Batrachyla antartandica*, *B. taeniata*, and *Hylorina sylvatica*. As a result, Pyron and Wiens (2011) transferred those species to *Eupsophus*. These authors also did not recover the *E. roseus* and *E. vertebralis* groups as monophyletic, because they found

E. calcaratus (of the *E. roseus* group) sister to *E. emiliopugini* (*E. vertebralis* group), and those species sister to the remaining *Eupsophus* (including the other species of the *E. vertebralis* group, remaining species of *E. roseus* group, and *Batrachyla antartandica*, *B. taeniata*, and *Hylorina sylvatica*).

Lynch (1978) presented one of the first pre-cladistic hypothesis including *Alsodes* and *Eupsophus* in a relatively broad context of taxon sampling, based mainly on osteological characters. In that paper, many topologies were proposed, none of which placed *Alsodes* particularly close to *Eupsophus*. Instead, *Alsodes* was closely related to *Telmatobius* or alternatively to *Insuetophrynus*. On the other hand, *Eupsophus* was related to *Thoropa* + *Batrachyla*, sister to *Hylorina* or sister to *Alsodes* + *Telmatobius*. Darst and Cannatella (2004) inferred relationships of hyloid frogs, and found *Alsodes* to be the sister group of *Telmatobius* (*Eupsophus* not included), using the mitochondrial genes *12S* and *16S*. Later, Correa et al. (2006), with a denser taxon sampling than Darst and Cannatella (2004), found a close relationship between both genera, recovering *Alsodes* as paraphyletic with respect to *Eupsophus* (multiple species of both genera included). The topology obtained by Correa et al. (2006) showed *A. nodosus* as sister taxon of *Eupsophus* plus the other species of *Alsodes*. Faivovich et al. (2005), Frost et al. (2006) and Grant et al. (2006) found both genera as sister taxa in different analyses including a much denser sequence sampling but minimal taxonomic sampling with only one exemplar for each genus included. Finally, as noted above, Pyron and Wiens (2011) found *Alsodes* to be sister to *Eupsophus* (although *Eupsophus* was paraphyletic with respect to *Hylorina* and two species of *Batrachyla*), based on available sequences in GenBank.

In this study we present a densely sampled phylogenetic analysis of *Alsodes* and *Eupsophus*. Our goals are to: (i) test the monophyly of both genera; (ii) test the sister group relationships of *Alsodes* and *Eupsophus;* (iii) infer relationships among species and test the monophyly of the proposed species groups; (iv) contribute to the resolution of taxonomic problems in *Alsodes* and *Eupsophus*; and (v) discuss implications of the results for our understanding of the evolution of karyology.

Material and methods

Taxon sampling

Multiple specimens of 13 of the 18 species of *Alsodes* were included in the analysis. They represent all species groups proposed so far: the monotypic *A. barrioi*, *A. nodosus*, and *A. norae* groups; and the *A. monticola* species group (*A. australis*, *A. coppingeri*, *A. gargola*, *A. hugoi*, *A. igneus*, *A. pehuenche*, *A. tumultuosus*, *A. vanzolinii*, *A. valdiviensis*, *A. verrucosus*), including the two subspecies of *A. gargola* (Cei, 1976). Among species not included are *A. laevis*, *A. monticola*, and *A. vittatus*, three species not collected since their original description (Cuevas and Formas, 2005a; Formas et al., 2008). Tissues of *A. montanus* and *A. kaweshkari* were not available. To our sample of *A. nodosus* we added sequences from GenBank produced by Correa et al. (2006).

The analysis included exemplars of the 13 species currently recognized in *Eupsophus* (including *E. antartandicus*, *E. taeniatus* and *E. sylvaticus*, recently transferred from *Batrachyla* and *Hylorina* to *Eupsophus* by Pyron and Wiens, 2011). Multiple specimens per species were generally included, in particular for *E. roseus* and *E. calcaratus*, which have a wide distribution and high levels of intraspecific variation. Sequences of *E. queulensis* produced by Veloso et al. (2005), Correa et al. (2006), and Méndez et al. (2006) were downloaded from GenBank. To our sequences of *E. sylvaticus* (previously in *Hylorina*), we added those produced by Correa et al. (2006). The inclusion of *E. antartandicus*, *E. taeniatus*, *E. sylvaticus* and *Batrachyla leptopus* allows testing of the monophyly of *Eupsophus* and *Batrachyla* as well.

Outgroups were selected on the basis of the results of Frost et al. (2006), Grant et al. (2006), and Pyron and Wiens (2011). Due to the extremely low support found by Pyron and Wiens (2011) for Alsodidae, we included species of most genera representing the families recovered by Pyron and Wiens (2011) as closely related to Alsodidae, which includes genera previously placed in Cycloramphidae (*sensu* Grant et al., 2006). Hylodidae was found by Frost et al. (2006) as related to some components of Cycloramphidae (*sensu* Grant et al., 2006), and recovered by Pyron and Wiens (2011) as sister to Alsodidae; we included *Crossodactylus schmidti* as an exemplar of this well supported family. Of Batrachylidae, we included two species of *Atelognathus* and *Batrachyla leptopus*, which are the three species employed by Pyron and Wiens (2011) to erect the family. Of the well supported families Ceratophryidae and Telmatobiidae, we included a single species of each (*Ceratophrys* and *Telmatobius*). We also included the two genera of Rhinodermatidae: *Insuetophrynus*, a monotypic genus related to *Alsodes* in many pre-cladistic papers (Barrio, 1970; Lynch, 1978), and *Rhinoderma darwinii*.

For the evaluation of character distribution around Alsodidae, we follow the topology of Frost et al. (2006), as modified by Grant et al. (2006), and that of Pyron and Wiens (2011). Although the hypotheses are quite different, none of them has high values of support for most groups, so we will employ both hypotheses for the discussion of character state distributions. We base the discussion of optimization at the *Alsodes* + *Eupsophus*

clade following these hypotheses, since the sampling of the present contribution was not designed to explore relationships among the families related to Alsodidae, as this would have demanded a much denser taxon sampling.

See Appendix 1 and Appendix S1 in Supporting Information for a complete list of GenBank numbers, voucher specimens and locality information. Most vouchers specimens from Instituto de Zoología, Universidad Austral de Chile (IZUA), which make up a large part of our analysis, were destroyed together with the complete IZUA Herpetological Collection during a fire that consumed the Emilio Pugín building on 3 December 2007, while the tissues where being processed. All remaining tissues and DNA extractions have been accessioned by IZUA (see Appendix 1).

Character sampling

The mitochondrial gene sequences produced for this project include portions of *cytochrome b*, *cytochrome oxidase I* (*COI*), *12S*, the intervening $tRNA^{Val}$, *16S*, and a fragment including the complete upstream section of *16S*, the intervening $tRNA^{Leu}$, *NADH dehydrogenase subunit 1* (*ND1*), and $tRNA^{Ile}$. The nuclear gene sequences produced include portions of *seven in absentia homolog 1*, *rhodopsin* exon 1 and *RAG-1*. All the primers employed are those of Faivovich et al. (2005), with the addition of 16S-frog and tMet-frog (fragment of $16S + tRNA^{Leu} + ND1 + tRNA^{Ile}$; Wiens et al., 2005).

No phenotypic dataset is available for *Alsodes* and *Eupsophus*. For this reason we make only general comments about a few morphological characters whose taxonomic distributions suggest that they are putative synapomorphies of some of the major clades. Similarly, our study indicates some noteworthy transformations in chromosome number during the evolutionary history of the *Alsodes* + *Eupsophus* clade. Understanding of chromosome homology of the chromosomes involved on these transformations is very poor, hence we only mention general changes involving chromosome numbers, without establishing transformation hypotheses involving specific chromosomes.

DNA extraction and sequencing

Whole cellular DNA was extracted from ethanol-preserved tissues with the DNeasy (Qiagen, Valencia, CA, USA) isolation kit. Amplification was carried out in a 25-μL volume reaction using puRe Taq Ready-To-Go PCR beads (Amersham Biosciences, Piscataway, NJ, USA) or a master mix using Fermentas Taq Polymerase and reagents. For all the amplifications, the PCR program included an initial denaturing step of 30 s at 94 °C, followed by 35 (mitochondrial gene fragments) or 45 (nuclear gene fragments) cycles of amplification (94 °C for 30 s; 48–64 °C for 30 s; 72 °C for 60 s), with a final extension step at 72 °C for 6 min. Polymerase chain reaction (PCR) amplification products were desalted and concentrated using either Ampure (Agencourt Biosciences, Beverly, MA, USA) or with EXO I/SAP, and labelled with fluorescent dye-labelled terminators (ABI Prism Big Dye Terminators ver. 1.1 cycle sequencing kits, Applied Biosystems, Foster City, CA, USA). The products were sequenced with an ABI 3730XL (Applied Biosystems) or sequenced by Macrogen. All samples were sequenced in both directions to check for potential errors. Chromatograms obtained from the automated sequencer were read and contigs were made using the sequence-editing software Sequencher 3.0 (Gene Codes, Ann Arbor, MI, USA). Complete sequences were edited with BioEdit (Hall, 1999). See Appendix 1 for a list of specimens and locality data.

Phylogenetic analysis

The phylogenetic analyses included treatment of sequences both as dynamic and static homology hypotheses. The consideration of sequences as dynamic homologies simultaneously with tree searches has been discussed and justified by Wheeler (1996, 2002) and De Laet (2005). The use of static alignments (multiple alignments) independently of tree searches is the most common approach in molecular phylogenetics, regardless of the omnipresent and often ignored problem of the lack of an optimality criterion to choose among competing alignments. We also performed a multiple alignment followed by static parsimony and Bayesian analyses.

