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A molecular phylogeny of the lizard genus *Phymaturus* (Squamata, Liolaemini): Implications for species diversity and historical biogeography of southern South America

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ABSTRACT

The lizard genus *Phymaturus* is widely distributed in Argentina and along the eastern edge of Chile between 25° and 45° south. We sampled 27 of the 38 currently recognized species plus 22 candidate species using two mitochondrial genes (cytb and 12S), four protein coding nuclear genes and seven anonymous nuclear loci, and present the first comprehensive molecular phylogenetic hypothesis for the clade. We recovered two large clades (the *palluma* or northern group and *patagonicus* or southern group) previously recognized on the basis of morphological and mitochondrial sequence evidence, and compared results obtained from concatenated-gene analyses with results of a coalescent-based species-tree approach (BEST). With both methods we identified four main clades within the *palluma* group (*mallimaccii*, *roigorum*, *verdugo*, and *vociferator*) and five main clades within the *patagonicus* group (*calcogaster*, *indistinctus*, *payuniae*, *somuncurensis*, and *spurcus*). We found several instances of non-monophyly with cytb and cases of incongruence between mitochondrial vs nuclear data for which we discuss alternative hypotheses. Although with lower support values, combined BEST results are more congruent with concatenated nuclear data than with combined concatenated analyses, suggesting that BEST is less influenced by demographic processes than combined concatenated analyses. We discuss the taxonomic, biogeographic and conservation implications of these results and how the future integration of phylogeographic and morphological approaches will allow the further testing of demographic and biogeographic hypotheses.

1. Introduction

Patagonia is the southernmost region of South America in Argentina and Chile, and includes a steppe with high plateaus and the southernmost extension of the Andean Cordillera. Until recently, it has been one of the most neglected regions for phylogenetic and phylogeographic research (Beheregaray, 2008), but there is now an increasing research focus across a variety of taxa. Studies of plants (Acosta and Premoli, 2009; Allnutt et al., 1999; Azpilicueta et al., 2009; Cosacov et al., 2010; Jakob et al., 2009; Muellner et al., 2005; Premoli et al., 2000; Pastorino and Gallo, 2002; Tremetsberger et al., 2009), mammals (Kim et al., 1998; Lessa et al., 2010; Palma et al., 2005; Smith et al., 2001; Himes et al., 2008), fishes (Ruzzante et al., 2006, 2008; Zemlak et al., 2008, 2010; Unmack et al., 2012), frogs (Nuñez et al., 2011); lizards (Avila et al., 2006; Breitman et al., 2011; Fontanella et al., 2012a,b; Morando et al., 2003, 2004, 2007; Victoriano et al., 2008), and freshwater crabs (Pérez-Losada et al., 2004; Xu et al., 2009), have emphasized the influences of Andean orogeny and Quaternary climatic events on present day distributions and evolutionary processes that shaped observed phylogeographic patterns.

A very distinct clade of lizards, widely distributed throughout Patagonia, northwestern Argentina, and the eastern edge of Chile is the genus *Phymaturus*. Members of this genus are strictly saxicolous, restricted to 25 to 45 degrees south latitude, and range from 300 to 4000 m in elevation. In Chile the genus is confined to the highlands of the western slopes of the Andes, and its geographic distribution is relatively small. In contrast it is widely distributed in Argentina, ranging from Puna environments in Catamarca province, extra-Andean mountain ranges (such as Famatina), pre-cordilleran mountain ranges in the north, high plateaus (Somuncura and Payunia), in an isolated mountain range (Auca Mahuida), and further south in north-central and northwestern Patagonia (Cei, 1986, 1993). The distribution of *Phymaturus* seems to be correlated with late Tertiary volcanic and basaltic environments (Cei and

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Castro, 1973), in a climate characterized by dry cold winters and sunny summers, and with a wide range of daily temperatures. Due to their reliance on rock outcrops with deep crevices for shelter, populations of *Phymaturus* species have patchy distributions.

All known species are herbivorous, saxicolous (Cei, 1986, 1993), and viviparous (with a report of facultative parthenogenesis in captivity, Chiszar et al., 1999). All that have been studied require 7–8 years to reach sexual maturity (Piantoni et al., 2006), and the majority of species exhibit a biennial reproduction cycle with litter sizes of one (Cabezas Cartes et al., 2010), two, or rarely three offspring (Habit and Ortiz, 1996; Ibargüengoytía, 2004), and perhaps some kind of parental care (Boretto and Ibargüengoytía, 2009).

First described by Gravenhorst (1837), the genus *Phymaturus* was poorly studied for more than 150 years, and only one species with two subspecies (*P. palluma palluma* (Molina) 1782 and *P. palluma patagonicus* (Koslowskyi) 1898), was included by Peters and Donoso Barros (1970) in their catalog of South American Squamata. However, studies carried out in the last 20 years have increased this to a total of 38 described species (Avila et al., 2011; Barbour, 1921; Cei, 1971; Cei and Castro, 1973; Cei and Roig, 1975; Cei and Videla, 2003; Corbalan et al., 2009; Etheridge, 1995; Koslowsky, 1898; Lobo and Quinteros, 2005a,b; Lobo and Abdala, 2007; Lobo et al., 2010b, 2012a,b; Nuñez et al., 2010; Pincheira-Donoso, 2004; Scolaro and Cei, 2003; Scolaro and Ibargüengoytía, 2007, 2008; Scolaro et al., 2008; Scolaro and Tappari, 2009; Scolaro and Pincheira Donoso, 2010), although three of these are of doubtful validity: *P. agilis* (see below, Lobo et al., 2012c), *P. gynechlomus* (see below, Lobo et al., 2010a; Lobo and Ehteridge, personal communication and *P. dorsimaculatus* (see below, Pincheira-Donoso et al., 2008). Etheridge (1995) provided a list of characters supporting monophyly of the genus, including wide and flattened head and body, lateral nuchal skin folds obscured by fat-filled pouches, and the tail with regular whorls of spinose scales. Etheridge (1995) also provided some morphological characteristics defining two main clades within *Phymaturus*, corresponding to the two subspecies recognized by Peters and Donoso-Barros: a *palluma* (northern) group and a *patagonicus* (southern) group. Later studies confirmed the monophyly of these two groups based on molecular (Espinoza et al., 2004; Morando, 2004) and morphological characters (Lobo and Quinteros, 2005b).

The *palluma* group occurs along both the eastern and western Andean slopes in Argentina and Chile between 25° and 40°S; in Argentina it also occurs in some extra-cordilleran basaltic plateaus: in Prepuna formations in Catamarca province, Famatina in La Rioja province, precordillera in San Juan, Payunia region in Mendoza and vocanic formations in Neuquén, including Auca Mahuida. This clade is defined by several synapomorphies (Etheridge, 1995), including the presence of non-imbricate supraciliary scales, five or more suboculars, two to four rows of lorilabials, mental narrower than rostral and usually in contact with infralabials, well-developed caudal spines, and two caudal annuli per segment. Determination of the type locality of *P. palluma,* the first described species of this genus, has been difficult but recently a review of all available evidence demonstrates that Cordón del Portillo (Mendoza province) is the type locality for this species (Lobo and Etheridge, unpublished).

Sixteen species were described within the *palluma* clade, but *P. gynechlomus* (Corbalan et al., 2009) is being synonymized with *P. palluma* (Lobo et al., 2010b; Lobo and Etheridge, in rev.), and Pincheira-Donoso et al. (2008) suggested that *P. dorsimaculatus* may be a synonym of *P. vociferator*. Five species occur in Chile (Pincheira-Donoso et al., 2008; Nuñez et al., 2010), but Lobo and Quinteros (2005b) and Lobo and Abdala (2007) have suggested that these include distinct populations that may represent undescribed species.

The *patagonicus* group extends from 35°S in southern Mendoza Province to 45°S (the Chubut – Santa Cruz Province border). It is found in extra-Andean volcanic formations from Payunia region in Mendoza, in mountain chains and plateaus in central Neuquen, volcanic plateaus south and southeast of Río Negro, including the Somuncurá plateau. This group's distribution overlaps with species of the *palluma* group in the Payunia region in Mendoza, and in Auca Mahuida and plateus formations in eastern Neuquén. Morphological synapomorphies for this group include: overlapping elongate and imbricate superciliaries, a single elongate subocular usually not fragmented, smooth rather than keeled caudal scales, and a fused and closed Meckel's groove (Cei, 1993; Etheridge, 1995). This group includes 21 described species, but recently *P. agilis* was proposed as synonymous with *P. spectabilis* (Lobo et al., 2012c), and *P. desuetus* was described from one individual from the distributional range of *P. spectabilis* (see Lobo et al., 2010b). This group comprises a significant element of the Argentinian Patagonian herpetofauna.

Previous phylogenetic studies on this genus have been based on morphological and chromosomal characters (Cei and Lescure, 1985; Etheridge, 1995; Lescure and Cei, 1991; Pereyra, 1992), but relationships were still poorly known until Lobo and Quinteros (2005b) published the first comprehensive phylogenetic analyses of *Phymaturus* using cladistic methods. This study included more than 100 characters (mainly external morphological and skeletal attributes), and 14 recognized species and eight allopatric populations that may (at that time) represent new species, most of them under the name *palluma*. Most recently Lobo et al. (2010b) described four new species and provided four morphologically based identification keys for species of the *patagonicus* group inhabiting different geographic areas, and one key for species of the *palluma* group. Nuñez et al. (2010) described four new species of the *palluma* group from Chile based on morphological characters, and provided an identification key for the five species distributed in this country. Very recently, Lobo et al. (2012d) presented an updated revision of the morphological variation known for *Phymaturus* and presented phylogenetic relationships based on 206 characters, for most of the described species plus nine populations/species that may represent new species within the *palluma* group.

During 10 years of intensive field work in Patagonia, we collected ten of 15 described species plus ten populations that may represent undescribed species of the *palluma* group, and 17 of the 21 described species plus 12 populations that may represent undescribed species of the *patagonicus* group, for which we have observed great inter- and intra-population color pattern variability. The objective of this study is to present the first comprehensive molecular phylogenetic hypothesis to include most of the described species of *Phymaturus*, and also several isolated populations that may represent candidate species. Based on specific habitat requirements and patchy distributions, *Phymaturus* species are predicted to have highly genetically-structured populations that may be susceptible to over-splitting by tree-reconstruction methods based on limited molecular data (Bond and Stockman, 2008). Here we present a mitochondrial gene tree from 406 individuals, in which following Vieites et al. (2009) we identified: (1) described species (individuals sampled from type localities); (2) "candidate species" (distinct allopatric lineages and/or color morphs); and (3) "deep conspecific lineages" for very distinct haplotypes from the same locality. For all these taxa we sequenced another mt gene (12S) and 10–11 nuclear genes (four protein coding and six/seven anonymous nuclear loci [ANL] – one ANL did not amplify for the *palluma* group); and based on combined analyses we estimated present phylogenetic hypotheses for the majority of recognized taxa. Although the sampling design of this study was not planned as a rigorous test of species delimitation, it is relevant to some aspects of this issue and therefore, we discuss the

molecular phylogenetic support for candidate new species and the validity of some recently described species.

With regard to the biogeographic history of Patagonia, published studies are now available for several clades of plants, rodents, insects, bird, fish, and lizards (e.g. Breitman et al., 2011, in press; Cosacov et al., in press; Díaz Gómez, 2009; Dominguez et al., 2006; Pardiñas et al., 2011; Sede et al., 2012; Sérsic et al., 2011; Zemlak et al., 2008; Unmack et al., 2012). Most of the lizard studies have focused on different clades of *Liolaemus* (the sister clade to *Phymaturus*), and the life-history features of *Phymaturus* contrast sufficiently with those of co-distributed *Liolaemus* that we expect results of this study to reveal "counter – examples" to some of the shared patterns emerging among several species groups of *Liolaemus* (Morando et al., 2007; Breitman et al., in press).

2. Materials and methods

2.1. Taxon sampling

From 2000 to 2010, we collected a total of 406 *Phymaturus* lizards (101 *palluma* group; 305 *patagonicus* group) from 84 localities. This data set includes 27 of 38 described species, of which 10 from a total of 16 represent described species for the *palluma* group, and 17 from a total of 21 represent described species for the *patagonicus* group. Appendix A summarizes the number of individuals sequenced per locality, distributional information and voucher specimens for all taxa used in this study. Vouchers are deposited in the LJAMM-CNP herpetological collection (http://www.cenpat.edu.ar/nuevo/colecciones03.html, Centro Nacional Patagónico [CENPAT], Puerto Madryn, Argentina), the Fundación Miguel Lillo (FML, San Miguel de Tucumán, Argentina), Museo de Ciencias Naturales [MCN] of Salta National University, and the herpetological collection of the Bean Life Science Museum, Brigham Young University (BYU). For described species we either used individuals from type localities, or in a few cases areas close to type localities (see details in Appendix A).