The rationale for using parsimony as an optimality criterion was advanced by Farris (1983), and discussed more recently by Goloboff (2003) and Goloboff and Pol (2005). Phylogenetic analyses using direct optimization were performed with POY4.1.1 (Varón et al., 2009, 2010), using equal weights for all transformations (substitutions and insertion/deletion events). Sequences of *12S*, *16S*, *ND1* and intervening tRNAs (valine, leucine, isoleucine) were preliminarily delimited in sections of putative homology (Wheeler et al., 2006), and equal-length sequences of nuclear protein-coding genes were considered as static alignments to accelerate the searches. Searches were performed using the command "Search". This command implements a search driven by building Wagner trees using random addition sequences (RAS), tree bisection–reconnection (TBR) branch swapping followed by ratchet (Nixon, 1999), and tree fusing (Goloboff, 1999). The command (Search) stores the shortest trees of each independent run and does final tree fusing using the pooled trees as a source of topological diversity. Ten 12-h runs of Search were implemented in parallel at the American Museum of

Natural History Cluster using 16 processors for a total of 1920 CPU hours. The resulting trees were submitted to a final round of swapping using iterative pass optimization (Wheeler, 2003a).

We performed a multiple alignment with ClustalX (Thompson et al., 1997) under default parameters. For the phylogenetic analysis we employed TNT (Willi Hennig Society edition; Goloboff et al., 2008) using New Technology with a search level 100 and requesting 100 independent hits of the best length, submitting the resulting trees to a round of TBR swapping. Two analyses were conducted, considering alternatively gaps as a fifth state and as missing data. Support estimation was performed by generating 50 RAS + TBR per replicate, keeping only one tree, for a total of 1000 replicates of parsimony jackknife, with 0.36 of removal probability (Farris et al., 1996). Trees were edited with WinClada (Nixon, 2002). Parsimony jackknife absolute frequencies were also estimated from the implied alignment (Wheeler, 2003b) with TNT, under the same parameters reported above for the static alignment.

Finally, we also performed Bayesian analysis. The multiple alignment employed for this analysis is the same used for the static parsimony analysis with TNT models for each gene being chosen with jModelTest ver. 0.1.1 (Posada, 2008), a modification of Modeltest (Posada and Crandall, 1998). The first, second, and third codon positions were treated as separate partitions for each protein-coding gene. Additionally, the regions of *12S*, $tRNA^{val}$, *16S*, $tRNA^{leu}$ and $tRNA^{ile}$ were also treated as separated partitions for model selection. The Akaike information criterion (AIC) was used to select the best fitting model for each gene (Pol, 2004; Posada and Buckley, 2004). Bayesian analyses were performed in MrBayes 3.1 (Huelsenbeck and Ronquist, 2001). Analyses consisted of four runs, each consisting of two replicate Monte Carlo Markov chains. Each run used four chains and default settings of priors (dirichlet for substitution rates and state frequencies, uniform for the gamma shape parameter and proportion of invariable sites, all topologies equally likely *a priori*, and branch lengths unconstrained:exponential). Three analyses using 10 million generations were first performed (with a burn-in fraction of 0.25). Evaluation of resulting parameters (using Tracer ver. 1.5, A. Rambaut and A. J. Drummond, available from http://beast.bio.ed.ac.uk/Tracer) showed that likelihood values appeared to stabilize before 4 million generations in some replicates. Consequently, we performed an additional run of 20 million generations, sampling every 1000 generations, and trees from the first 4 million generations were discarded as burn-in in this analysis. In addition, stationarity was evaluated considering (i) the similarity in topologies, posterior probabilities, and likelihoods between trees; and (ii) the average standard deviation of split frequencies between runs within each pair of searches. The 16 million post-burn-in trees had a mean log-likelihood of –65 828.88.

Results

The analysis using direct optimization in POY resulted in 24 equally parsimonious trees of length 14 232 using equal weights for substitutions and insertion/deletion events. *Eupsophus* as recently redefined by Pyron and Wiens (2011) is polyphyletic, with *E. antartandicus*, *E. sylvaticus* and *E. taeniatus* recovered in a clade with *Batrachyla leptopus* (Fig. 1). The remaining species of *Eupsophus*, and *Alsodes*, are each monophyletic (with 100% jackknife support), and these two genera are sister taxa (100% jackknife support). Most clades within the ingroup have parsimony jackknife support values ≥ 95% with a few exceptions discussed below. Strict consensus for outgroups is shown in Fig. 1, and one of the 24 topologies for the ingroup is shown in Fig. 2.

Within *Alsodes*, we found *A. monticola* group paraphyletic with respect to the monotypic *A. barrioi*, *A. norae* and *A. nodosus* groups. Our results also indicate the non-monophyly of previous groupings suggested by Cei (1980) and Cuevas (2008), based on the grounds of the nuptial pad ornamentation on the first digit, and karyotype and distribution, respectively.

Eupsophus contains the two major classic clades (i.e. excluding *Batrachyla antartandica*, *B. taeniata* and *Hylorina sylvatica*), corresponding to the *E. vertebralis* and the *E. roseus* groups, whose monophyly is corroborated in our analyses (Fig. 2), both with 100% jackknife support. Our results indicate that *Eupsophus queulensis* Veloso et al., 2005 is a junior synonym of *E. septentrionalis* Ibarra-Vidal et al., 2004 (see Appendix 2 for further discussion). Additionally, we recovered *E. calcaratus* as polyphyletic, with an exemplar identified as *E. calcaratus* from Termas de Epulafquen (also known as Termas de Lahuenco, Neuquén Province, Argentina) nested within the Chilean samples of *E. roseus* (see Appendix S3 for discussion).

In the ingroup, conflict is restricted to a polytomy composed of *A. igneus* + *A. barrioi* + *A. g. gargola* (including unidentified specimens, and one assigned to *A. australis*) + *A. g. neuquensis* + *A. norae* (Figs 2 and 3). This group accounts for the 24 alternative topologies of the direct optimization analysis. Two of them are at the species level (see Fig. 3), while the remaining topologies are alternative positions of the specimens attributed here to *A. g. gargola* (see Appendix 2 for further discussion).

For the analysis using static alignments (TNT) and considering gaps as fifth state, we obtained 108 equally parsimonious trees of length 14 729. The analysis performed with gaps considered as missing data resulted

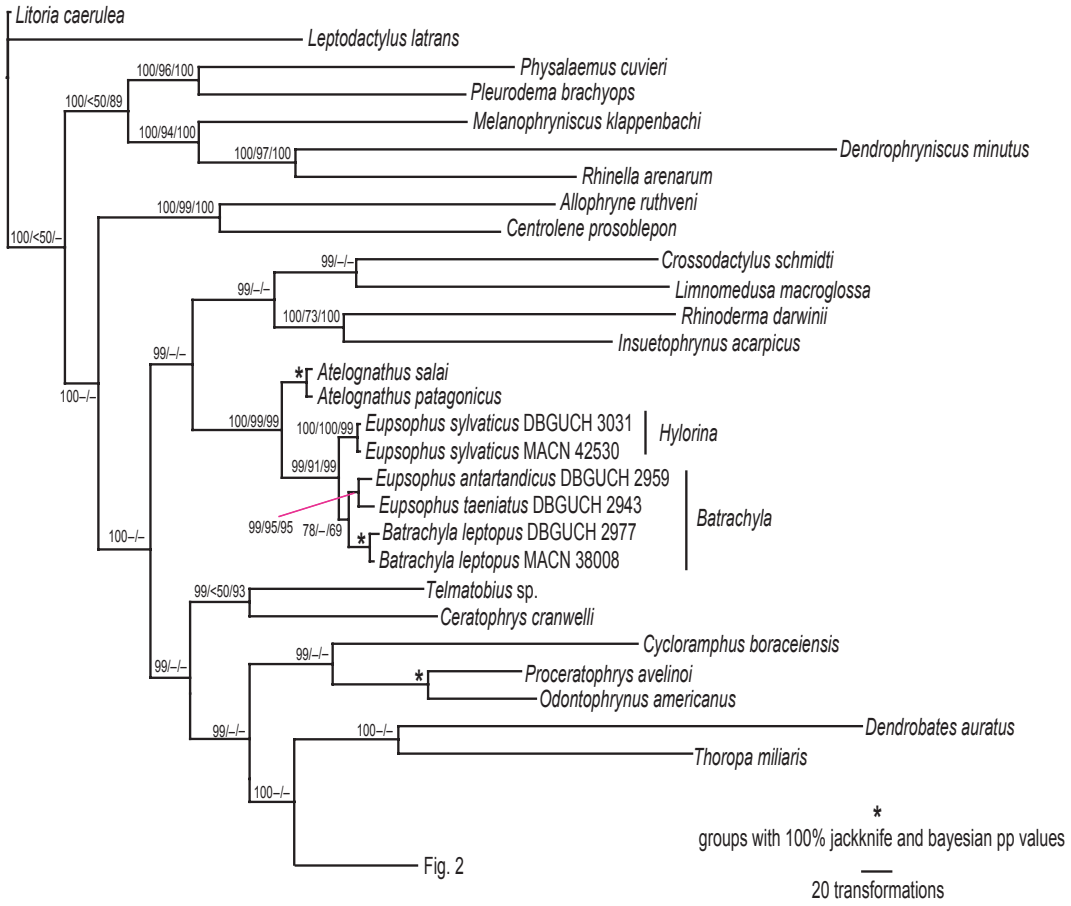

Fig. 1. Strict consensus of the 24 most parsimonious trees, found using direct optimization, under equal weights for all transformations (substitutions and insertion/deletion events). Numbers on nodes from left to right and separated by "/" indicate: (i) parsimony jackknife absolute frequency estimated for the implied alignment of one of the 24 most parsimonious trees; (ii) parsimony jackknife absolute frequency estimated for the static alignment analysed with parsimony in TNT with gap as fifth state; (iii) Bayesian posterior probability from the analysis of the static alignment. Asterisks indicate groups with 100% for both parsimony jackknife frequencies and Bayesian posterior probability; "–" denotes groups not recovered in the analysis with the static alignment on TNT or in the Bayesian analysis. Branch lengths are proportional to the number of unambiguous parsimony transformations; not all loci are available for all terminals. The two generic changes proposed in this paper are indicated on the right side of the tree. See Appendix 1 for complete locality data.

in an identical ingroup species level topology to the two previously mentioned analyses, with 36 equally parsimonious trees of length 14 278.