2.2. Gene sampling strategy

Based on a ML and Bayesian tree for all mtDNA cytochrome b (cytb) non-redundant haplotypes (see below), we selected 51 terminal taxa (bold voucher numbers in Appendix A) for subsequent sequencing of other markers (following the subsampling design described by Morando et al., 2003; see below). These 51 terminals (*palluma* group = 20; *patagonicus* group = 31) represent: (1) 27 described species (*P. videlai* was only included in the cytb tree, because it was described after the collection of this data set and we did not have evidence at that time for this population representing a different species); and (2) other populations that, based on the cytb results and/or color pattern, could not clearly be assigned to described species but represented localities that were geographically distant and/or isolated from type localities. We labeled these terminals as *P. sp.* followed by a number, to identify them as candidate species (Appendix A). Our treatment of these taxa as separate *P. sp.* units is not an endorsement of their recognition as distinct species, but simply a proposal for candidate species status (Vieites et al., 2009) that deserve further integrative studies to assess their taxonomic status.

2.3. Laboratory procedures

Total genomic DNA was extracted from liver/muscle tissues preserved in 96% ethanol with Qiagen Dneasy tissue extraction kit. Three μl of extraction product were electrophoresed on 1% agarose gel to estimate the quality and amount of genomic DNA, and sample dilutions were performed where necessary prior to polymerase chain reaction (PCR) amplification. Double-stranded PCR-amplified products were checked by electrophoresis on a 1% agarose gel (the size of the target region estimated using a molecular-weight marker), purified using a vaccum drier, and directly sequenced using the Perkin Elmer ABI PRISM BigDye Cycle Sequencing Ready Reaction (PE Applied Biosystems, Foster City, CA). Excess BigDye was removed with plate sephadex columns (Princeton Separations Inc.), and sequences were run on an ABI

Table 1
Summary of gene regions used in this study. Length of aligned matrices in base pairs, (*palluma*/*patagonicus*); N: number of samples sequenced with one outgroup: (*palluma* 21/*patagonicus* 31); missing data (in%) (*palluma*/*patagonicus*); #number of indels (length in base pairs) (*palluma*/*patagonicus*); S: number of variable sites (parsimony informative) (*palluma*/*patagonicus*); H: number of haplotypes (*palluma*/*patagonicus*); Pi: per-site nucleotide diversity (*palluma*/*patagonicus*); D: Tajima's *D* (D)**0.05, ***$p < 0.001$;G–C: G–C content; GB: GenBank accession numbers. *UNPHASED.

Locus	Length (bp)	N (20/31)	Missing%	# Indels (bp)	S	H	Pi	D	G–C	GB
Cytb	830/829	20/31	–	–	112(67)/167(118)	20/31	0.042/0.055	−0.06/−0.29	0.44/0.412	JX969009–JX969028/ JX969029–JX969059
12S	851/847	20/31	1.13/0.11	3(1) – 3(3)/7(1)	57(24)/95(66)	19/29	0.018/0.030	−0.80/−0.27	0.44/0.44	JX969060–JX969079/ JX969080–JX969110
cmos	523	17/30	3.19/0.02	–	1(1)/3(2)	2/4	0.0007/0.0015	0.1399	0.45/0.43	JX969517–JX969533/ JX969534–JX969563
NT3*	541	20/31	0.12/0.39	–	12(12)	14/3	0.006/0.0006	0.57/−0.28	0.41/0.40	JX969595–JX969614/ JX969564–JX969594
PRLR*	533	20/31	0.03/–	–/1(15)	13(10)/15(15)	12/18	0.0039/0.007	−0.98/0.19	0.44/0.44	JX969497–JX969516/ JX969466–JX969496
PNN	1004	18/30	3.28/1.93	–/1(24)	3(3)/15(15)	4/11	0.0007/0.002	0.14/−0.95	0.47/0.48	JX969420–JX969436/ JX969437–JX969465
Phy38	741/735	21/29	0.51/2.12	1(1) – 1(3)/–	19(17)/18(16)	15/13	0.005/0.005	−0.18/0.08	0.48/0.48	JX969123–JX969142/ JX969143–JX969170
Phy41	582/576	21/31	2.7/2.57	1(1) – 1(2)/1(2)	9(9)/22(21)	10/22	0.003/0.006	−0.15/−0.98	0.45/0.45	JX969315–JX969334/ JX969335–JX969364
Phy60	926/917	16/32	3.44/2.16	1(7)/3(2) – 1(4) – 1(1–4)	16(14)/34(33)	15/25	0.007/0.007	0.097/-0.41	0.33/0.45	JX969171–JX969185/ JX969186–JX969215
Phy64	629/631	13/26	2.92/6.16	–/1(1–3) – 1(4) – 1(2) – 1(1)	16(14)/274(47)	15/22	0.007/0.031	0.09/−2.55***	0.33/0.34	JX969111–JX969122/ JX969365–JX969389
Phy84	618/616	21/32	1.52/0.74	1(2)/–	13(12)/46(45)	10/30	0.005/0.018	−0.08/0.39	0.35/0.36	JX969216–JX969235/ JX969236–JX969266
Phy87	–/737	–/30	–/1.99	–/	–/28(27)	–/20	–/0.006	–/−0.838	–/0.40	–/JX969390–JX969419
Phy89	632	20/30	2.05/0.16	1(4) – 1(1)/1(2)	57(19)/25(23)	17/19	0.010/0.008	−2.12**/−0.054	0.42/0.44	JX969267–JX969285/ JX969286–JX969314

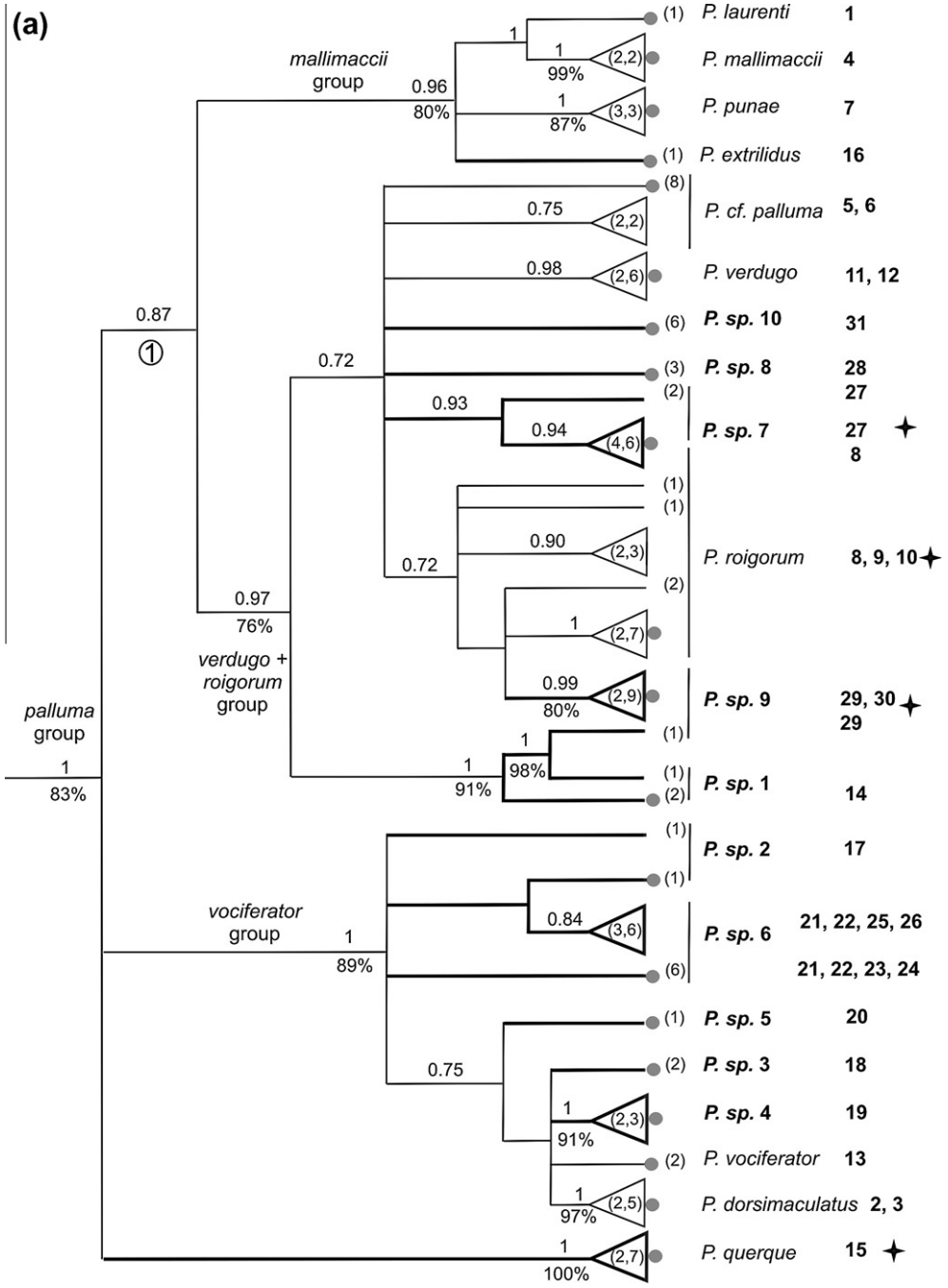

Fig. 1. Cytb gene tree for the non-redundant haplotypes for *Phymaturus*. (a) Part of the cytb gene tree corresponding to the *palluma* group. Numbers above branches represent posterior probabilities (>0.70), numbers below branches represent ML bootstrap support values (>70%). Numbers in parentheses within triangles represent the number of different haplotypes ($n = 117$) and number of sampled individuals, respectively, and on branch tips the number of sampled individuals sharing that haplotype. Dots on terminals represent taxa that were sampled for further 12S and nuclear gene sequencing. Darker branches correspond to candidate species; numbers after species names represent localities in Fig. 2 and Table 1, and stars represent localities where species from the *palluma* and *patagonicus* groups are found in syntopy. (b) Part of the cytb gene tree corresponding to the *patagonicus* group; numbers and symbols are as in Fig. 1a. Numbers in circles and capital letters on branches denote nodes that are used to describe results.

PRISM 3730XL automated DNA analyzer (PE Applied Biosystems, Foster City, CA) at the DNA Sequencing Center at BYU. Sequences are deposited in GenBank (accession numbers in Table 1).

2.4. Molecular markers

Mitochondrial cytb and 12S gene regions were amplified via Polymerase Chain Reactions (PCR) following Morando et al. (2003). For cytb, a ~800 bp fragment was amplified for all 407 individuals (406 ingroup plus one individual of *Liolaemus elongatus* – LJAMM-CNP 6169 – a member of the sister genus as outgroup), using the light-strand primers GluDGL (Palumbi, 1996) and the heavy-strand primer Cytb 3 (Palumbi, 1996); Cytb 2 (Palumbi, 1996) and Cyt.F.1 (Whiting et al., 2003) were used as internal sequencing primers. For a subset of 51 terminals (*palluma* group = 20; *patagonicus* group = 31; dots in Fig. 2), we amplified the following 11–12 gene regions: the mitochondrial 12S rRNA fragment, four nuclear protein-coding genes (NPCG), and seven

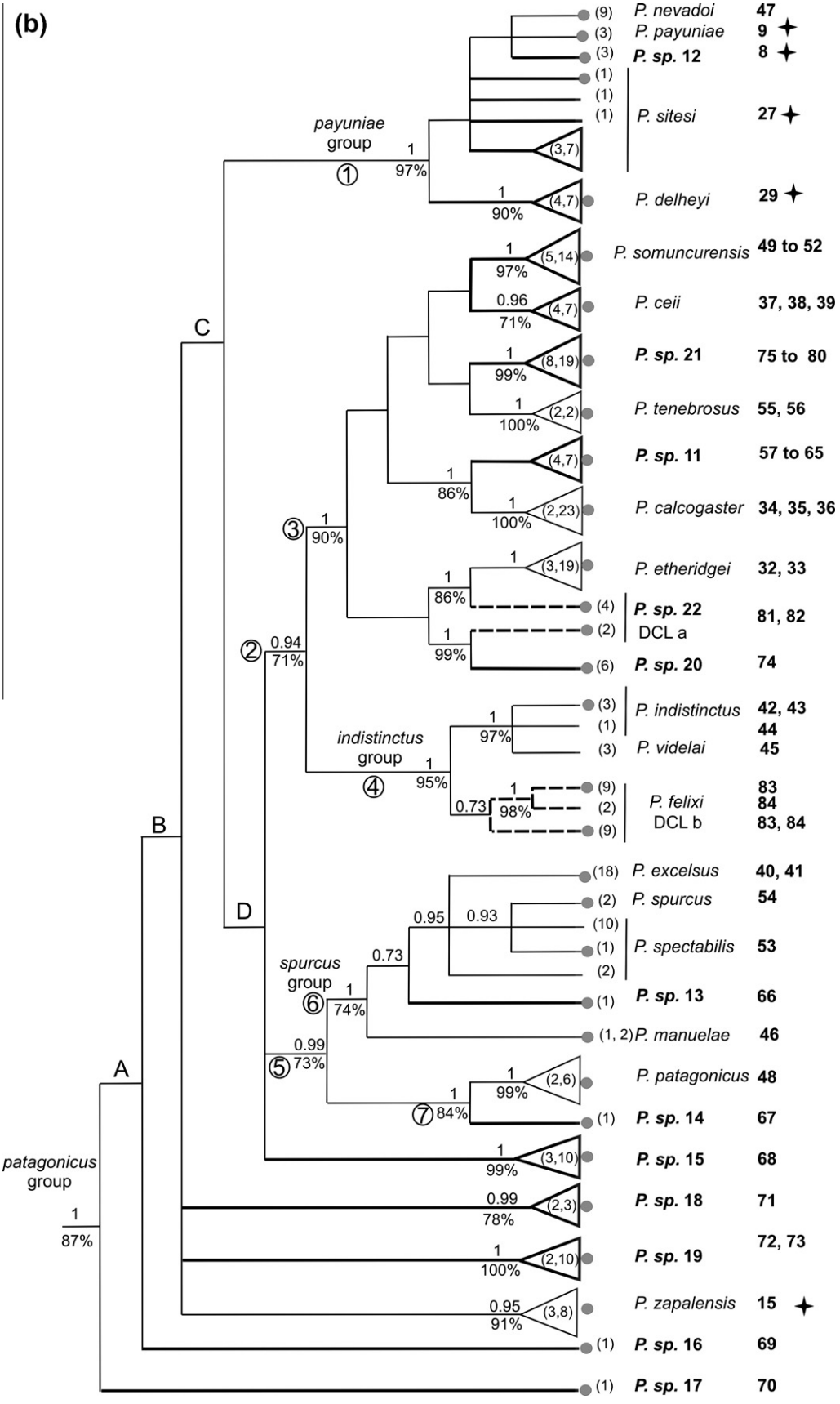

Fig. 1. (*continued*)

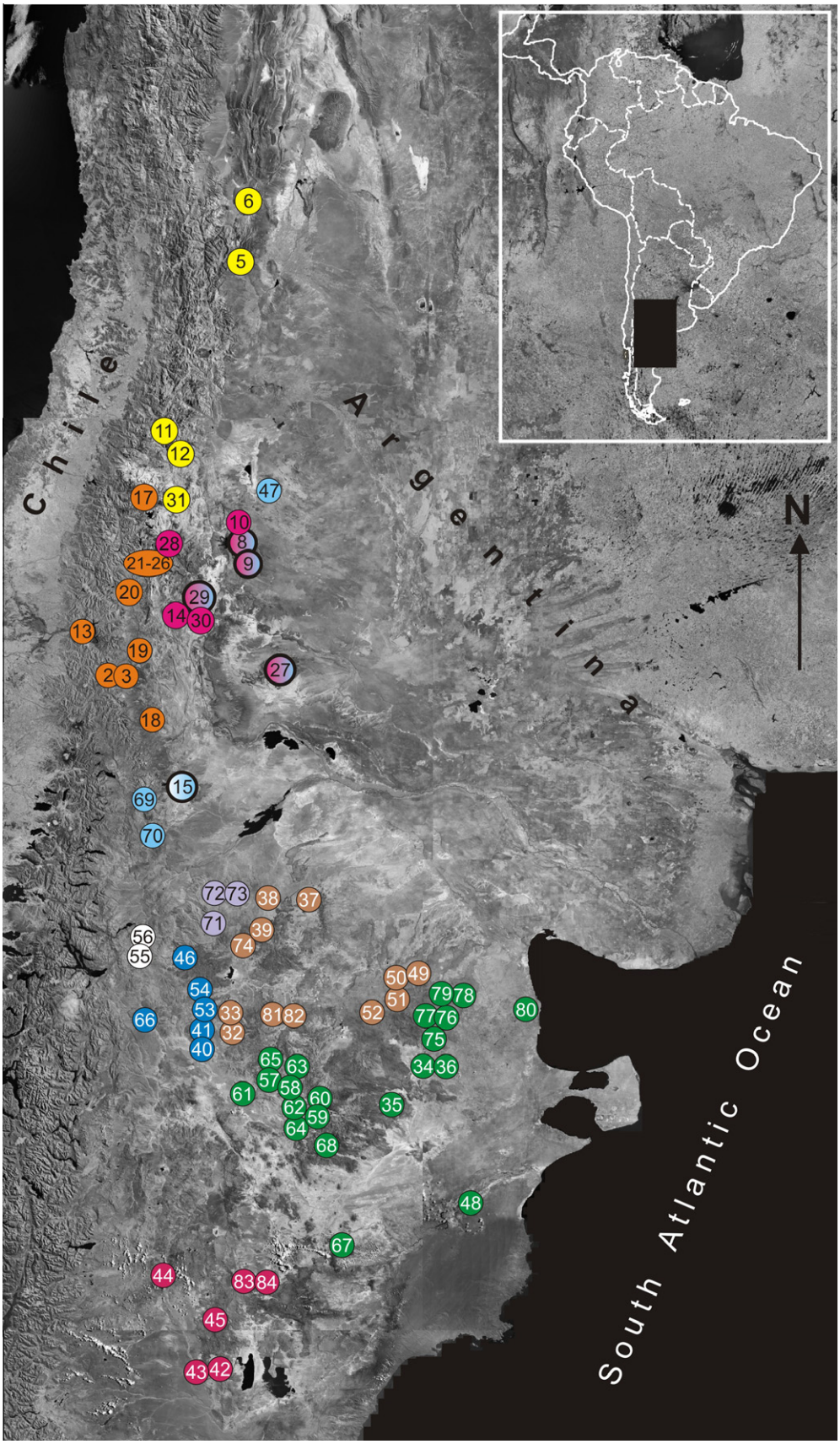

Fig. 2. Distribution map for species and candidate species of *Phymaturus* sampled for this study. Localities 1, 4, 7, 16 from the *mallimaccii* group are not shown as they are distributed further north in Argentina. *Phymaturus palluma* group: yellow = *verdugo* group, orange = *vociferator* group, pink = *roigorum* group; *P. patagonicus* group: brown = *somuncurensis* group, light blue = *payuniae* group, purple = *P. spp*. 21 and 22, blue = *spurcus* group, green = *calcogaster* group, red = *indistinctus* group. Circles with darker lines and two colors = sympatric localities between species of the *palluma* and *patagonicus* groups.

anonymous nuclear loci (Anonymous Nuclear Loci, ANL). We used the primers of Wiens et al. (1999) to amplify ~856 bp of 12S, and for the four NPCG regions we used: c-mos (primers G73 and G78 from Saint et al. (1998)), and PLRL, NT3, and PNN primers from Townsend et al. (2008).

To develop the ANL, we assembled a genomic library from one individual of *Phymaturus* following the general protocol of Noonan and Yoder (2009). Total genomic DNA was extracted using the Qiagen extraction kit, followed by restriction digests with HaeIII and DraI separately, of 5 μl of genomic DNA (50 μl total volume), and then ligated fragments with SNX primers. Ligated products were PCR amplified and visualized on a 1% agarose gel, and fragments of 800-1500 bp were excised and purified using the QIAGEN Gel Extraction kit, eluting with water. These selected fragments were then ligated into 0.5 μl of Blunt vector, transformed into competent *Escherichia coli* one shot TopoTA cells (Invitrogen), grown for 1.15 h at 37 °C and plated on agar plates containing Xgal. After ~15 h, colonies were individually picked with a pipette tip, cells were lysed, and plasmid inserts were amplified using the M13F and M13R primers.

A total of 202 colonies was amplified and run on a 1% agarose gel to check amplification and determine product size. Details of fragment size variation of the fragments and success rates will be published elsewhere in a comparative framework (Camargo et al., 2012a,b). We sequenced 202 colonies, and verified a subset of 45 fragments via Blast search on GenBank for this work. We used the megablast (highly similar sequences) and the more dissimilar sequences (discontiguous megablast) criteria. Those sequences that resulted on "non significant similarity was found" output were considered as ANL. Primers were developed for 27 loci that met ANL criteria in the searches, and these were tested on a subset of 15 species. Amplification employed a standard touchdown cycle: 95 °C–1.5 min; 10 [95 °C–35 s, 63 °C–35 s (−0.5 °C/cycle), 72 °C–1 min]; 10(95 °C–35 s, 58 °C–35 s, 72 °C–1 min); 15(95 °C–35 s, 52 °C–35 s, 72 °C–1 min); 72–10 min, and standard reaction conditions [1.0 μl $MgCl_2$, 2.0 μl dNTP, 2.0 μl Buffer, 1.0 μl each primer, 1.0 μl DNA template, 0.1 U *Taq* polymerase (Sigma); 14.5 μl total volume] for all loci. Of the 27 loci tested, we chose seven (Phy 38, 41, 60, 64, 84, 87 and 89) that amplified cleanly and were phylogenetically informative among the 15 test species, and amplified/sequenced these ANL for the remaining samples (Appendix B for primer sequences).

2.5. Sequence alignments

Sequences were edited and aligned using the program Sequencher 4.5 (Gene Codes Corp. Inc. 1995), and coding regions were translated into amino acids for confirmation of alignment. In cytb data, no indels or missing data were present. The alignment for the 12S data set was straightforward as there were few indels (all very small, Table 1) and missing data (coded as "?"). For the nuclear genes, heterozygous sites were coded using ambiguity codes. One ANL marker has 35% and 16% missing data for the *palluma* and *patagonicus* groups (Phy64), respectively; the others were almost complete (Table 1 summarizes information on fragment length, patterns of variation, and the number of terminals sequenced for each marker).

2.6. Data subset and model selection

All 13 markers were analyzed separately to identify possible contaminants, if necessary new extractions were prepared and re-sequenced. We first analyzed the following data subsets: (1) all 117 unique cytb haplotypes; (2) both mtDNA genes combined (cytb + 12S) for the subset of 51 selected terminals (see below). Considering that (a) the *palluma* and *patagonicus* clades are diagnosed by multiple morphological synapomorphies (Etheridge, 1995), (b) that previous morphological (Lobo et al., 2012b,d) and molecular (Espinoza et al., 2004; Morando, 2004) works also found support for the monophyly of these two clades, (c) that results from analyses (1) and (2) also recovered both clades with strong support, (d) that ANL markers are *Phymaturus* specific (do not amplify on *Liolaemus*) and (e) for computational efficiency; we performed other analyses separately for each of the two groups for the following data subsets: (3) both mtDNA genes combined (cytb + 12S), (4) all nuclear combined (NPCG + ANL); and (5) all data combined (mtDNA + nuclear genes).

The "all nuclear" and "all loci" analyses were performed in traditional concatenated analyses, but because this approach may not be reliable for inferring species relationships due to coalescent error (Carstens and Knowles, 2007; Kubatko and Degnan, 2009), we also repeated several of the above analyses using a model-based "species tree" method. We used the Bayesian method BEST (Liu and Pearl, 2007; Edwards et al., 2007) to estimate the posterior distribution of species trees from the posterior distribution of gene trees, for the "all nuclear" and "all loci" data sets.

Nucleotide substitution models were selected for each data partition using jModelTest 0.1.1 (Posada, 2008; Guindon and Gascuel, 2003). For the two mtDNA markers, a model with nst = 6 and rates = invgamma was used for the complete data set as well as for both groups separately. For the *palluma* group, most of the nuclear genes conformed to a model nst = 6 rates = equal, but for one ANL (Phy41) we used nst = 2 rates = equal, and for another (Phy64) we used nst = 6 rates = gamma. For the *patagonicus* group five nuclear loci (Phy41, 38, 87, NT3, PNN) conformed to the model nst = 6 rates = equal, the protein coding PRLR locus used nst = 2 rates = gamma, ANL markers Phy60, Phy64 and cmos used nst = 6 rates = gamma, Phy84 used nst = 6 rates = invgamma, and Phy89 used nst = 2 rates = equal (see Appendix C for details).