Bayesian analysis of the static matrix recovered similar results for the ingroup, with two exceptions (see Appendix S2 for resulting Bayesian topology and the Bayesian posterior probabilities). One of the discrepancies is the presence of a different resolution within the clade of *Alsodes igneus* + *A. barrioi* + *A. g. gargola* + *A. g. neuquensis* + *A. norae*. In this case, *A. norae* is sister taxon of a group (with 91% posterior probability, Pp) composed of *A. barrioi* + *A. igneus* (85% Pp) and *A. g. gargola* + *A. g. neuquensis* (90% Pp). The second difference with the Bayesian analysis is the position of *E. septentrionalis* + *E. queulensis*. This clade is recovered as sister of *Eupsophus* sp. 2 + *E. roseus* (96% Pp), instead of sister to this clade plus *E. nahuelbutensis* + *E. contulmoensis* as depicted in Fig. 2. Nevertheless, the association of *E. contulmoensis* + *E. nahuelbutensis* with *Eupsophus* sp. 2 + *E. roseus* has a moderate to low jackknife support in the parsimony analysis (73 and 88% for the static alignment with TNT considering gaps as fifth state and for the direct optimization analysis, respectively; Fig. 2).

Discussion

Outgroup relationships

The topology among outgroups is not congruent with previous hypotheses (Correa et al., 2006; Frost et al., 2006; Grant et al., 2006; Pyron and Wiens, 2011). While the sampling was not designed to resolve relationships outside of the *Alsodes* + *Eupsophus* clade, there are some interesting points that merit comment.

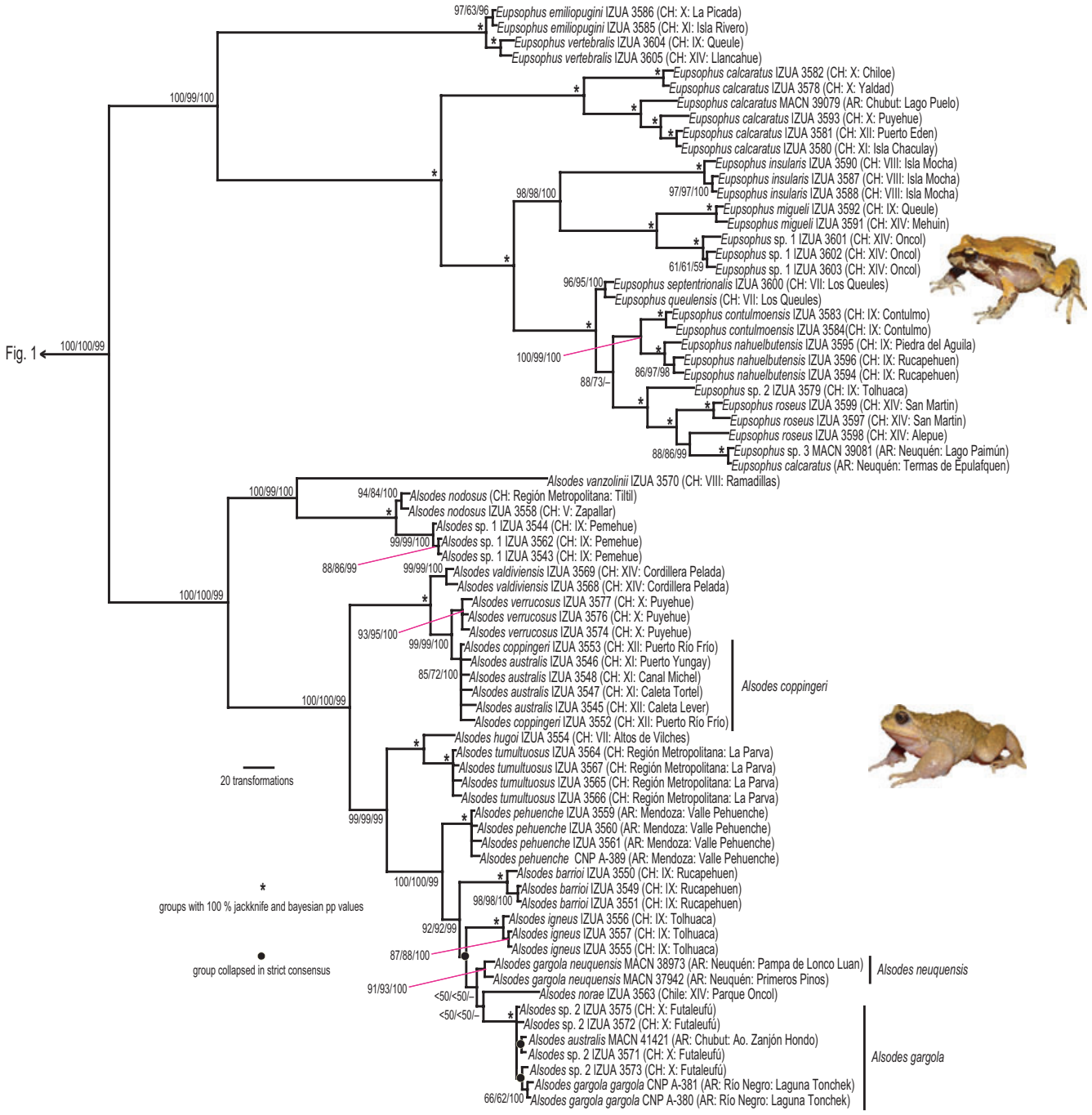

Fig. 2. Topology of the *Alsodes* + *Eupsophus* clade in one of the 24 most parsimonious trees found using direct optimization, under equal weights for all transformations (substitutions and insertion/deletion events). Numbers on nodes from left to right and separated by "/" indicate (i) parsimony jackknife absolute frequency estimated for the implied alignment of one of the 24 most parsimonious trees; (ii) parsimony jackknife absolute frequency estimated for the static alignment analysed with parsimony in TNT with gap as fifth state; (iii) Bayesian posterior probability from the analysis of the static alignment. Asterisks indicate groups with 100% for both parsimony jackknife frequencies and Bayesian posterior probability; "–" denotes groups not recovered in the analysis with the static alignment on TNT or in the Bayesian analysis. Branch lengths are proportional to the number of unambiguous parsimony transformations; not all loci are available for all terminals. Abbreviations: AR, Argentina; CH: Chile; roman numerals indicate the administrative regions of Chile. For the sake of clarity, taxonomic changes proposed in this paper for *Alsodes* are indicated on the right side of the tree. See Appendix 1 for complete locality data.

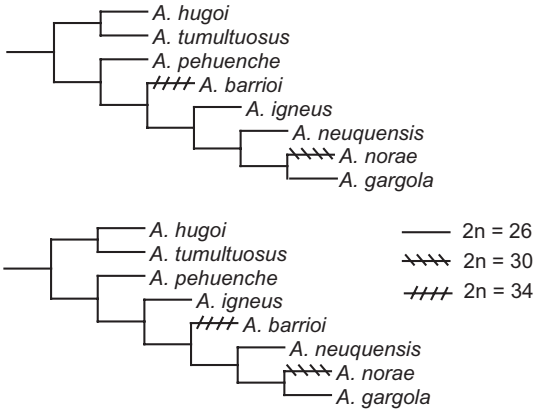

Fig. 3. Condensed topologies representing the species as terminals (and not the individual specimens sampled), with the taxonomy proposed in this paper for a clade of *Alsodes*. We show two of the 24 topologies found in the analysis at the species level (remaining topologies are alternative positions of the specimens within *A. gargola*). Chromosome numbers are optimized over the branches. See text for literature sources of chromosome number data, with the exception of *A. neuquensis* (B.L.B., pers. obs.).

As stated above, we recovered *Eupsophus sylvaticus* sister to *E. antartandicus*, *E. taeniatus* and *Batrachyla leptopus* (99% jackknife support). Based on this result, and the discussion detailed in the next section, we transfer *E. antartandicus* and *E. taeniatus* back to *Batrachyla*, and *E. sylvaticus* back to *Hylorina* (which is resurrected from the synonymy of *Eupsophus*). Bayesian analysis reached identical topological results (99% Pp for *Hylorina* + *Batrachyla* species), while the analysis of the static matrix on TNT, with gaps considered as fifth state, recovered *H. sylvatica* in a polytomy with *B. antartandica* + *B. taeniata*, and *B. leptopus* (91% jackknife support). The analysis with TNT considering gaps as missing data recovered the same topology as POY and Bayesian analysis, with 80% jackknife support for *Hylorina* + *Batrachyla* species, and very low support (< 50%) for the monophyly of our exemplars of *Batrachyla*. Finally, *Insuetophrynus* is recovered as sister taxon of *Rhinoderma* in all the analyses performed: direct optimization (100% jackknife support; Fig. 1), TNT (61 and 73% jackknife support with gaps considered as missing data and as fifth state, respectively), and Bayesian (100% Pp).

On the supposed paraphyly of Eupsophus

Pyron and Wiens (2011) found *Eupsophus* paraphyletic with respect to *Batrachyla antartandica*, *B. taeniata*, and *H. sylvatica*, and transferred those species to *Eupsophus*. However, with the exception of advancing a new taxonomic arrangement, this heterodox result went undiscussed, without any mention of the large amount of evidence supporting the monophyly of *Eupsophus* as previously delimited. When analysing the sequences from GenBank employed by Pyron and Wiens (2011), it is evident that their topology can be attributed to the inclusion of chimeric sequences under the names of *Batrachyla taeniata* and *Hylorina sylvatica*.