2.7. Phylogenetic analyses

We used the complete cytb data set (765 bp, no missing data) from 407 individuals and ran Collapse 1.2 (Posada, 2004) to generate a matrix of 117 non-redundant haplotypes. We used jModeltest v0.1.1 (Guindon and Gascuel, 2003; Posada, 2008) with the corrected Akaike information criterion to select a model of molecular evolution for this matrix (nst = 6 rates = invgamma). With this dataset (data subset 1: cytb non-redundant haplotypes) we performed ML analyses using RAxML (Stamatakis, 2006, ver 7.2.8) with 1000 rapid bootstraps, and two separate analyses with MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003) with 10.0×10^6 generation runs and a sample frequency = 1000, to estimate a tree of non-redundant haplotypes (Fig. 1a and b). Based on this tree, we selected 51 terminal taxa (gray dots in Fig. 1a and b) for subsequent sequencing of the other 12 markers (12S mtDNA and all nuclear regions). We ran a combined mtDNA analysis for these 51 terminal taxa (data subset 2) using RAxML with 1000 bootstrap replicates. Both the cytb gene tree for unique haplotypes (data subset 1) and the combined mtDNA gene tree for the 51 terminals (data subset 2), strongly support the morphological and previous molecular evidence for the two *Phymaturus* clades (*palluma* and *patagonicus*); thus we prepared separate matrices for these two groups for further phylogenetic analyses using a species from each clade as the outgroup to the other. The *palluma* group includes 20 terminals (10 described species) rooted to *P. patagonicus*, and the *patagonicus* group includes 31 terminals (16 described species, it was not possible to include *P. videlai* in these analyses) rooted to *P. mallimaccii*.

Phylogenetic analyses for all separate genes were based on maximum parsimony (MP) and Bayesian inference methods; and for the three data subsets (combined mtDNA, combined nuclear,

all genes) were based on maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference methods. Bayesian analyses were conducted using MrBayes v3.1.2, ML analyses were run using RAXML (ver 7.2.8), and MP bootstrap analyses were performed using PAUP* 4b5 (Swofford, 2002). Bayesian analyses on individual gene regions were run for 5.0×10^6 generations and for the four partitions for 10.0×10^6 generations and default priors. Two replicate searches were run for each Bayesian analysis. Trees generated prior to reaching stationarity were discarded as burn-in, and stationarity was assessed based on a plateau in a plot of log-likelihood values over time, and based on the standard deviation of split frequencies between the two replicate searches. The percentage of samples that recover any particular clade on this tree represents that clade's posterior probability; we consider $P \geqslant 95\%$ as evidence of significant support for a clade (Huelsenbeck and Ronquist, 2001; Huelsenbeck and Rannala, 2004). MP searches were performed with all characters equally weighted with 10,000 replicates and 10,000 pseudoreplicates for non-parametric bootstrap analyses (Felsenstein, 1985). ML search for the combined mtDNA data subset were as follow: -f a -x 12345 -p 12345 -#1000 -m GTRGAMMA -s infile -n outfile; while for the combined nuclear and all genes datasets we used: -f a -x 12345 -p 12345 -#1000 -q infilepart -s infile -n outfile -m GTRGAMMA. The level of genetic variation was not high for any of the markers (Table 1), thus no extra partitions (e.g. by codon) were considered (Weisrock et al., 2012). We performed standard Bayesian concatenated analyses for the combined mtDNA, combined nuclear and all genes data subsets, all of which were run for 10.0×10^6 generations and default priors. Nodes were considered well supported if PP and ML bootstrap values were $\geqslant 0.95$ and 70, respectively.

The BEST "species tree" estimation (v2.2; Liu and Pearl, 2007; Liu et al., 2008) was implemented for the all nuclear combined (data subset 4) and the all data combined (data subset 5), for the *palluma* and *patagonicus* groups. All analyses were run for 15 days (the upper limit permitted on the BYU supercomputer facility) and sampled every 10,000 generations. We used the same models of molecular evolution selected for concatenated ML and Bayesian analyses unlinking all parameter estimations, with the following settings: Brlenspr = clock:uniform; Thetapr = invgamma (3,0.003); GeneMuPr = uniform (0.2,1.8); PoissonMean = 5; PropTemp = 0.05, and setting the marker as haploid for mtDNA genes. Some runs were prematurely terminated and others seemed to converge after 100 million generations, but ESS values were not >200. Therefore, we also performed runs with simpler models of molecular evolution for nuclear genes and with different priors (Thetapr = invgamma [3,0.03 and 0.3]), and after checking for convergence (Tracer v1.5.0, Rambaut and Drummond, 2009) we used a burnin = 50% of the samples. We tested all nuclear genes for recombination using RDP: Recombination Detection Program v3.44 (Heath et al., 2006; Martin and Rybicki, 2000), and found no evidence of recombination.

3. Results

3.1. Sequence data and patterns of variation

The cytb and 12S mtDNA data matrices were complete. We were unable to obtain sequence data for some species from some nuclear genes (see Appendix C for details). Patterns of variability in the gene regions included in this study are sumarized in Table 1. The mtDNA gene regions were more variable than all nuclear regions, and within the latter, the ANL were more variable than the NPCG. Indel number was low in all markers, highest in the 12S region and low to absent in all other genes. All regions in both genomes showed sufficient haplotype variability to be phylogenetically informative (column H, Table 1).

3.2. Phylogenetic analyses

3.2.1. Cytb gene tree

Fig. 1 presents the Bayesian cytb gene tree for all non-redundant haplotypes of *Phymaturus*, with the northern *palluma* group depicted in Fig. 1a, and the southern *patagonicus* group in Fig. 1b. The general topology was very similar to the one obtained in the ML analyses, no statistically significant incongruences were found. We identify on each clade the five localities where members of both groups co-occur (Fig. 2, locs. 8, 9, 15, 27 and 29), in northern Neuquén and southern Mendoza provinces (stars in Fig. 1a and b). No morphological evidence of hybridization between *palluma* and *patagonicus* species groups was found in these localities, nor did cytb show evidence of introgression between the two species groups.

The Bayesian tree for the *palluma* group (Fig. 1a, PP = 1, BS = 83%) has a basal polytomy that includes *P. querque* (Fig. 2, loc. 15), the most southerly distributed species of this group, the *vociferator* group, and node 1 [*mallimaccii* + (*verdugo* + *roigorum*)], although the latter is not strongly supported and is shown to facilitate description of this topology. Uncorrected cytb distances among these clades range from 4.5% to 5%. The *vociferator* group (PP = 1, BS = 89%) includes two described species (*P. vociferator* and *P. dorsimaculatus*) and five candidate species, two of which (*P. sp.* 2 and *P. sp.* 6) are paraphyletic. *Phymaturus sp.* 2 (Fig. 2, loc. 17) from Laguna del Maule (Chile), is geographically isolated from the six localities (Fig. 2, locs. 21–26) where *P. sp.* 6 is found in northern Neuquén Province (Argentina). The other three candidate species (*P. spp.* 3–5; Fig. 2, locs. 18–20) are geographically isolated, but there is no support for relationships among terminals of this clade.

Within the *verdugo* + *roigorum* group (PP = 0.97, BS = 76%) there are three described species (*P. cf. palluma*, *P. roigorum*, *P. verdugo*) and five candidate species (*P. spp.* 1, 7, 8, 9, and 10). Although this clade's geographic distribution is wide and includes isolated and geographically distant areas extending from northern (Fig. 2, locs. 5 and 6) to southern Mendoza (Fig. 2, locs. 11 and 12), crossing the Colorado River to northern Neuquén (Fig. 2, locs. 14, 28, 29 and 30), and from the isolated area of Auca Mahuida (Fig. 2, loc. 27), in general we do not recover strongly supported lineages corresponding to described or candidate species. Lastly, the northernmost *mallimacci* group (Fig. 1a, PP = 0.96, BS = 80%), includes four described species *P. laurenti*, *P. mallimaccii*, *P. punae*, and the recently described *P. extrilidus*.

The tree corresponding to the *patagonicus* group (Fig. 1b) does not have statistical support for any of the deepest relationships (nodes A–D, again presented for ease of interpretation), but in the more nested region of the tree, some geographically concordant clades are recovered with strong support. One of these is the *payuniae* group (node 1; PP = 1, BS = 97%) confined to the northernmost part of the *patagonicus* group distribution (Fig. 2, light blue localities), and includes four described and one candidate species, *P. sp.* 12, with no support for monophyly of *P. sitesi*. Another moderately supported clade (node 2, PP = 0.94, BS = 71%) recovers as sister clades 3 (PP = 1, BS = 90%) and 4 (PP = 1, BS = 0.95%), which include eight named species and several other differentiated haplotypes. Node 3 includes *P. somuncurensis*, *P. ceii*, *P. tenebrosus*, *P. calcogaster*, *P. etheridgei* and four other lineages, one of which, candidate species *P. sp.* 22, includes divergent haplotypes that fit our criteria of a Deep Conspecific Lineage (Fig. 1b, DCLa, locs. 81 and 82), with an uncorrected pairwise difference of 2.47%. Within the *patagonicus* group, examples of low pairwise

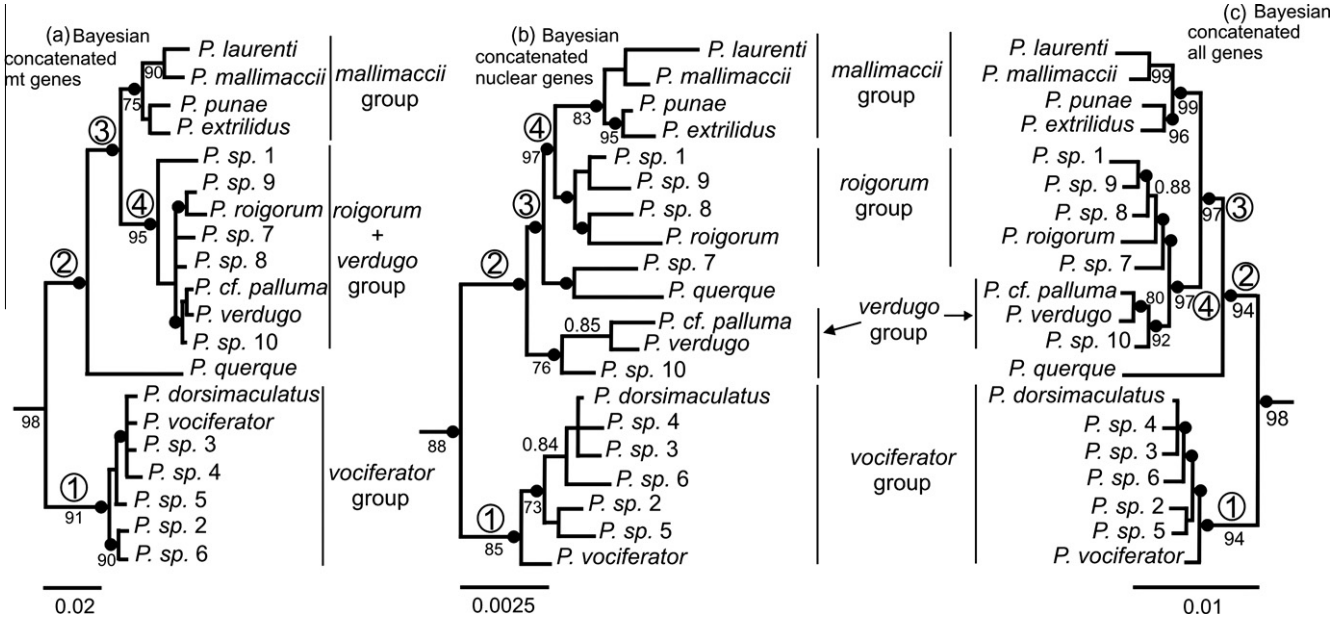

Fig. 3. Multigene concatenated Bayesian trees for the *P. palluma* group, including: (a) all mt genes; (b) nuclear genes; and (c) all genes. Numbers above branches represent posterior probabilities between 0.84 and 0.94, numbers below branches represent ML boostrap support values, dots on root nodes represent posterior probability values between 0.95 and 1.00; numbers within circles designate nodes of interest.

cytb genetic distances between described species range from 0.91% (*P. nevadoi* vs *P. payuniae*), 1.30% (*P. somuncurensis* vs *P. ceii*) and 1.69% (*P. manuelae* vs *P. spurcus*).