The terminal labelled *Batrachyla taeniata* by Pyron and Wiens (2011) is composed of sequences of *E. calcaratus*, *B. taeniata*, and *Homo sapiens*. The nuclear sequences *CXCR4*, *NCX1*, *RAG-1*, *SLC8A3* included by Pyron and Wiens (2011), produced and attributed to *Batrachyla taeniata* by Roelants et al. (2007), are here considered to be product of a misidentification of *E. calcaratus*. This assertion is based on comparison of the fragments from GenBank of *12S*, *16S* and *ND1*, produced from the same voucher by Van Bocxlaer et al. (2009) with our sequences from six specimens of this species. This comparison shows the smallest uncorrected *p* distance with the sequences of *E. calcaratus* (Tables 1 and 2). Note that we did not sequence the nuclear fragments employed by Pyron and Wiens (2011), so no comparison of those specific sequences with ours was carried out (the region of their *RAG-1* sequences do not overlap with ours). Nevertheless, the fact that different mitochondrial fragments (GenBank accession numbers FJ882752 and FJ882753) produced from the same voucher (MVZ 164828) are assignable to *E. calcaratus* is considered here enough evidence to infer that those nuclear sequences belong to *E. calcaratus* as well. This voucher corresponds to a specimen collected in the 1970s in Puerto Blest, Argentina (data obtained by consulting the online catalogue of the MVZ, Museum of Vertebrate Zoology at the University of California at Berkeley, inasmuch as in Roelants et al., 2007 and Van Bocxlaer et al., 2009 only "Argentina" was provided as locality data). At this locality, both *Eupsophus calcaratus* and *B. taeniata* are present (Barrio and Rinaldi de Chieri, 1971; Úbeda, 2000), so it is possible that the source of error is due to a misidentified specimen or a tissue incorrectly labelled. The other sequences of *B. taeniata* employed by Pyron and Wiens (2011) are fragments of *12S*, *16S*, and *cytochrome b*, all produced by different authors. From these, the only problematic one is that of *cytochrome b* (GenBank AY389147). When a BLAST search is performed, it results in identity values of 94% for *H. sylvatica* (AY389143); 92% for several *Homo sapiens* sequences, *E. migueli* (AY389145), and *E. contulmoensis* (AY389149); and 91% for *E. insularis* (AY389150) and *E. roseus* (AY389144), among several additional *H. sapiens* sequences. Similarly, the *cytochrome b* sequence of *E. nahuelbutensis* (AY389141) results in an identity value of 99% with *H. sapiens* sequences. It is evident that all these sequences are contaminated with *H. sapiens*, and should not be used in future studies. It must be noted that these sequences of *cytochrome b* of *E. contulmoensis*, *E. insularis*, *E. migueli*, *E. nahuelbutensis*,

Table 1
Percentage uncorrected pairwise distances between the overlapping regions *12S*, *tRNA^{val}*, and contiguous region of *16S* from our six specimens of *Eupsophus calcaratus* and the sequences erroneously attributed to *Batrachyla taeniata* by Van Bocxlaer et al. (2009). The number of *B. taeniata* is the GenBank accession number of the sequence

	1	2	3	4	5	6	7
1-*E. calcaratus* IZUA 3581 (CH: XII: Puerto Eden)	–						
2-*E. calcaratus* IZUA 3582 (CH: X: Chiloe)	2	–					
3-*E. calcaratus* IZUA 3593 (CH: X: Puyehue)	0.7	2.1	–				
4-*E. calcaratus* IZUA 3578 (CH: X: Yaldad)	2.1	0.1	2.2	–			
5-*E. calcaratus* IZUA 3580 (CH: XI: Isla Chaculay)	0.2	2.1	0.8	2.2	–		
6-*E. calcaratus* MACN 39079 (AR: Chubut: Lago Puelo)	0.9	2	0.9	2	0.8	–	
7-*B. taeniata* FJ882752	1	2.2	0.5	2.3	0.9	1	–

Table 2
Percentage uncorrected pairwise distances between the overlapping regions of *16S*, *tRNA^{leu}*, *ND1* and *tRNA^{ile}* from five specimens of *Eupsophus calcaratus* (specimen from Isla Chaculay, Chile, unavailable for part of this region) and the sequences erroneously attributed to *Batrachyla taeniata* by Van Bocxlaer et al. (2009). The number of *B. taeniata* is the GenBank accession number of the sequence

1-*E. calcaratus* IZUA 3581 (CH: XII: Puerto Eden)	–					
2-*E. calcaratus* IZUA 3582 (CH: X: Chiloe)	3.8	–				
3-*E. calcaratus* IZUA 3593 (CH: X: Puyehue)	1.3	4.4	–			
4-*E. calcaratus* IZUA 3578 (CH: X: Yaldad)	3.7	0.2	4.3	–		
5-*E. calcaratus* MACN 39079 (AR: Chubut: Lago Puelo)	1.1	3.6	1.8	3.5	–	
6-*B. taeniata* FJ882753	1.2	4.3	0.6	4.2	1.7	–

E. roseus and *H. sylvatica* were also employed by Pyron and Wiens (2011), probably influencing the topology for *Eupsophus*, which is not congruent with that of Correa et al. (2006) and the one obtained here. Finally, most sequences included by Pyron and Wiens (2011) under the name of *E. calcaratus* must be assigned to *E. roseus* (see Appendix S3 for discussion).

Similarly, sequences named *Hylorina sylvatica* by Pyron and Wiens (2011) are a chimera composed of *H. sylvatica*, *Insuetophrynus acarpicus*, and *Homo sapiens*. As discussed previously, at least a fragment of the sequence of *cytochrome b* is a contamination with *H. sapiens*. The other sequence attributed to *H. sylvatica* that deserves discussion is that of *12S* (GenBank AY389153). The most similar sequences are those of *I. acarpicus*, for which we found values of 1.3 and 1.5% of uncorrected *p* distance when compared with that of Correa et al. (2006) and the one produced here, respectively. Without any doubt it is not *H. sylvatica*, since the values of uncorrected *p* distance are of 12.1 and 12.3% when compared with the sequences of *H. sylvatica* of Correa et al. (2006) and our sequences, respectively. The source of these differences is not clear. They may be due to intraspecific variation, or problems with sequencing (note that this region is identical in both our sequence of *I. acarpicus* and that of Correa et al.). This last sequence, belonging to *I. acarpicus* and incorrectly attributed to *Hylorina*, was unfortunately the only fragment assigned to *Hylorina* included by Frost et al. (2006) and Grant et al. (2006), and was recovered in both analyses as sister taxon of *Alsodes* + *Eupsophus*. It is not surprising that the position of *Hylorina* in those studies is not congruent with the results of Correa et al. (2006) and the one obtained here, as theirs turns to be a mislabelled *I. acarpicus*. All problematic sequences from *Batrachyla*, *Eupsophus* and *Hylorina* (AY389141, AY389144–AY389145, AY389147–AY389149, AY389153) are unpublished sequences produced by José J. Nuñez. Upon the findings reported here, he has withdrawn them from GenBank.

Finally, the sequences employed by Pyron and Wiens (2011) for *Batrachyla antartandica* are two fragments (*12S* and *16S*) produced by Correa et al. (2006), which are the same as those employed here. *Batrachyla antartandica* is recovered nested in *Eupsophus* with the topology *H. sylvatica* + (*B. antartandica* + *B. taeniata*) by Pyron and Wiens (2011). This result may be due to the fact that the sequences of *B. antartandica* are of the same gene region of those correctly identified as *B. taeniata* and partly as *H. sylvatica*. When the problematic sequences incorrectly assigned to *B. taeniata* and *H. sylvatica* are not included, the species of *Batrachyla* and *H. sylvatica* are recovered as monophyletic (Correa et al., 2006; this paper). Clearly Pyron and Wiens's (2011) results involving at least the former Cycloramphidae *sensu* Grant et al. (2006) need to be re-evaluated.

Relationships of Batrachyla *and* Hylorina

The topologies recovered here are similar to those found by Correa et al. (2006), who recovered *H. sylvatica* as either sister of the clade of *B. antartandica*, *B. leptopus* and *B. taeniata* (in the maximum likelihood tree), or nested within *Batrachyla* (in the parsimony

analysis). In conclusion, all the analyses performed here, and that of Correa et al. (2006), agree on the monophyly of *H. sylvatica* and *Batrachyla* with high support values.

It has not escaped us that the monotypic *Hylorina* Bell, 1843 and *Batrachyla* Bell, 1843 could well be considered synonyms according to our topological results and those of Correa et al. (2006). This would stem from *Hylorina* being nested within *Batrachyla* (Correa et al., 2006), in which case the taxonomic changes would be mandatory, or from the sister group relationship of the monotypic *Hylorina* with *Batrachyla*, in which case the synonymy would just be justified on the grounds of the low information content of avoidable monotypic groups (e.g. Frost et al., 2006). The present analysis includes three of the five currently recognized species of *Batrachyla* (missing species are *B. nibaldoi* and *B. fitzroya*), hence we prefer to defer any taxonomic decision until a more complete taxonomic sampling is available.

The Insuetophrynus + Rhinoderma *clade*

The result of a sister group relationship of the Patagonian *Insuetophrynus* and *Rhinoderma* was also obtained by Correa et al. (2006) and Pyron and Wiens (2011), but it went largely undiscussed in both studies. For *Insuetophrynus*, Barrio (1970) reported the epicoracoids fused at the midline and forming a well developed crest at this level. *Rhinoderma* presents epicoracoids fused throughout their length (Kaplan, 2004). Following Lynch (1971, 1978) this type of pectoral girdle is not present in the different groups that were alternatively related to *Rhinoderma* and/or *Insuetophrynus* (Correa et al., 2006; Frost et al., 2006; Grant et al., 2006; Pyron and Wiens, 2011), so the fusion of epicoracoids is a putative synapomorphy for Rhinodermatidae (composed of *Insuetophrynus* and *Rhinoderma*).

Major patterns in Alsodes and Eupsophus

Alsodes is monophyletic, as previously reported by Formas et al. (2008) and Pyron and Wiens (2011). As discussed earlier, *Eupsophus* was found to be polyphyletic in the sense of Pyron and Wiens (2011), since *Batrachyla antartandica*, *B. taeniata* and *Hylorina sylvatica* were recovered with *Batrachyla leptopus*, and distantly related to the species commonly included in *Eupsophus*. These and *Alsodes* are strongly supported as sister taxa, a relationship previously obtained in the analyses of Faivovich et al. (2005), Frost et al. (2006) and Grant et al. (2006).

Our results show important similarities to the topology obtained by Correa et al. (2006) in their parsimony analysis (note that they did not show the consensus of their 26 optimal trees; they stated that these trees "showed similar topologies in the case of major clades, and among those trees the differences involved mainly terminal nodes and the relative positions of some clades within Hyloidea", so it is uncertain if the coincidences reported here actually are shared with their strict consensus). The only important exception is that Correa et al. (2006) found *Alsodes* paraphyletic with respect to *Eupsophus*, with *A. nodosus* as sister taxon of *Eupsophus* + their remaining species of *Alsodes* (*A. "monticola"*, *A. tumultuosus*, *A. barrioi*, and *Alsodes* sp.). This result of Correa et al. (2006) is probably due to a less dense taxonomic and character sampling, as in our analysis *A. nodosus* (the sequence produced by them) is recovered in a group composed of (*A. vanzolinii* + (*A. nodosus* + an undescribed species of *Alsodes*)), sister to the remaining species of *Alsodes*. However, the internal composition of their parsimony tree of *Eupsophus* and *Alsodes* (with the exception of *A. nodosus*) is identical to the one recovered here when pruning terminals not included by Correa et al. (2006). The only apparent difference comes from the specimen identified as *A. monticola* in Correa et al. (2006), which, based on arguments discussed in Appendix S3, we consider provisionally as *A. verrucosus*.

Alsodes

Cei (1980) recognized two species groups in *Alsodes*; one with "simple" cornified nuptial pad ornamentation on the first digit, and the other having a more "complex" spiny structure. In the former group, Cei (1980) included *A. gargola* and *Alsodes* sp. (using the name *A. monticola*, based on material from two localities from Neuquén Province, Argentina; the identity of this material is under study). In the group with "complex" spiny ornamentation, Cei (1980) included *A. pehuenche*, *A. nodosus* and "other Chilean species"; known Chilean species of *Alsodes* at that time were *A. laevis*, *A. montanus* and *A. tumultuosus*. Cuevas and Formas (2001) included in the group with complex ornamentations *A. australis*, *A. hugoi* and *A. kaweshkari*. On the other hand, *A. valdiviensis* was included by Formas et al. (2002) in the group of "simple" spiny ornamentations. Our results show that these groups are polyphyletic, with *A. nodosus* and *A. pehuenche* of the "complex" spiny group distantly related, as it happens in the "simple" spiny group, with *A. valdiviensis* closely related to *A. verrucosus*, but in a clade distant from *A. gargola*. It is relevant to consider that Cei (1980) did not define precisely the meaning of complex and simple ornamentations. The only comment offered by him is that the species having complex spiny ornamentations have strong black thorny excrescences. On this basis, it can be inferred that the group with simple ornamentations has poorly keratinized nuptial pads on the thumb, as at that time was believed to occur in *A. gargola* and *A. monticola* (probably in reference to *A. verrucosus*).

Cuevas and Formas (2001) and Formas et al. (2002) did not comment on the character states nor redefine them. Therefore it is evident that they need to be carefully defined and, if actually different states, their taxonomic distributions need to be reassessed before discussing them in a phylogenetic context.

Formas and Vera (1983) recognized groups based on chromosome numbers $2n = 22$ (the *A. nodosus* group, monotypic), $2n = 34$ (the *A. barrioi* group, monotypic) and $2n = 26$ (the *A. monticola* group, five species at that time, now 11). A fourth monotypic group was recognized for *A. norae* with $2n = 30$ (Formas et al., 2008). Our results indicate that the 26-chromosome group (*A. monticola* group) is paraphyletic with respect to the monotypic *A. nodosus* ($2n = 22$) and *A. barrioi* groups ($2n = 34$). Additionally, *A. norae* ($2n = 30$) is nested within species of $2n = 26$. Most notably, the polyphyly of the exemplars of *A. australis* and the para/polyphyly of *A. gargola* suggests important taxonomic problems in this genus. See Appendix S3 for a discussion of these points. For reasons developed there and in Appendix 2, in the remainder of this paper, we consider *Alsodes neuquensis* Cei, 1976 a full species.

The groups erected by Cuevas (2008) on grounds of karyotype and distribution are not monophyletic. The ''Coastal Range'' group (*A. barrioi*, *A. nodosus*, *A. norae*, *A. valdiviensis* and *A. vanzolinii*) is polyphyletic since most components are only distantly related (Fig. 2) and nested within the group that Cuevas (2008) characterized as presenting an Andean distribution (32° to 48°S) and conservative in terms of chromosome morphology ($2n = 26$; FN = 52).

When our topology of *Alsodes* is compared with that of Pyron and Wiens (2011), there are some differences and points that deserve discussion. First, *A. nodosus* and *A vanzolinii* are not recovered as sister taxa, a result obtained here if we prune *Alsodes* sp. 1 (not sampled by them). This could be attributed to differences of taxon and character sampling. *Alsodes vanzolinii* is highly autapomorphic, having a notably long branch (see Fig. 2), and limited evidence was included by Pyron and Wiens (only a fragment of 304 bp of *cytochrome b*) for this species. Second, their sequence of *A. australis* was produced by Formas et al. (2008) and is here considered to belong to *A. coppingeri* (see Appendix S3). Third, their sequences of *A. kaweshkari* are identical to specimens from Futaleufu (Chile), considered here to belong to *A. gargola*, a situation that raised doubts about the sequence and tissues attributed to *A. kaweshkari* (see Appendix S3 for further discussion). Fourth, the sequences employed by Pyron and Wiens (2011) as *A. gargola* are here considered to belong to *A. neuquensis* (see Appendix 2). Finally, the terminal *A. monticola* of Pyron and Wiens (2011) is a chimera between the sequence of *16S* produced by Correa et al. (2006) and that of Nuin and do Val (unpublished; GenBank number AY143351) for the *12S* sequence. The specimen of Correa et al. (2006) is tentatively considered here to belong to *A. verrucosus* (see above and Appendix S3), while the identity of that produced by Nuin and do Val is not clear, since *A. monticola* was restricted to its type locality and not collected since its description (Formas et al., 2008). Unfortunately, neither voucher number nor locality data is provided for this *12S* fragment. In conclusion, we cannot discard the possibility that the terminal named as *A. monticola* by Pyron and Wiens (2011) is composed of sequences from different species. Based on these arguments, the internal topology found by Pyron and Wiens (2011) for *Alsodes* must be evaluated with caution.

Finally, one of the most remarkable characteristics of *Alsodes* is the presence of round, spiny pectoral patches (Formas et al., 1998). Round patches are also present, with a different morphology, in *Insuetophrynus* (Barrio, 1970). Although the exact position of *Insuetophrynus* + *Rhinoderma* is not clear, the alternative topologies suggested for this monotypic genus (Correa et al., 2006; Pyron and Wiens, 2011; this study) in relation to *Alsodes* allow us to consider two independent origins for this structure.

Eupsophus

With the recovery of *Eupsophus antartandicus*, *E. taeniatus* and *E. sylvaticus* in Batrachylidae (here transferred to *Batrachyla* and *Hylorina*), we obtained as monophyletic the two major classic groups recognized within *Eupsophus*, the *E. vertebralis* and *E. roseus* species groups. They were erected on the basis of chromosome numbers, morphometrics, allozymes, and advertisement calls (Formas et al., 1983; Fernández de la Reguera, 1987; Formas, 1991, 1992; Formas and Brieva, 1994), although the polarities of several of these characters are not clear. Besides the molecular evidence presented here, and chromosome number (chromosome evolution is discussed in a separate section below ''Chromosome diversity and evolution''), there are several character states that, if corroborated in the context of a total evidence analysis, could be putative phenotypic synapomorphies for *Eupsophus*, and which are worth discussing.

Burton (1998a,b) suggested that a putative synapomorphy for *Eupsophus* comes from the myology of the manus, as a ventral origin of the mm. flexores teretes (FT) of Digits III and IV with respect to the mm. transversi metacarporum (TM) 1 and 2 is present in the genus. A dorsal origin of the FT III and IV with respect to the TM 1 and 2 was reported in *Alsodes* and in the different taxa to which the *Alsodes* plus *Eupsophus* clade has been related (i.e. Frost et al., 2006; Grant et al., 2006; Pyron and Wiens, 2011).

Larval chondrocrania provide several putative synapomorphies for *Eupsophus* to be tested in a total

evidence analysis: These include (i) the absence of crista parotica of the otic capsule; (ii) connection of the processus muscularis through a ligament (as opposed to a cartilaginous connection) to the larval neurocranium; (iii) short trabecular horns, compared with the total length of the chondrocranium (10.5 to < 20%); (iv) the absence of cartilaginous projections along ceratobranchialia (Vera Candioti et al., 2005, 2011; Cárdenas-Rojas et al., 2007; Nuñez and Úbeda, 2009).

In *Eupsophus*, eggs are laid in water, in small hollows in the ground or holes at the end of flooded tunnels, and the complete development of the endotrophic tadpoles takes place there (Formas and Vera, 1980; Úbeda and Nuñez, 2006). Among related groups, endotrophic larvae have also been reported in *Zachaenus parvulus* (Lutz, 1944) and *Cycloramphus stejnegeri* (Heyer and Crombie, 1979; Lavilla, 1991). The two species of *Rhinoderma* also present endotrophy at least during part of their development, since they have partial (*R. rufum*) or complete (*R. darwinii*) larval development within the male vocal sac (Formas et al., 1975; Jorquera et al., 1981). Considering the position of these genera in the hypotheses of Frost et al. (2006), Grant et al. (2006) and Pyron and Wiens (2011), both the endotrophic development (as anticipated by Formas, 1992) and the oviposition site can be regarded as putative synapomorphies of *Eupsophus*.

The contrasting colour in the upper third portion of the iris, with this portion being usually golden to reddish, could be a putative synapomorphy of the *Eupsophus roseus* group. This region of the iris has the same colour as the lower part in the two species of the *E. vertebralis* group and in *Alsodes* species, as well as in *Limnomedusa*, sister to the *Alsodes* + *Eupsophus* clade in the hypothesis of Pyron and Wiens (2011).

Chromosome diversity and evolution

The diversity of chromosome number in *Alsodes* + *Eupsophus* is unique when compared with more inclusive groups (see Green and Sessions, 2007 for a review), and considering that the clade includes only 28 species, it is matched by few hyloid clades. On the basis of the topologies of Grant et al. (2006) and Pyron and Wiens (2011), we infer that the plesiomorphic chromosome number of *Alsodes* + *Eupsophus* is $2n = 26$.