The three southernmost distributed species of *Phymaturus* are recovered as a clade (node 4, the *indistinctus* group, PP = 1, BS = 95%), with *P. felixi* representing a second example of DCL (Fig. 1b, DCL b, locs. 83 and 84), with an uncorrected pairwise difference of 1.56%. Node 5 (PP = 0.99, BS = 73%) recovers the *spurcus* (node 6, PP = 1, BS = 74%) and *patagonicus* groups (node 7; PP = 1, BS = 84%) as sister taxa. The former is confined to a small area in southwestern Rio Negro province, with some of the described species in very close geographic proximity. The *patagonicus* group (node 7) includes two terminals (*P. patagonicus* + *P. sp.* 14); several other candidate species (15–19) and *P. zapalensis*, are recovered as strongly supported clades, but without statistical support for their relationships.

Based on this gene tree (Figs. 1a and b), we selected a subset of terminals for subsequent 12S and nuclear gene sequencing. We do not have any further sequences of *P. videlai* (Fig. 1b, node 4), due to its very recently discovery and description as a new species.

3.2.2. Concatenation analyses

3.2.2.1. MtDNA ML gene tree for Phymaturus. ML analyses including all 51 *Phymaturus* terminals with cytb + 12S concatenated gene data, recovered the *palluma* and the *patagonicus* groups with 98% bootstrap support (BS), and showed almost identical results as Bayesian analyses based on these two groups analyzed separately; thus we included bootstrap support values on trees depicting these results (see next sections).

3.2.2.2. Phymaturus palluma group.

3.2.2.2.1. MtDNA gene tree. Bayesian analysis of the concatenated mitochondrial markers (cytb and 12S) recovered a more fully resolved tree (Fig. 3a) than the cytb gene tree, especially with regard to the deep nodes, and there are no statistically significant conflicts between them. At the deepest levels, the *vociferator* group (node 1; PP = 0.97, BS = 91%) is the sister clade to all other species and terminals that are recovered in a well resolved second group (node 2; PP = 0.98, BS = 61%). Within the *vociferator* group, the clade (*P. spp.* 2 and 6) is distinct from all other terminals (PP = 0.98, BS = 90%), and a second moderately supported clade recovers a polytomy that includes *P. dorsimaculatus*, *P. vociferator*, *P. spp.* 3 and 4 (PP = 0.97, BS = 62%); the placement of *P. sp.* 5 is not resolved. At node 2 (PP = 0.98, BS = 61%), *P. querque*, the southernmost distributed species of the *palluma* group, is recovered with moderate support as the sister taxon of the (*mallimaccii* + (*roigorum* + *verdugo*)) clade (node 3; PP = 0.90, BS = 64%), and within the *mallimaccii* clade (PP = 1, BS = 75%) *P. laurenti* is sister to *P. mallimaccii* (BS = 90%) and *P. punae* is sister to *P. extrilidus* (BS = 62%). The *roigorum* + *verdugo* clade (node 4; PP = 1, BS = 95%) includes two moderately supported clades, the (*P. sp.* 9 + *P. roigorum*) (PP = 0.92) and the (*P. palluma*, *P. verdugo,* and *P. sp.* 10) (PP = 0.99, BS = 67%).

3.2.2.2.2. Nuclear combined. Analyses of the concatenated nuclear genes (Fig. 3b) recovered one well-supported clade, the *vociferator* (node 1; PP = 1, BS = 88%) group, and several other clades nested in node 2 (PP = 0.98). Within the *vociferator* group, *P. vociferator* is recovered as the basal terminal, and *P. dorsimaculatus* nested within a well-supported clade containing five different lineages (*P. spp.* 2–6; PP = 1, BS = 85%). The second large clade (node 2, PP = 0.98) recovers the *verdugo* group (PP = 0.99, BS = 76%) as the sister clade of all others. The small clade of (*P. querque* + *P. sp.* 7) (PP = 0.99), is sister (node 3; PP = 1) to the *roigorum* (PP = 0.98) + *mallimaccii* (PP = 1, BS = 83%) groups (node 4; PP = 1, BS = 97%). The major strongly supported difference between the mtDNA and nuclear topologies is the placement of the *verdugo* group (mtDNA = nested within a paraphyletic *roigorum* group, vs sister to a larger group [node 3] that includes the *roigorum* group as sister to the *mallimaccii* group).

3.2.2.2.3. All genes combined (mtDNA + nuclear). The Bayesian analyses for all genes combined (Fig. 3c) recovered similar results to the concatenated nuclear genes with two exceptions. First, the bayesian well-supported (*P. querque* + *P. sp.* 7) clade of the nuclear gene tree is not recovered in the "all genes" topology; *P. querque* is instead recovered as sister (node 2) to the (*mallimaccii* + (*verdugo* + *roigorum*)) clade (node 3), as in the mtDNA gene tree (com-

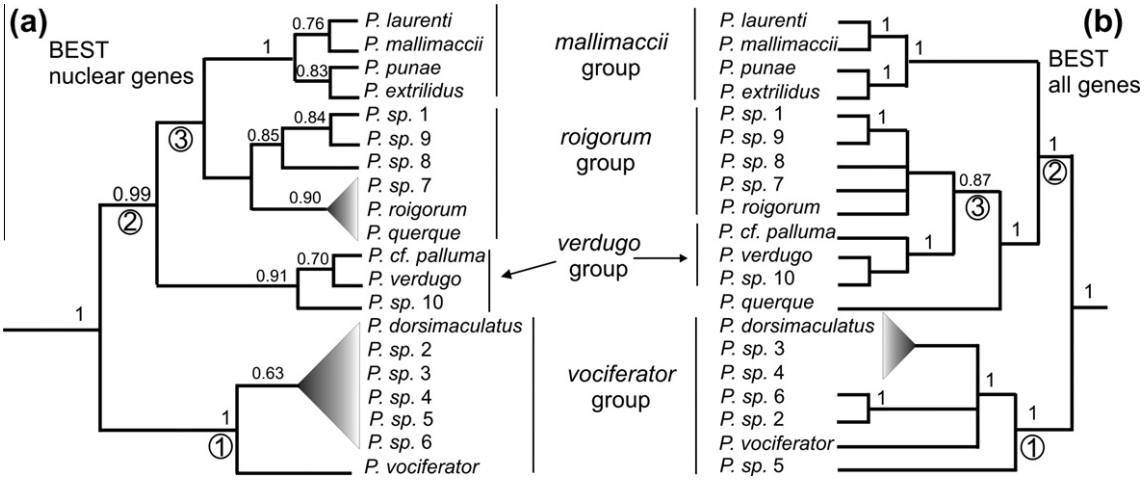

Fig. 4. BEST analyses of the *P. palluma* group; (a) consensus tree from only nuclear BEST analyses and (b) consensus tree from all-genes BEST analyses. Numbers above branches represent posterior probabilities >0.50.

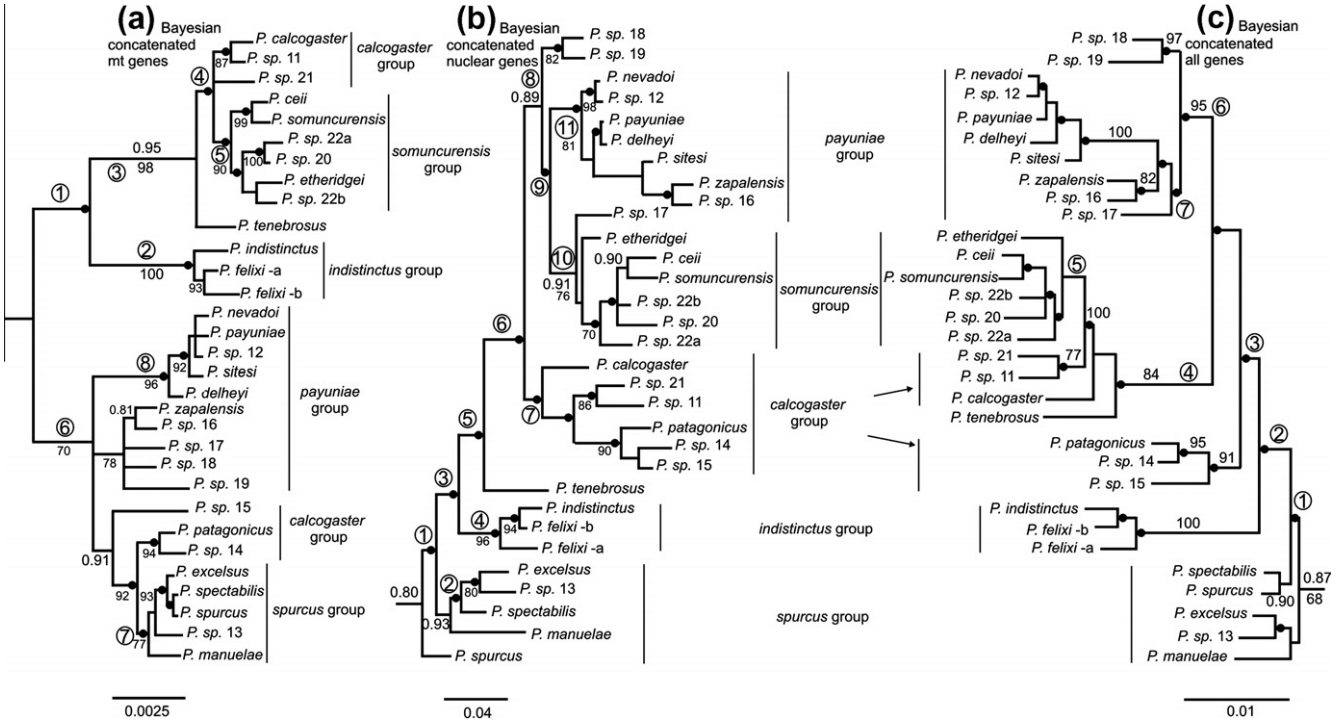

Fig. 5. Multigene concatenated Bayesian trees for the *P. patagonicus* group, including: (a) all mt genes; (b) nuclear genes; and (c) all genes. Numbers above branches represent posterior probabilities between 0.80 and 0.94, numbers below branches represent ML boostrap support values, dots on root nodes and numbers within circles are as in Fig. 3.

pare 3a and 3c topologies). Second, the *verdugo* group is recovered as sister (node 4, PP = 1, BS = 97%) to a moderately supported *roigorum* clade (PP = 0.98) rather than sister to *roigorum* + *mallimaccii* groups. In this regard the all genes topology is more similar to the mtDNA gene tree, which recovers the *verdugo* group as part of an unresolved *roigorum* group (Fig. 3a vs 3c topologies).

3.2.2.2.4. BEST analyses. To obtain a BEST species trees for the nuclear data set, we started 27 different analyses (different parameter combinations), but only one run based on simple models of molecular evolution reached 91.420.000 generations with an ESS Lnl = 551.536/351.437. Perhaps missing data (Appendix B) and limited sampling for some species caused premature termination of some runs for this data set in some analyses. However, as most topologies were similar to those obtained with the concatenated analyses, we report these results as schematic species tree hypotheses (Fig. 4a), because we do not rely on other parameter estimates (besides the topology) as they had ESS values <200. Although support values were generally lower, the BEST analysis (Fig. 4a) recovered a very similar topology to the Bayesian all nuclear analysis (Fig. 3b). The *vociferator* clade is less resolved, but *P. vociferator* is recovered as the sister terminal of the other species (node 1, PP = 1). Similarly, the *verdugo* clade is recovered as sister to all other terminals (node 2, PP = 0.99), but BEST and Bayesian analyses differ in placement of *P. querque* and *P. sp.* 7; BEST recovers *P. roigorum* with these terminals with some support (Fig. 4a; PP = 0.90), in contrast to their well-supported position external to the *roigorum* group in the Bayesian topology (Fig. 3b).