Alsodes has a remarkably variable karyotype, including haploid number, chromosome morphology, and C-band pattern. Although most species have a known karyotype, the extensive variation makes it difficult to establish homologies, and therefore to interpret these variations in a phylogenetic context, beyond some evident patterns discussed below. There are four species of *Alsodes* that show a chromosome number that deviates from $2n = 26$: *A. nodosus* and an undescribed species here referred to as *Alsodes* sp. 1 ($2n = 22$); *A. norae* ($2n = 30$); and *A. barrioi* ($2n = 34$). Our results indicate unambiguously that all these transformations in chromosome number arose independently from the ancestral $2n = 26$ karyotype. None of the most parsimonious trees, nor the topology found in the Bayesian analysis, recovers an *A. barrioi* + *A. norae* clade as monophyletic (Fig. 3), indicating that their increase in chromosome number would have arisen independently. What we find striking is that all the transformations in chromosome number during the evolutionary history of *Alsodes*, whether reductions or increases, involved multiples of two haploid chromosomes. While differences in multiples of two haploid chromosomes are known in other anuran groups (e.g. Ceratobatrachidae, Microhylidae; see Green and Sessions, 2007), we are not aware of other cases of independent transformations in a phylogenetic context involving the *repeated* evolution of multiples of two haploid chromosomes.

The group that is sister to the remaining *Alsodes* is composed of *A. vanzolinii* + (*A. nodosus* + *Alsodes* sp. 1). Within this group, *A. vanzolinii* has $2n = 26$, while *A. nodosus* and *Alsodes* sp. 1 share a $2n = 22$ karyotype (J.J.N., pers. obs.). Considering the topology obtained for *Alsodes* + *Eupsophus* and the relationships of both genera with related groups (following the topologies of Grant et al., 2006 or Pyron and Wiens, 2011), the reduction by four chromosomes can be considered a synapomorphy of this pair of sister species, and the only case of chromosome number reduction from the ancestral $2n = 26$ to $2n = 22$ chromosomes during the evolutionary history of *Alsodes*. Cuevas and Formas (2005b) pointed out that *A. barrioi* and *A. norae* (as *A.* aff. *valdiviensis*) share secondary constrictions and NORs in the long arm of pair 4, however, none of our most parsimonious trees recovers *A. barrioi* and *A. norae* as sister groups.

Among *Eupsophus*, the *Eupsophus vertebralis* species group has $2n = 28$ chromosomes, whereas the *E. roseus* group has $2n = 30$. In this context of an inferred $2n = 26$ ancestral karyotype, the transformation from $2n = 26$ to $2n = 28$ chromosomes is interpreted as a putative synapomorphy of *Eupsophus*, and the transformation from $2n = 28$ to $2n = 30$ is considered a putative synapomorphy of the *E. roseus* group (if the character states are considered additive, otherwise the optimization is ambiguous). This hypothesis must be tested with additional evidence regarding homology, as at this point the specific pairs involved in the rearrangements remain unknown.

A trans-Andean origin?

When the distribution of the species of the *Alsodes* + *Eupsophus* clade is optimized on the optimal cladograms, in accordance with the *cis*-Andean (east to

the Andes) and *trans*-Andean (west to the Andes) range, there is a pattern that suggest a *trans*-andean origin and diversification of this group (Fig. 4a). The presence of *cis*-Andean species or populations could be explained as multiple independent ingressions from *trans*-Andean clades. The *Eupsophus* species present on both sides of the Andes are *E. calcaratus*, *E. emiliopugini*, *E roseus* and *E. vertebralis*. However, all populations present east of the Andes are restricted to the eastern Andean slopes and valleys of the Andes, in close association with the Valdivian forest, and not reaching precordilleran areas (Basso and Úbeda, 1999; Úbeda et al., 1999; Úbeda, 2000). Particularly, *E. emiliopugini* and *E. vertebralis* present a wide distribution west of the Andes, and their presence on the Argentina side of the Andes is restricted to only one known population for each species on intermediate areas (Lago Puelo and Puerto Blest, respectively, see Fig. 4b). Lago Puelo is a peculiar lake (at only 200 m of altitude) because it drains to the Pacific Ocean, and we consider on this ground that the species has a trans-Andean distribution. Interestingly, *E. calcaratus* and *E. roseus* show a similar distributional pattern to *E. emiliopugini* and *E. vertebralis* west of the Andes, with the difference that *E. calcaratus* is widely distributed in the eastern slopes of the Andes (Fig. 4c).

Alsodes pehuenche and *A. gargola* are the two species of *Alsodes* present on both sides of the Andes (Fig. 4e). *Alsodes pehuenche* has a peculiar distribution, restricted to a few square kilometres in a high-altitude valley that bisects the Cordillera de los Andes (Corbalán et al., 2010). *Alsodes gargola* has previously been known only for the east of the Andes; however, specimens from Futaleufú (Chile) are considered here to belong to this species, representing the first record for this area. This locality is in a very low-altitude valley (a pass at only 350 m) that bisects the mountain range. *Alsodes gargola* and *A. neuquensis* are the only species of the genus with a clear and wide *cis*-Andean distribution, reaching to the east precordilleran areas. The two species not sampled in this study (*A. kaweshkari* and *A. montanus*) have a *trans*-Andean distribution (Formas et al., 1998; Correa et al., 2008).

Finally, *Alsodes* and *Eupsophus* may prove to be an interesting lineage for the study of recent Patagonian biogeography and phylogeography, particularly in relation to glaciations and local refugia (i.e. Nuñez et al., 2011). Glaciation developed from the late Miocene (ca. 6 Myr) in multiple events with different intensities. Particularly, Pleistocene glaciation can be arranged into five major episodes since the Great Patagonian Glaciation (ca. 1 Myr) to the Last Glacial Maximum (ca. 25 kyr; Rabassa et al., 2011). The existence of a great number of endemic species of both genera that are restricted to particular areas suggests that glaciation may have had an important role in the diversification and distribution of these species. Several species of both genera occur in the Coastal Range, composed of non-Andean mountains along the Pacific coast (four *Alsodes* and four *Eupsophus* species in the Cordillera de Nahuelbuta, Cordillera Pelada and Cordillera de Mahuidanchi mountains, and low-altitude areas near to those localities in Coastal Range, Fig. 4c, d–e), and to a lesser degree also on the western (two *Alsodes* and one *Eupsophus* species, Fig. 4c–e) and eastern slopes of the Andes (two species of *Alsodes*, Fig. 4a). This is congruent with hypothesized regions of forest refugia. Pollen records suggest that deciduous taxa from the southern South American temperate forests survived last glaciations in refugia located in three main regions: the Nahuelbuta Mountains (in Coastal Chile, at 37°40′S), the Central Valley (in Chile), and both slopes of the Andes at latitudes north of 39°S (Heusser, 1984; Villagrán, 1991). Unfortunately, due to the absence of a fossil record related to the group (the material assigned to *Eupsophus* by Schaeffer, 1949 does not belong neither to *Eupsophus* nor *Alsodes*; Nicoli, 2012), we do not find it possible or reasonable to estimate divergence times in our phylogenetic hypothesis.

A clade with unusually low nucleotide distances

The clade composed of *A. barrioi*, *A. gargola*, *A. igneus*, *A. neuquensis* and *A. norae* shows very low values of nucleotide divergence as expressed by the uncorrected *p*-distances of *COI* and a fragment of *16S* (Tables 3 and 4). Those distances are markedly under the threshold level, if any, of the genetic divergence (uncorrected *p*-distances) proposed to identify amphibian candidate species. For example, Fouquet et al. (2007) suggested a minimum of 3% of genetic divergence for *16S* to identify Neotropical species, while Padial et al. (2009) found levels of divergences in *Pristimantis* (a Neotropical genus) between 3 and 22%, with a mean of 15%. In the case of *COI*, Vences et al. (2005) found values of genetic divergences in mantellid frogs around 10–14%. As pointed out by Padial et al. (2009), difficulties arise when a threshold is imposed, since magnitudes of divergences vary from lineage to lineage. Our results of low distances are most striking when considering the high chromosome diversity of this group. *Alsodes norae* and *A. barrioi* have $2n = 30$ and $2n = 34$, respectively (and they are not sister taxa), while the other species have $2n = 26$. Particularly, *A. norae*, *A. neuquensis* and *A. gargola* show the lowest average distances within this group (between 0 and 0.1% for *16S*; between 1.78 and 2.24% for *COI*). In the case of the *16S* fragment, also *A. barrioi* shows extremely low distances (between 0 and 0.2%), with *A. igneus* being the only species with higher values of divergence within the clade (although still low, 0.4–0.8%). Not surprisingly, most phylogenetic conflict within the ingroup is restricted to this clade.

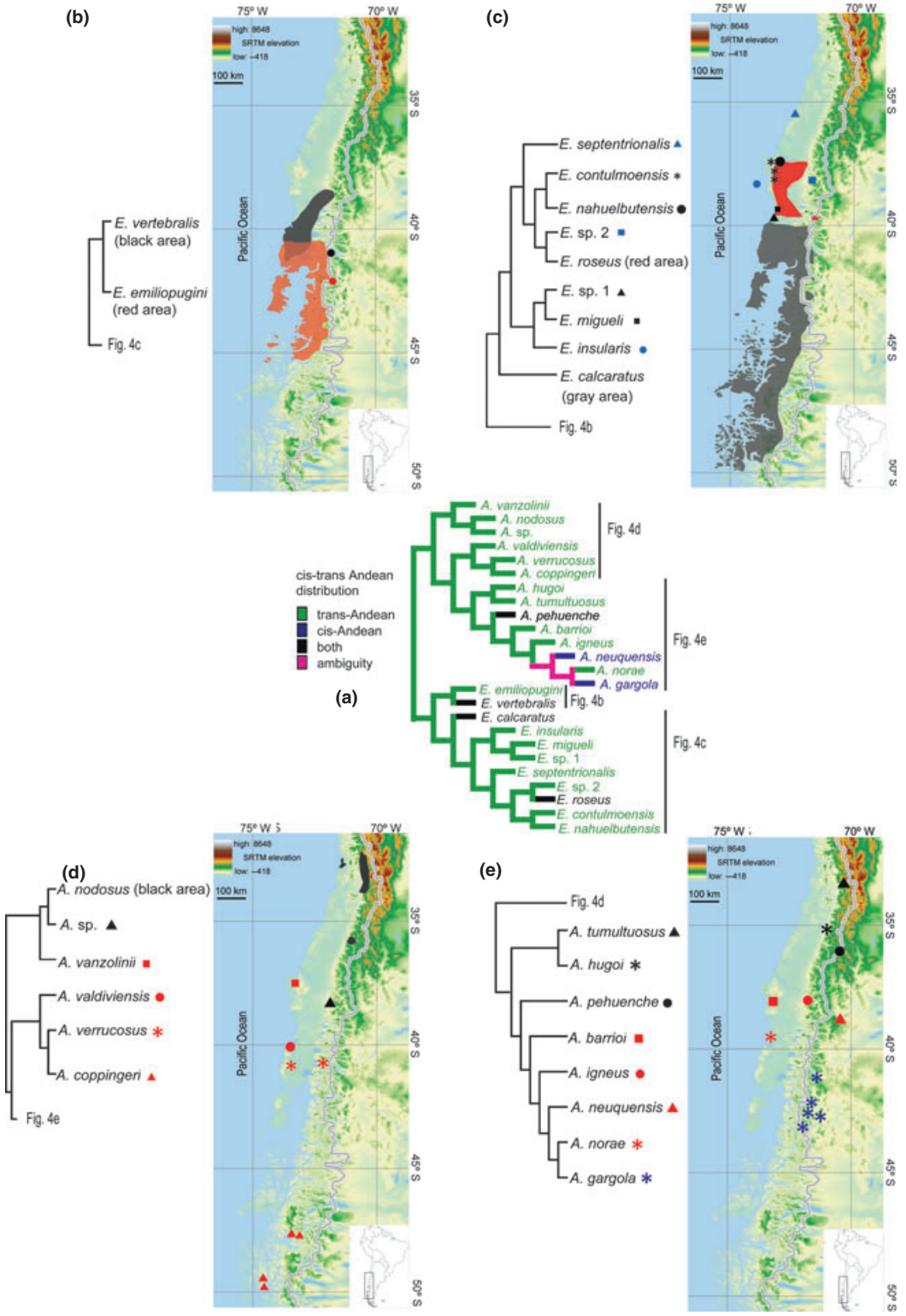

Fig. 4. Distribution of the species of *Alsodes* and *Eupsophus* (b–e), and optimization of those distributions according to *cis*- and *trans*-Andean ranges (a) in one of the optimal cladograms (the ingroup topology is nearly constant in all analyses, with minor differences, irrelevant for our inferences). For the sake of clarity, *Alsodes* and *Eupsophus* clades are divided into two clades each (b–e). See Appendix S4 for the literature sources of species distributions, and for specific considerations for the optimization of the *cis*- and *trans*-Andean distribution. Among species not sampled (besides those known only from type material and not collected since original description) are *A. montanus* and *A. kaweshkari*, both with *trans*-Andean distribution (Formas et al., 1998; Correa et al., 2008).

Table 3
Percentage uncorrected pairwise distances between sequences of *cytochrome oxidase I* (COI) from *Alsodes barrioi*, *A. igneus*, *A. gargola*, *A. neuquensis* and *A. norae*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1-*A. barrioi* IZUA 3549 (CH: IX: Rucapehuen)	–													
2-*A. barrioi* IZUA 3550 (CH: IX: Rucapehuen)	0.5	–												
3-*A. barrioi* IZUA 3551 (CH: IX: Rucapehuen)	0	0.5	–											
4-*A. igneus* IZUA 3555 (CH: IX: Tolhuaca)	3.4	2.9	3.4	–										
5-*A. igneus* IZUA 3556 (CH: IX: Tolhuaca)	3.4	2.9	3.4	0.4	–									
6-*A. igneus* IZUA 3557 (CH: IX: Tolhuaca)	3.4	2.9	3.4	0	0.4	–								
7-*A. norae* IZUA 3563 (Chile: XIV: Reserva Forestal Oncol)	3.4	3.5	3.4	3.2	2.9	3.2	–							
8-*A. g. neuquensis* MACN 37942 (AR: Neuquén: Primeros Pinos)	2	2.2	2	1.7	1.4	1.7	1.7	–						
9-*A. g. neuquensis* MACN 38973 (AR: Neuquén: Pampa de Lonco Luan)	2.6	2.8	2.6	2.3	2	2.3	2	2.3	–					
10-*Alsodes* sp.2 IZUA 3571 (CH: X: Futaleufú)	2.6	2.8	2.6	3.1	2.8	3.1	2.2	1.4	2	–				
11-*Alsodes* sp.2 IZUA 3572 (CH: X: Futaleufú)	2.8	2.9	2.8	3.2	2.9	3.2	2.3	1.6	2.2	0.2	–			
12-*Alsodes* sp.2 IZUA 3573 (CH: X: Futaleufú)	2.6	2.8	2.6	3.1	2.8	3.1	2.2	1.4	2	0	0.2	–		
13-*Alsodes* sp.2 IZUA 3575 (CH: X: Futaleufú)	2.8	2.9	2.8	3.2	2.9	3.2	2.3	1.6	2.2	0.2	0.2	0.2	–	
14-*A. australis* MACN 41421 (AR: Chubut: Ao. Zanjón Hondo)	2.6	2.8	2.6	3.1	2.8	3.1	2.2	1.4	2	0	0.2	0	0.2	–

For purposes of clarity and comparison, we use for the specimens the same names as in Fig. 2. In consequence, *A. gargola* is represented here by the specimens 10–14 (topotypes not available for this region), and *A. neuquensis* is named here as subspecies of *A. gargola* (specimens 8 and 9).

Table 4
Percentage uncorrected pairwise distances between sequences of a fragment of *16S* from *Alsodes barrioi*, *A. igneus*, *A. gargola*, *A. neuquensis* and *A. norae*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1-*A. barrioi* IZUA 3549 (CH: IX: Rucapehuen)	–															
2-*A. barrioi* IZUA 3550 (CH: IX: Rucapehuen)	0.2	–														
3-*A. barrioi* IZUA 3551 (CH: IX: Rucapehuen)	0	0.2	–													
4-*A. igneus* IZUA 3555 (CH: IX: Tolhuaca)	0.6	0.8	0.6	–												
5-*A. igneus* IZUA 3556 (CH: IX: Tolhuaca)	0.4	0.6	0.4	0.2	–											
6-*A. igneus* IZUA 3557 (CH: IX: Tolhuaca)	0.6	0.8	0.6	0	0.2	–										
7-*A. norae* IZUA 3563 (Chile: XIV: Parque Oncol)	0	0.2	0	0.6	0.4	0.6	–									
8-*A. g. neuquensis* MACN 37942 (AR: Neuquén: Primeros Pinos)	0.2	0.4	0.2	0.8	0.6	0.8	0.2	–								
9-*A. g. neuquensis* MACN 38973 (AR: Neuquén: Pampa de Lonco Luan)	0	0.2	0	0.6	0.4	0.6	0	0.2	–							
10-*A. g. gargola* CNP A–380 (AR: Río Negro: Laguna Tonchek)	0	0.2	0	0.6	0.4	0.6	0	0.2	0	–						
11-*A. g. gargola* CNP A-381 (AR: Río Negro: Laguna Tonchek)	0	0.2	0	0.6	0.4	0.6	0	0.2	0	0	–					
12-*Alsodes* sp.2 IZUA 3571 (CH: X: Futaleufú)	0	0.2	0	0.6	0.4	0.6	0	0.2	0	0	0	–				
13-*Alsodes* sp.2 IZUA 3572 (CH: X: Futaleufú)	0	0.2	0	0.6	0.4	0.6	0	0.2	0	0	0	0	–			
14-*Alsodes* sp.2 IZUA 3573 (CH: X: Futaleufú)	0	0.2	0	0.6	0.4	0.6	0	0.2	0	0	0	0	0	–		
15-*Alsodes* sp.2 IZUA 3575 (CH: X: Futaleufú)	0	0.2	0	0.6	0.4	0.6	0	0.2	0	0	0	0	0	0	–	
16-*A. australis* MACN 41421 (AR: Chubut: Ao. Zanjón Hondo)	0	0.2	0	0.6	0.4	0.6	0	0.2	0	0	0	0	0	0	0	–

The fragment employed is delimited by the primers *16S Ar-Wilkinson2* (ca. 570 bp). For purposes of clarity and comparison, we use for the specimens the same names as in Fig. 2. As a result, *A. gargola* is represented here by specimens 10–16, and *A. neuquensis* is named here as subspecies of *A. gargola* (specimens 8–9).

Did different developmental modes affect the responses to past geological/climatic events?

A final noteworthy point of our results is the differences in branch lengths between *Alsodes* and *Eupsophus* (Fig. 2). Internal branches of *Alsodes* are notably shorter than in *Eupsophus*. This result does not seem to be a problem of sampling—extant species, at least—since it covers most known diversity and distribution ranges for the species of both genera. Although no hypothesis can be advanced at this time, it is a curious scenario to be considered in future studies.

Differences in the mode of reproduction between the genera may be an interesting starting point to consider. *Eupsophus* has endotrophic larvae developed in small, water-filled holes, while *Alsodes* has exotrophic larvae. This could have resulted in non-equivalent processes of diversification and expansion/retraction of distributional ranges due to different responses to glaciations and/or pre-Quaternary events. As expected, and evidenced by the comparison of several phylogeographic studies from Patagonian species (Sérsic et al., 2011), it has been noted that different events may have operated at different levels across different taxa. This will remain as an interesting scenario to be considered and tested as additional evidence from phylogeographic studies and the fossil record (for calibration points for the estimation of divergence times) become available.

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Note added in proof

While this paper was in press, Nuñez et al. (2012) described as *Eupsophus altor* the species that we included in this paper as *Eupsophus* sp. 1

Supporting Information

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

Appendix S1. Genbank numbers of the sequences employed in this study.

Appendix S2. Results of the Bayesian analysis.