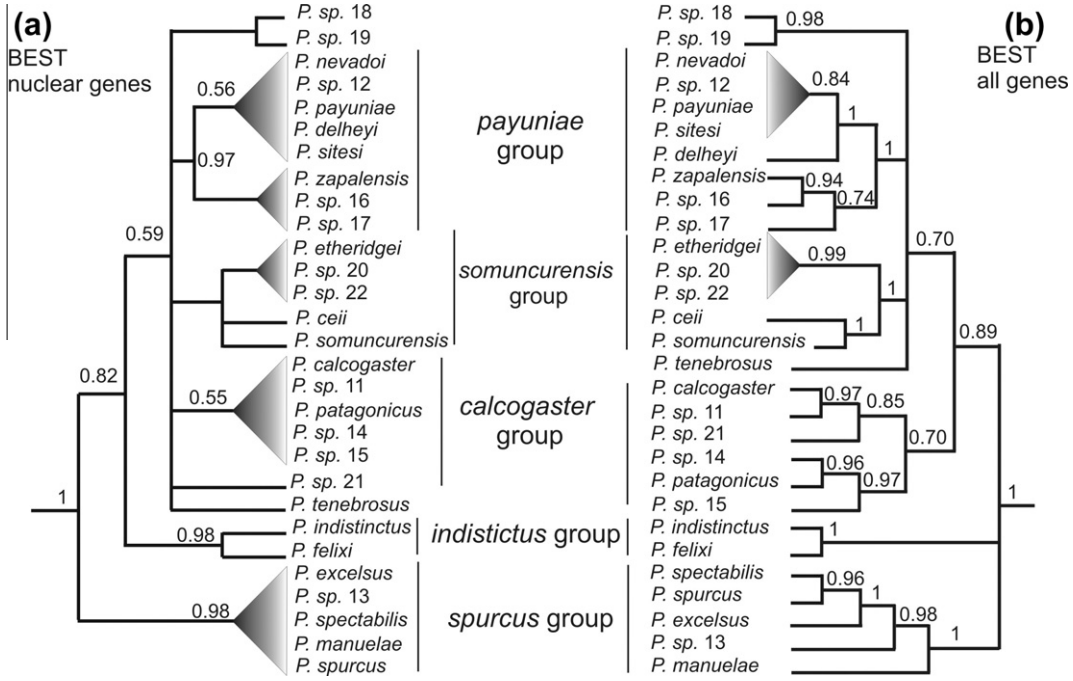

Fig. 6. BEST analyses of the *P. patagonicus* group; (a) consensus tree from only nuclear BEST analyses and (b) consensus tree from all-genes BEST analyses. Numbers above branches represent posterior probabilities >0.50.

The BEST species trees obtained for all genes, based either on models of evolution for nuclear genes as selected by jModeltest, or with simple models, were obtained from runs up to 164,340,000 generations and attained LnL ESS value = 172.121/238.944; none of the trees we saved had strongly supported nodes incongruent with trees recovered from the "nuclear only" data set. We illustrate a BEST species tree (Fig. 4b) that is similar topologically to the Bayesian tree (Fig. 3c) in that: (1) the *vociferator* clade (node 1) is well supported and recovered as the sister group to another well-supported clade (node 2) that includes all other terminals (Figs. 3c vs 4b); and (2) the *roigorum* and *verdugo* clades are recovered as sister groups (node 3) but with less support in the BEST tree. Major differences between BEST and Bayesian topologies include: (1) the position of *P. querque*: not the sister taxon to all other terminals subtended by node 2 in BEST, while sister to these in the Bayesian topology (Fig. 3c vs 4b); and (2) well supported but conflicting placement of several terminals within the *vociferator* clade (see node 1 in both topologies).

3.2.2.3. Phymaturus patagonicus group.

3.2.2.3.1. MtDNA gene tree. The mtDNA concatenated gene tree (Fig. 5a; PP and ML values for this, the "nuclear" and the "all genes" trees are as in Fig. 3) recovers two main clades, but with weak support. Node 1 (PP = 1), which includes the *indistinctus* group (node 2, PP = 1, BS = 100%, with two divergent lineages of *P. felixi*) as sister to a well-supported node 3 (PP = 0.95, BS = 98%), within which *P. tenebrosus* is sister to all other terminals (node 4, PP = 1). The *somuncurensis* group (node 5) is recovered with strong support (PP = 1, BS = 90%), but the *calcogaster* group is not recovered as a clade. The second main clade (node 6, BS = 70%) has no Bayesian support and includes species from the *payuniae*, *calcogaster* and *spurcus* groups. Only the *spurcus* group (node 7, PP = 0.99, BS = 77%) and the northern part of the *payuniae* clade (node 8, PP = 0.99, BS = 96%) are strongly supported. This concatenated mitochondrial (cytb and 12S) gene tree is more fully resolved than the cytb gene tree (Fig. 1b), especially with regard to the deep nodes, and there are no statistically significant conflicts between them.

3.2.2.3.2. Nuclear combined. Analyses of the *patagonicus* combined nuclear genes (Fig. 5b) recovered poorly-supported deep nodes but several well-supported nested clades. These well-supported clades include node 4 (the *indistinctus* group, PP = 1, BS = 96%), the (*P. excelsus* + *P.* sp. 13) clade, the *payuniae* group (node 11, PP = 1, BS = 81%), and a few more nested nodes. Although only supported by a high Bayesian PP value (1), the basal position of most of the species of the *spurcus* group as the sister clade to all others (node 1) contrasts sharply with its nested position in the mtDNA tree (Fig. 5a).

Within the *indistinctus* group (node 4, PP = 1, BS = 96%), the most southerly distributed species of the genus (Fig. 2, locs. 42, 43, 44, 45, 83, 84), the two lineages within *P. felixi* are paraphyletic with respect to *P. indistinctus* (PP = 1, BS = 94%). Within the *calcogaster* group (node 7, PP = 0.95), distributed east and south of the Somuncura Plateau, *P. calcogaster* is the most basal taxon (PP = 1), *P. sp.* 21 is sister to *P. sp.* 11 (PP = 1, BS = 86%) and *P. patagonicus* is sister (PP = 1, BS = 90%) to (*P. sp.* 14 + *P. sp.* 15). Species distributed north and west of the Somuncura Plateau are recovered in the *somuncurensis* group (node 10, PP = 0.91, BS = 76%) with moderate support. *Phymaturus sp.* 22 had very divergent cytb haplotypes (DCL a; Fig. 1b) that are recovered as paraphyletic with nuclear genes (PP = 1). Node 8 recovers two lineages (*P. sp.* 18 and *P. sp.* 19, PP = 1, BS = 82%), that are not related to any described species, as sister to (*payuniae* + *somuncurensis*) groups (node 9, PP = 1). The northernmost distributed group of the *patagonicus* clade is the *payuniae* group (node 11, PP = 1, BS = 81%), that with this data set is more inclusive than with the cytb gene tree (Fig. 1b). The two northernmost distributed lineages (*P. nevadoi* + *P. sp.* 12, Fig. 2, locs. 47 and 8) of this group are sister taxa (PP = 1, BS = 98%) and basal to the rest of the species group. The two lineages just to the south (*P. payuniae* + *P. delheyi*; Fig. 2 locs. 9 and 29) are recovered as sister (PP = 0.97) and basal to *P. sitesi* (the next lineage to the south, Fig. 2, loc. 27) + (*P. zapalensis* + *P. sp.* 16), the

two southernmost distributed species of this group (Fig. 2, locs. 15 and 69).

3.2.2.3.3. All genes combined (mtDNA + nuclear). The Bayesian all genes analyses (Fig. 5c) do not recover the *spurcus* group as a clade; instead three of these species (*P. manuelae* + (*P. excelsus* + *P. sp.* 13)) are recovered as a clade basal to the rest of the *patagonicus* group (node 1, PP = 1), followed by (*P. spectabilis* + *P. spurcus*) (PP = 0.9). The ML all genes analyses although recovers this group as a clade and basal to all the others in the *patagonicus* group, it does not have bootstrap support. Node 2 recovers the *indistinctus* group (PP = 1, BS = 100%), with *P. felixi* as paraphyletic, as sister to the clade subtended by node 3 (PP = 1). Node 3 recovers three species of the *calcogaster* group (*P. patagonicus, P. sp.* 14 and *P. sp.* 15) as sister to the rest of the *patagonicus* group (PP = 1, BS = 91%). The other three species of the *calcogaster* group are recovered within node 4, which includes *P. tenebrosus* as basal taxon and a clade nesting the *somuncurensis* group (node 5). Relationships within the *somuncurensis* group are identical to those recovered in the nuclear-only tree. Node 6 recovers the *payuniae* group (node 7, PP = 1) including *P. sp.* 17 (the southernmost distributed of this group; Fig. 2, loc. 70) as the most basal taxa (PP = 1), as sister to *P. sp.* 18 + *P. sp.* 19 (PP = 1, BS = 95%). Phylogenetic relationships within the *payuniae* group differ substantially from the nuclear-only approach, as the topology seems to be exactly the reverse of the divergent events depicted in Fig. 5b.

3.2.2.3.4. BEST analyses. The BEST nuclear-only species tree, based either on models of evolution for nuclear genes as selected by jModeltest, or with simple models, were obtained from runs up to ~111 million generation (LnL ESS values for simple models = 551.536/351.437). In general, both sets of saved trees recovered very similar topologies for relationships among main clades, albeit with some weakly-supported differences. The BEST nuclear tree (Fig. 6a) is less resolved than the concatenated tree (Fig. 5b), but there are three statistically supported differences between them: (1) the *spurcus* group is recovered as monophyletic with strong support (PP = 0.98); (2) the two divergent lineages within *P. felixi* are recovered as sister taxa (shown as a single branch in Fig. 6a); and (3) *P. sp.* 17 is recovered within the *payuniae* group.

BEST analyses with all genes were run for up to ~164 million generations, with simpler models of evolution for nuclear genes (LnL ESS values = 172.121/238.944), and recovered more nodes with high support values than those run with more complex models of evolution (as selected by jModeltest). A third run with 127 million generations, but with theta prior = 0.03 and simpler models of evolution, attained LnL ESS values >200, and recovered a topology similar to the previous two analyses but with considerably lower support values at all nodes (results available upon request to the first author). Possibly some missing data and/or only one individual for some species made it difficult for BEST to attain ESS values above 200, thus we do not rely on other BEST parameter estimates but focus on the topology. Topologies in all runs showed no significant differences, so we only include schematic trees (trees re-drawn from the original to emphasize topology and ignore branch lengths due to low ESS values) for BEST species tree estimation. The BEST all genes tree (Fig. 6b) resolved four main differences compared with the concatenated all genes tree (Fig. 5c). BEST recovers: (1) a well-supported and well-resolved *spurcus* clade (PP = 1); (2) two divergent lineages within *P. felixi* are recovered as sister terminals (a single line again); (3) the *calcogaster* group is weakly recovered as a clade (PP = 0.70); and (4) *P. sp.* 17 is sister to (*P. zapalensis* + *P. sp.* 16); PP = 0.74).

3.2.2.3.5. Evidence of hybridization within the patagonicus group. Three species within the *spurcus* group, *P. excelsus*, *P. spectabilis* and *P. spurcus,* have very restricted and geographically proximate distributions (locs. 40, 41, 53 and 54, Fig. 2), and *P. spurcus* is sympatric with *P. excelsus* at loc. 40 (Fig. 2) according to Lobo and Quinteros (2005a). All individuals from these species were almost identical in both mtDNA genes (cytb and 12S). For three ANL (Phy89, Phy84, Phy38) and one NPCG (PLRL) that were informative for this issue, we were able to obtain extra sequences (sample sizes = 13, 12, 11, 11, respectively), for which some individuals were fixed for different nucleotides, while others were heterozygous in the same positions, suggesting either evidence of hybridization–introgression or retention of ancestral polymorphisms. A detailed study comparing morphology, color pattern, and additional genetic markers is beyond the scope of this work, but it is the focus of ongoing research modeled after the study of Olave et al. (2011).

4. Discussion

4.1. Non-monophyletic mtDNA gene trees and species limits

Recovery of para- or polyphyletic patterns in a gene tree, with respect to accepted species boundaries, reveals aspects of the biology of the focal group of interest that have important consequences for evolutionary inference (Funk and Omland, 2003). These authors documented that 23% of surveyed animal species were characterized by non-monophyletic mitochondrial gene trees, most likely due to: (1) inaccurate taxonomy; (2) mtDNA introgression between species; or (3) incomplete lineage sorting. Incomplete lineage sorting is expected when species divergence is recent and/or population sizes were very large, whereas introgression occurs when interspecific hybridization results in transfer of mtDNA across species boundaries. Interspecific gene flow is likely to be common because 10% of animal species are estimated to hybridize in nature (Mallet, 2007), and in at least one well-studied species rich clade of animals (freshwater fishes ["darters"] of the clade Etheostominae), 12.5% of all species (sampled 245 of 248 species) were characterized by heterospecific mtDNA (Near et al., 2011). Both asymmetrical mtDNA gene flow and incomplete sorting may produce similar gene tree topologies (Wakeley, 1996; Holder et al., 2001). However, mtDNA should sort more rapidly to reciprocal monophyly than nuclear DNA due to its smaller effective population size (Avise et al., 1987; Moore, 1995).

The cytb gene tree including the unique haplotypes from all sampled individuals (Figs. 1a and b) recovered some non-monophyletic patterns and some unresolved, potentially non-monophyletic patterns based on currently accepted taxonomy and candidate species (as used by Morando et al. (2003)). Although most of these non-monophyletic patterns are not statistically supported by ML bootstrap values, we devote some discussion to a consideration of these patterns and their possible causes.

Most of the observed non-monophyly or unresolved patterns are within the *palluma* group (Fig. 1a). As an example, the *vociferator* clade shows a basal polytomy including haplotypes from the northernmost distributed species of this group (Fig. 1a: *P. spp.* 2 and 6, Fig. 2: locs. 17 and 21–26, respectively). This area is a hypothetized refugium for vertebrates (Sérsic et al., 2011), and the remaining species from this group are located further south at the presumed margin of the last maximum glacial extension, and coincident with a hypothetized post-LGM vertebrate dispersal route (Sérsic et al., *op. cit.*). It is possible that the northern taxa (*P. spp.* 2 and 6) persisted in a refugial area, while the southern species dispersed and diverged more recently from a reduced number of individuals in an advancing front (Hewitt, 2000), thus quickly attaining cytb monophyly; which is consistent with expectations under models of recent speciation via peripheral isolation (Harrison, 1991; Omland, 1997). Alternatively all of these terminals correspond to one widespread but geographically highly structured species with almost no interpopulation gene flow. These alterna-

tives yield very clear and largely non-overlapping genetic predictions: (1) if nuclear markers such as microsatellites reveal little or no gene flow between these taxa, hypotheses of recent speciation events are supported and not enough time has elapsed to attain cytb reciprocal monophyly; or (2) evidence of gene flow indicates a widely distributed species with highly structured populations.

Other such patterns are observed in the (*verdugo* + *roigorum*) clade, one unresolved pattern is between *P. cf. palluma* haplotypes, the most northern distributed individuals from this clade (Fig. 2, locs. 5 and 6); the second case is one individual from *P. roigorum* (from loc. 8), recovered within *P. sp.* 7 while the third case is *P. roigorum* paraphyly with respect to *P. sp.* 9. The last case is one individual of *P. sp.* 9 recovered within *P. sp.* 1; these two species are in very close proximity and on this basis we suggest the likely (but not exclusive) cause is introgressive hybridization. An alternative explanation for these patterns within the (*verdugo* + *roigorum*) clade is that all these terminals constitute one geographically structured species.

Within the *patagonicus* group (Fig. 1b), there are three cases of non-monophyly/unresolved patterns. In one case within the *payuniae* group, *P. sitesi* is unresolved with respect to *P. nevadoi*, *P. payuniae* and *P. sp.* 12. *Phymaturus sitesi* (Fig. 2, loc. 27) is geographically isolated from the other three more nested species (Fig. 2, locs. 8, 9 and 47), and again there are two possible explanations for this pattern: either all of these terminals comprise one highly geographically structured species, or *P. sitesi* has a larger population size and maintains higher genetic diversity, while the three nested species are recently diverged and founded from small ancestral populations that rapidly sorted to mtDNA monophyly.

The second non-monophyletic pattern is observed with *P. sp.* 22 (DCL a) haplotypes, two of which are closely related to *P. sp.* 20 and the remaining 4 are more closely related to *P. etheridgei* than with each other. Limited taxonomic knowledge may be one cause for this pattern; although *P. sp.* 22 individuals differ from the other terminals in this clade in coloration, intensive studies are needed to exclude the alternative possibility that *P etheridgei* and *P. sp.* 20 are sympatric in this area but differ very little in color patterns. Lastly, a third non-monophyletic pattern is in the *spurcus* group; *P. spectabilis* is paraphyletic with respect to *P. excelsus* and *P. spurcus*, both of which are restricted to small geographic areas. Evidence suggests that hybridization may be one of the most plausible explanations for the paraphyly of *P. spectabilis*; the mtDNA haplotypes are identical or very similar among these species, while color patterns are very different. Further, nuclear genes suggest some level of differentiation between some of these taxa and some level of hybridization in others, so again integrative studies are needed to assess species limits and ongoing or historical interactions.

4.2. Phylogenetic relationships and evolutionary processes

Our results present the first comprehensive molecular hypothesis for phylogenetic relationships for the genus *Phymaturus*, including the majority of described species and 22 candidate species. Previous molecular studies centered on the family level relationships included only two (Schulte et al., 2000), seven (Espinoza et al., 2004: *palluma* group = 4 and *patagonicus* group = 3), and 12 species (Morando, 2004: six species from each group). Based on morphological data, Lobo and Quinteros (2005b) presented phylogenetic hypotheses for five or possibly six different species from the *palluma* group, and 11 species from the *patagonicus* group. Recently Lobo et al. (2012d) presented and updated morphologically based phylogenetic study of the genus, including 17 described species for the *patagonicus* group and 10 for the *palluma* group. Although the terminals included in Lobo et al. (2012d) are not exactly the same as those used in this work, the majority of described species are included in both contributions, the main difference being that Lobo et al. (*op. cit.*) included nine candidate species for the *palluma* group, while we included 10 candidate species for this group plus 12 for the *patagonicus* group. We first discuss our main results, and then the main congruences and incongruences between the hypotheses presented by Lobo et al. (2012d) and ours.

For the *palluma* group we find strong evidence for three clades: *vociferator*, *verdugo*, and *mallimaccii* groups, and moderate support for the *roigorum* group (Figs. 3 and 4), with the southernmost distributed clade (*vociferator*) as the most basal one, and the northernmost distributed one (*mallimaccii*) as highly nested. The Bayesian trees show some strongly supported topological differences that are weakly or not supported by ML analyses; two of which are in the *palluma* group: 1-The placement of *P. querque* with mtDNA as sister to the ((*verdugo* + *roigorum*) + *mallimaccii*) clade in the mtDNA gene tree (Fig. 3), contrasts sharply with its placement within the *roigorum* group and sister to *P. sp.* 7 (its geographically nearest neighbor; Fig. 2, locs, 15 and 27 respectively) in the nuclear tree. A similar contrasting pattern is also recovered with the BEST analyses (Fig. 4). 2-The placement of the *verdugo* group, which is recovered with the *roigorum* group in the mtDNA tree (Fig. 3a), while with nuclear genes, the *roigorum* group is closer to the *mallimaccii* group and the *verdugo* group is external to these (Figs. 3b and 4a). Since we used only one individual per species or candidate species, and given that some species boundaries are not well defined (previous section), it is possible that some incongruent results between partitions and methods may be due to incomplete lineage sorting or given the high frequency of mtDNA introgression in animals (Funk and Omland, 2003), gene flow between some of these taxa is an alternative explanation and nuclear genes may be showing the real sister relationship between these taxa.

In general, within the *patagonicus* group we found strong evidence for three groups: *indistinctus*, *payuniae*, and *spurcus* (Figs. 5 and 6), and moderate support for the *somuncurensis* and *calcogaster* groups, with no clear support for the placement of *P. tenebrosus* and *P. spp.* 18 and 19 within any of these five groups. The composition of the *calcogaster*, *somuncurensis*, *spurcus*, and *payuniae* clades was different with the alternative data subsets. Phylogenetic relationships among these groups with the concatenated nuclear and all genes analyses (Figs. 5b and 5c respectively) are generally resolved and congruent, with few exceptions. First, the *spurcus* group is not recovered as a clade with either analysis. Second, the *calcogaster* group is not recovered as a clade in the all genes analyses, and third the topological position of *P. sp.* 18 + *P. sp.* 19 is slightly different. In contrast BEST analyses recover the *spurcus* group as a clade (Figs. 6a and b), and the *calcogaster* group, although with low support, is also recovered as a clade in all genes analyses (Fig. 6b).

Some Bayesian statistically significant incongruent topologies found between datasets are weakly or not supported by ML bootstrap values, but some of these patterns are also observed in the BEST results. Thus, we discuss four notable points of incongruence between the mtDNA and the nuclear analyses that may represent interesting aspects of these species evolutionary histories. First, the *calcogaster* group, strongly supported as a clade with nuclear data (Fig. 5b), in the mtDNA tree (Fig. 5a) is split into two groups of species, with *P. calcogaster* and *P. spp.* 11 and 21 closely related with the *somuncurensis* group, and P. *patagonicus* and *P. spp.* 14 and 15 sister to the *spurcus* group in a different part of the tree. The all genes analyses (Fig. 5c) also fail to recover this clade, while the BEST analysis is less influenced by the mtDNA locus and although with low support, recovers these species as a clade (Fig. 6b).

Second, the topological position of the *spurcus* clade in the mtDNA tree as the sister clade to part of the *calcogaster* species

Table A1
Number of individuals of all ingroup taxa used in this study; locality numbers in parentheses, underlined localities are type localities, numbers in bold represent localities (five) where different species from the *patagonicus* and *palluma* groups are in sympatry. Locality numbers match those in Fig. 2. Numbers under the N column give the number of lizards sequenced for cytb gene, from each locality and LJAMM-CNP collection number. Numbers in bold are individuals used for phylogeny reconstruction for the other markers.

Species	Locality	N	Coord.
Palluma group			
P. dorsimaculatus	(2) Neuquén. Ñorquin. Copahue	4 (982/**983**/1200/01)	37°49′S, 71°05′W
	(3) Neuquén. Ñorquin. Cascada del Agrio	1 (3285)	37°48′S, 70°55′W
P. extrilidus	(16) San Juan. Calingasta. Private Reserve Don Carmelo. Sierra de las Invernadas	1 (**538**)	30°56′S, 69°05′W
P. mallimaccii	(4) La Rioja. Famatina. Road to Mina La Mejicana	2 (2002/**2035**)	28°54′S 67°42′W
P. cf. *palluma*	(5) * Mendoza. Vallecitos. 2418 m	3 (2719/99/**2800**)	32°59′S, 69°20′W
	(6) Mendoza. Las Heras. Provincial Road 319. 17 km W Santa Clara	7 (2708/93–97/2801)	32°09′S, 69°04′W
P. laurenti	(1) Catamarca. Belén. Quebrada de Randolfo	1 (**5857**)	26°51′S, 66°44′W
P. querque	(**15**) Neuquén. Catan Lil. Provincial Road46, 9.5 km SW entrance National Park Laguna Blanca	7 (**8060**–66)	39°08′S, 70°25′W
P. punae	(7) San Juan. Iglesia. Llanos de la Lagunita	3 (**2699**/2701/02)	29°24′S 69°25′W
P. roigorum	(**8**) Mendoza. Malargüe. Provincial Road 183, Payun Liso volcano base	10 (7965–74)	36°29′S, 69°22′W
	(**9**) Mendoza. Malargüe. 22.8 km E junction to Provincial Road183, 13.8 km E junction Puesto El Clavado, Altiplanicie del Payun	2 (**4434**/35)	36°39′S, 69°16′W
	(10) Mendoza. Malargüe. Provincial Reserve La Payunia, Pampas Negras, ~ 5 km S Cerro Fortunoso	3 (7912–14)	36°20′S, 69°22′W
P. verdugo	(11) Mendoza. Malargüe. Provincial Road226. 11.4 km S Termas del Azufre	4 (**5792**–95)	35°17′S 70°24′W
	(12) Mendoza. Malargüe. 43 km S Termas del Azufre	2 (5806/07)	35°29′S, 70°14′W
P.vociferator (CH)	(13) Chile. Laguna del Laja, Nacional Park Laguna del Laja, Antuco	2 (3431/**3432**)	37°23′S, 71°22′W
Candidate species – palluma group			
P. sp. 1	(14) Neuquén. Chos Malal. Tromen	3 (**5190**/91/7692)	37°05′S; 70°07′W
P. sp. 2	(17) Chile. Laguna del Maule. Chile	2 (3440/**3442**)	35°57′S, 70°34′W
P. sp. 3	(18) Neuquén. Loncopue. Provincial Road 33. 23,9 km SE Loncopue	2 (5389/**5390**)	38°08′S, 70°26′W
P. sp. 4	(19) Neuquén. Ñorquin. Provincial Road 21. 20 km S El Cholar	3 (**5314**/15/16)	37°34′S, 70°38′W
P. sp. 5	(20) Neuquén. Minas. Provincial Road 45. 28.5 km NW junction Provincial Road 43	1 (**5285**)	36°55′S, 70°55′W
P. sp. 6	(21) Neuquén. Minas. Las Olletas. 7 km N Aguas Calientes	2 (5259/**5260**)	36°39′S 70°35′W
	(22) Neuquén. Minas. Los Tachos	3 (6400/01/02)	36°41′S 70°32′W
	(23) Neuquén. Minas. Paso Malo, Arroyo Covunco	1 (6407)	36°41′S 70°30′W
	(24) Neuquén. Minas. Puesto Viejo de los Castillo, near Humazo del Covunco.	1 (6410)	36°39′S 70°32′W
	(25) Neuquén. Minas. Piedras amarillas	4 (6429/30/31/38)	36°41′S 70°35′W
	(26) Neuquén. Minas. Arroyo Covunco, near Carrizo bridge	1 (6459)	36°40′S 70°28′W
P. sp. 7	(**27**) Neuquén. Auca Mahuida	7(10369/460/557/559/**10560**/61/62)	37°45′S, 68°56′W
P. sp. 8	(28) Neuquén. Chos Malal. Domuyo volcano	3 (6162/63/**6175**)	36°25′S, 70°25′W
P. sp. 9	(**29**) Neuquén. Chos Malal. Provincial Road 37, Butaco stream	9 (**5222**/23/24/29/7649–7653)	36°59′S; 70°00′W
	(30) Neuquén. Chos Malal. Provincial Road37. El Escorial. 41.1 km N junction National Road 40	1(10329)	37°03′S, 70°04′W
P. sp. 10	(31) Mendoza. Malargüe. National Road 145, 17.3 km E Pehuenche pass, 23 km W Las Loicas	6 (**7900**–7905)	35°57′S, 70°14′W
Patagonicus group			
P. calcogaster	(34) Chubut. Telsen. Provincial Road 8 and Quelé Cura	4 (6551/52/53/3278)	42°13′S, 66°21′W
	(35) Chubut. Telsen. Junction to Vaca Lagoon. 16.2 km Provincial Road 4.	11 (6855/**6856**/57/8125–32)	42°30′S 67°21′W
	(36) Chubut. Telsen. Provincial Road 8, 55.2 km junction Provincial Road 5	8 (8138/39/40/42–46)	42°11′S 66°22′W
P. ceii	(37) Río Negro. 25 de Mayo. Provincial Road 8, 18 km S San Antonio del Cuy	7 (1584/**1915**/1916/2727/28/6851/52)	40°17′S, 68°27′W
	(38) Río Negro. El Cuy. Provincial Road 6, 45.4 km SW junction Provincial Road 8	6 (6803/20–22/45/46)	40°17′S, 68°55′.W
	(39) Río Negro. El Cuy. Provincial Road 6, 8.4 km S Colan Conue	5 (6804–08)	40°44′S, 69°09′W
P. delheyi	(**29**) Neuquén. Chos Malal. Provincial Road 37, cruce en arroyo Butaco	7 (**5221**/7654–59)	36°59′S, 70°00′W
P. etheridgei	(32) Río Negro. 25 de Mayo. Provincial Road 76, 57 km S junction National Road 23	10 (3681/88/89/**5897**/3589–92/6548/49)	41°45′S, 69°21′W
	(33) Río Negro. 25 de Mayo. Provincial Road 76, 37 km S junction National Road 23, South of Ingeniero Jacobacci	9 (3638–45/3707)	41°35′S, 69°22′W
P. excelsus	[40] Río Negro. Ñorquinco. 1 km NW Ojo de Agua	9 (2136/37/2355/56/**2265**/66/2652–54)	41°32′S 69°51′W
	(41) Río Negro. Ñorquinco. 2.2 NE Ojo de Agua	8 (3535/36 3622–27)	41°32′S, 69°51′W
P. felixi P. DCL b	(83) Chubut. Paso de Indios. Provincial Road 24, 110 km S Paso de Indios	22 (**3717**/3823/24/**3825**–30/32–37/82/83)	44°31′S, 69°11′W