Appendix S3. Taxonomic comments.

Appendix S4. Literature sources and comments on the optimization of the distribution of the *Alsodes* and *Eupsophus* species in Fig. 4.

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Appendix 1

Locality data (GenBank numbers for these specimens are provided as additional Supporting Information). Vouchers are adult specimens, unless indicated as T (tissue) or D (DNA extraction).

Collection abbreviations are as follows: CNP (Centro Nacional Patagónico, Puerto Madryn, Argentina); IZUA (Instituto de Zoología, Universidad Austral de Chile, Valdivia, Chile);

MACN (Museo Argentino de Ciencias Naturales ''Bernardino Rivadavia'', Buenos Aires, Argentina).

Alsodes sp.: IZUA 3543: Chile: IX Región: Pemehue (T). *Alsodes* sp.: IZUA 3544: Chile: IX Región: Pemehue (T). *Alsodes* sp.: IZUA 3562: Chile: IX Región: Pemehue (D). *A. coppingeri*: IZUA 3545: Chile: XII Región: Caleta Lever (D). *A. coppingeri*: IZUA 3546: Chile: XI Región: Puerto Yungay (T). *A. coppingeri*: IZUA 3547: Chile: XI Región: Caleta Tortel (T). *A. coppingeri*: IZUA 3548: Chile: XI Región: Canal Michel (T). *A. coppingeri*: IZUA 3552: Chile: XII Región: Última Esperanza: Puerto Río Frío (D). *A. coppingeri*: IZUA 3553: Chile: XII Región: Última Esperanza: Puerto Río Frío (T). *A. barrioi*: IZUA 3549: Chile: IX Región: Rucapehuén (D). *A. barrioi*:

IZUA 3550: Chile: IX Región: Rucapehuén (T). *A. barrioi*: IZUA 3551: Chile: IX Región: Rucapehuén (T). *A. gargola*: IZUA 3571: Chile: X Región: Futaleufú (T). *A. gargola*: IZUA 3572: Chile: X Región: Futaleufú (T). *A. gargola*: IZUA 3573: Chile: X Región: Futaleufú (D). *A. gargola*: IZUA 3575: Chile: X Región: Futaleufú (D). *A. gargola*: CNP A-380: Argentina: Río Negro: Cerro Catedral: Laguna Tonchek. *A. gargola*: CNP A-381: Argentina: Río Negro: Cerro Catedral: Laguna Tonchek. *A. gargola*: MACN 41421: Argentina: Chubut: Futaleufú: Arroyo tributario Arroyo Zanjón Hondo, sendero Co. Alto El Petiso. *A. hugoi*: IZUA 3554: Chile: VII Región: Altos de Vilches (D). *A. igneus*: IZUA 3555: Chile: IX Región: Tolhuaca (D). *A. igneus*: IZUA 3556: Chile: IX Región: Tolhuaca (T). *A. igneus*: IZUA 3557: Chile: IX Región: Tolhuaca (T). *A. neuquensis*: MACN 38973: Argentina: Neuquén: Aluminé: Pampa de Lonco Luan sobre ruta provincial 13. *A. neuquensis*: MACN 37942: Argentina: Neuquén: Aluminé: Arroyo 10 km W Primeros Pinos. *A. nodosus*: IZUA 3558: Chile: V Región: Valparaiso: Petorca: Zapallar (T). *A. norae*: IZUA 3563: Chile: XIV Región: Parque Oncol (D). *A. pehuenche*: IZUA 3559: Argentina: Mendoza: Valle Pehuenche (D). *A. pehuenche*: IZUA 3560: Argentina: Mendoza: Valle Pehuenche (T). *A. pehuenche*: IZUA 3561: Argentina: Mendoza: Valle Pehuenche (T). *A. pehuenche*: CNP A-389: Argentina: Mendoza: Ruta Prov. 224, 1.2 km E Paso Pehuenche. *A. tumultuosus*: IZUA 3564: Chile: Región Metropolitana: Santiago: La Parva (T). *A. tumultuosus*: IZUA 3565: Chile: Región Metropolitana: Santiago: La Parva (D). *A. tumultuosus*: IZUA 3566: Chile: Región Metropolitana: Santiago: La Parva (T). *A. tumultuosus*: IZUA 3567: Chile: Región Metropolitana: Santiago: La Parva (T). *A. valdiviensis*: IZUA 3568: Chile: XIV Región: Cordillera Pelada (D). *A. valdiviensis*: IZUA 3569: Chile: XIV Región: Cordillera Pelada (T). *A. vanzolinii*: IZUA 3570: Chile: VIII Región: Ramadillas (D). *A. verrucosus*: IZUA 3574: Chile: X Región: Parque Nacional Puyehue (T). *A. verrucosus*: IZUA 3576: Chile: X Región: Puyehue: (T). *A. verrucosus*: IZUA 3577: Chile: X Región: Parque Nacional Puyehue (D). *Eupsophus calcaratus*: IZUA 3593: Chile: X Región: Parque Nacional Puyehue (T). *E. calcaratus*: IZUA 3578: Chile: X Región: Yaldad (T). *E. calcaratus*: IZUA 3580: Chile: XI Región: Isla Chaculay (D). *E. calcaratus*: IZUA 3581: Chile: XII Región: Puerto Edén (T). *E. calcaratus*: IZUA 3582: Chile: X Región: Chiloe (T). *E. calcaratus*: MACN 39079: Argentina: Chubut: Cushamen: Próximo a Los Hitos, Lago Puelo. *Eupsophus* sp.: IZUA 3579: Chile: IX Región: Parque Nacional Tolhuaca (T). *E. contulmoensis*: IZUA 3583: Chile: IX Región: Contulmo (T). *E. contulmoensis*: IZUA 3584: Chile: IX Región: Contulmo (T). *E. emiliopugini*: IZUA 3585: Chile: XI Región: Isla Rivero (D). *E. emiliopugini*: IZUA 3586: Chile: X Región: La Picada (D). *E. insularis*: IZUA 3587: Chile: VIII Región: Isla Mocha (T). *E. insularis*: IZUA 3588: Chile: VIII Región: Isla Mocha (T). *E. insularis*: IZUA 3590: Chile: VIII Región: Isla Mocha (T). *E. migueli*: IZUA 3591: Chile: XIV Región: Mehuín (T). *E. migueli*: IZUA 3592: Chile: IX Región: Queule (T). *E. nahuelbutensis*: IZUA 3594: Chile: IX Región: Rucapehuén (T). *E. nahuelbutensis*: IZUA 3595: Chile: X Región: Piedra del Águila (T). *E. nahuelbutensis*: IZUA 3596: Chile: X Región: Rucapehuén (T). *E. roseus*: IZUA 3597: Chile: XIV Región: San Martín (T). *E. roseus*: IZUA 3598: Chile: XIV Región: Alepue (T). *E. roseus*: IZUA 3599: Chile: XIV Región: San Martín (T). *E. roseus*: MACN 39081: Argentina: Neuquén: Huiliches: sendero a Base Volcán Lanín, próximo a Lago Paimún. *E. septentrionalis*: IZUA 3600: Chile: VII Región: Los Queules (T). *Eupsophus* sp.: IZUA 3601: Chile: XIV Región: Parque Oncol (T). *Eupsophus* sp.: IZUA 3602: Chile: XIV Región: Parque Oncol (T). *Eupsophus* sp.: IZUA 3603: Chile: XIV Región: Parque Oncol (T). *E. vertebralis*: IZUA 3604: Chile: IX Región: Queule (T). *E. vertebralis*: IZUA 3605: Chile: XIV Región: Llancahue (D). *Hylorina sylvatica*: MACN 42530: Argentina: Neuquén: RN231 y Río Totoral. *Insuetophrynus acarpicus*: IZUA 3606: Chile: XIV Región: Mehuín (D).

Appendix 2

Taxonomic conclusions.

The subspecies of Alsodes gargola

Alsodes gargola was described by Gallardo (1970) from Cerro Catedral, Río Negro Province, Argentina. Later, Cei (1976) described a subspecies, *A. g. neuquensis*, from Pampa de Lonco Luan, a plateau in central Neuquén Province (250 km N of type locality of the nominal subspecies) based on one male and two females. Minor morphological differences (more slender body, larger snout, smoother skin) with the nominal subspecies were reported by Cei (1976). Those differences are not evident when considering a large series of specimens (B.L.B., pers. obs.). On the other hand, it is relevant to note that *A. g. gargola* and *A. g. neuquensis* are not recovered as sister taxa in any of the most parsimonious trees, since we recovered the single topology of *A. neuquensis* + (*A. norae* + *A. g. gargola*). One of the few differences found between our parsimony-based analyses (direct optimization and analysis of the static alignment) and our Bayesian analysis was the sister group relationship between *A. g. gargola* and *A. g. neuquensis* (90% Pp) on the later. On the basis of these results, we tentatively consider *A. neuquensis* to be a distinct, full species. Note that any attempt to consider *A. neuquensis* a synonym of *A. gargola* would require the inclusion of *A. barrioi*, *A. igneus* and *A. norae* in the synonymy of the latter as well. Although *A. barrioi*, *A. igneus* and *A. norae* have differences in chromosome number or chromosome morphology with *A. gargola*, we consider it legitimate to raise doubts concerning their status and suggest that a reassessment of their differences, and those of *A. neuquensis* (if any), with *A. gargola* is in order.

Eupsophus queulensis *and* E. septentrionalis

The original descriptions of *Eupsophus septentrionalis* Ibarra-Vidal et al., 2004 and *E. queulensis* Veloso et al., 2005; seem to refer to the same species. In addition to this, the material was collected at the same locality. Veloso et al. (2005) did not mention the work of Ibarra-Vidal et al. (2004), probably because the former was in press when the paper of Ibarra-Vidal et al. (2004) was published. The percentage uncorrected *p*-distance of *16S* between the exemplars of *E. quealensis* and *E. septentrionalis* is 0.54%. Moreover, they were recovered as sister taxa in the analysis. On the basis of these observations, we consider *E. queulensis* Veloso et al., 2005 a junior synonym of *E. septentrionalis* Ibarra-Vidal et al., 2004.