(*continued on next page*)

Table A1 (*continued*)

Species	Locality	N	Coord.
	(84) Chubut. Paso de Indios. Provincial Road 24 104.7 km N junction Provincial Road 23	5 (9172/73/74/87/88)	44°31′S, 69°10′W
P. indistinctus	(42) Chubut. Rio Senguer. Provincial Road 20. San Bernardo Sa. 19 km W Los Manantiales	2 (**2124**/2393)	45°27′S, 69°42′W
	(43) Chubut. Pampa Lehman	1 8198	45°26′S, 69°52′W
	(44) Chubut. Co. Ferraroti	1 8196	44°28′S, 70°08′W
P. manuelae	(46) Río Negro. Pilcaniyeu. National Road 23, 4.8 km SE Comillo	2 (**5448**/5589)	41°02′S, 70°12′W
P. nevadoi	(47) Mendoza. Malargüe. Provincial Road 180. 31 km S La Ventana	9 (**4431**/32/33/7933–38)	35°55′S, 68°36′W
P. patagonicus	(48) Chubut. Gaiman. National Road. 25 40 km SW Dolavon	6 (**3205**–3210)	43°27′S, 66°07′W
P. payuniae	(**9**) Mendoza. Malargüe. 22.8 E junction Provincial Road 183. Altiplanicie del Payun. 1731m	3 (4436/**4437**–38)	36°39′S, 69°16′W
P. sitesi	(**27**) Neuquén. Auca Mahuida	10 **10367**/368/465/466/468/467/554/555/556/561/	37°45′S, 68°56′W
P. somuncurensis	(49) Río Negro. Valcheta. Somuncurá Plateau	4 (**4453**–56)	41°11′S, 66°53′W
	(50) Río Negro. Valcheta. Cerro Corona Chico (Somuncurá Plateau)	1 (6022)	41°22′S, 66°56′W
	(51) Río Negro. 9 de Julio. 65.6 km El Rincón police office, between Co. Corona Grande and Co. Corona Chico	7 (6826–32)	41°23′S 66°57′W
	(52) Río Negro. 9 de Julio. Provincial Road 8, 20.6 N Provincial Road 5	2 10957/58	41.58225 67.95637
P. spectabilis	(53) Rio Negro. Ñorquinco. 17.4 NE Ojo de Agua. 28 SE Ing. Jacobacci	13 (**3600**–03/05/3617–3621/3634–36)	41°26′S, 69°46′W
P. spurcus	(54) Río Negro. 25 de Mayo. National Road 23. in front of Huanuluan Ranch, 25.1 km W Ing. Jacobacci	3 (**3586**/3629/30)	41°21′S, 69°48′W
P. tenebrosus	(55) Río Negro. Pilcaniyeu. National Road 40. 2.7 km S San Pedro Ranch	1 (**5426**)	40°52′S 70°34′W
	(56) Río Negro. Pilcaniyeu. National Road 40. Co. Alto	1 8681	40°46′S 70°34′W
P. videlai	(45) Chubut. Sarmiento. Buen Pasto	3 9084/85/86	45°05′S, 69°28′W
P. zapalensis	(**15**) Neuquén. Catan Lil. Provincial Road 46, 9.5 km SW entrance Nacional Park Laguna Blanca	8 (**8067**–74)	39°08′S, 70°25′W
Candidate species – patagonicus group			
P. sp. 11	(57) Chubut. Telsen. Provincial Road 67, 16 a 17.7 km N Gan Gan (2 km junction Cañada Leona)	23 (3407/**3408**/6771–78/6901/7477–88)	42°24′S 68°15′W
	(58) Chubut. Telsen. Provincial Road 67, 31.4 km N Gan Gan (El Lloradero)	5 (3446–50)	42°23′S 68°09′W
	(59) Chubut. Telsen. Provincial Road 4, 2 km E Gan Gan	5 (5592/6746–49)	42°31′S 68°01′W
	(60) Chubut. Telsen. Provincial Road 4, 18 km E junction Provincial Road 11, 15 km E Gan Gan	2 (6101/02)	42°30′S 67°58′W
	(61) Chubut. Gastre. Cerro Navidad, Navidad project, 3 km S Provincial Road 4, 40 km W Gan Gan	1 (6106)	42°24′S 68°49′W
	(62) Chubut. Telsen. Provincial Road 67, 20.7 km N Gan Gan	9 (6492/94/7608–14)	42°24′S 68°10′W
	(63) Chubut. Telsen. Provincial Road 67, road to Talagapa, 53.1 km N Gan Gan	3 (7508–10)	42°13′S, 68°14′W
	(64) Chubut. Telsen. Provincial Road 67. 22, 6 km S Gan Gan	2 (8187/88)	42°41′S, 68°13′W
	(65) Chubut. Telsen. Provincial Road 67. 11.2 km S Rio Negro Chubut border	1 (10977)	42°04′S, 68°27′W
P. sp. 12	(**8**) Mendoza. Malargüe. Provincial Road 183, Payun Liso volcano	3 (**7975**–77)	36°29′S, 69°22′W
P. sp. 13	(66) Río Negro. Ñorquinco. Nacional Road 1s40, 2.5 km N Chenqueniyen	5 (3504–**3507**/08)	41°33′S, 70°40′W
P. sp. 14	(67) Chubut. Paso de Indios. Provincial Road 27 y Provincial Road 53, in front of Cerro El Sombrero	6 (**3459**–3464)	44°09′S, 68°14′W
P. sp. 15	(68) Chubut. Telsen. Provincial Road 67. 36 km S Gan Gan	10 (**8190** a 95/8199–8202)	42°52′S, 68°03′W
P. sp. 16	(69) Neuquén. Catan Lil. Provincial Road 46. La Jardinera stream. 25 km E Rahue	2 (5378/**5379**)	39°23′S, 70°43′W
P. sp. 17	(70) Neuquén. Catan Lil. 2 km W Nacional Road 40, near El Salitral	1 **8916**	39°47′S, 70°38′W
P. sp. 18	(71) Río Negro. El Cuy. Provincial Road 67. 20 km S Mencue	3 (5547/**5549**/50)	40°34′S, 69°44′W
P. sp. 19	(72) Río Negro. El Cuy. Provincial Road 67. 19,2 km NE Mencue	1 (**5541**)	40°19′S, 69°26′W
	(73) Río Negro. El Cuy. Provincial Road 67, 37.9 km SW junction Provincial Road 6 y 74	9 (6995–7003)	40°18′S, 69°22′W
P. sp. 20	(74) Río Negro. 25 de Mayo. Provincial Road 6, 64 km NE Ing. Jacobacci	6 (6542/**6543**–46/6842)	40°53′ 69°17′
P. sp. 21	(75) Río Negro. Valcheta. Arroyo Verde and Provincial Road 5	1 (6554)	41°45′S, 66°30′W

Table A1 (*continued*)

Species	Locality	N	Coord.
	(76) Río Negro. Valcheta. Road to Establecimiento La Polvareda	4 (3233/**3234**–3236)	41°33′S, 66°29′W
	(77) Río Negro. Valcheta. Road to Somuncurá Plateau	2 (3237/38)	41°34′S, 66°29′W
	(78) Río Negro. Valcheta. Road to Establecimiento La Polvareda	5 (3259–63)	41°33′S, 66°30′W
	(79) Río Negro. Valcheta. Road to Cecchi Ranch, 24.8 km W Arroyo Los Berros	4 (3317–20)	41°28′S, 66°19′W
	(80) Rio Negro. San Antonio. Quebrada de las sierras Grandes. Sierra Grande	3 (8203/04/05)	41°36′S, 65°22′W
P. sp. 22 *P.* DCL a	(81) Río Negro. 25 de Mayo. Provincial Road 5, ~50 km NW El Cain	2 (6256/**6257**)	41°30′S, 68°35′W
	(82) Río Negro. 25 de Mayo. Provincial Road 5, 40 km SE Maquinchao	4 (**6538**/39/36/41)	41°30′S, 68°33′W

LJAMM-CNP vouchers donated to other herpetological collections: *P. calcogaster*, 8145: BYU 48818; *P. ceii*, 1584: FML 08394, 1916: MACN 910, 2727: MACN 914, 2728: MACN 915; *P. delheyi*, 5221: MLP.S 2611, 7654: MLP.S 2609, 7656: MLP.S 2610; *P. dorsimaculatus*, 1200: FML 07831, 1201: FML 07832; *P. excelsus*, 2265: MACN 1590; *P. indistinctus*, 2393: MACN 1482; *P. mallimaccii*, 2002: MACN 1484, 2035: MACN 810; *P. roigorum*, 7967: BYU 48734, 7972: BYU 48735, 7973: BYU 48736, 7974: BYU 48737; *P. sitesi*, 10465: BYU 12591, 10554: MLP.R 2606, 10555: MLP.R 2607; 10556: MLP.R 2608, 10561: MLP.R 2605; *P. sp.* 1, 5191: BYU 47971; *P. sp.* 11, 7477: BYU 48738, 7478: BYU 48739, 7479: BYU 48740, 7480: BYU 48741, 7481: BYU 48742, 7487: BYU 48743, 7488: BYU 48744; *P. sp.* 18, 5450: BYU 47970; *P. sp.* 19, 6995: MLP.S 2635.

* The precise type locality is unknown but evidence suggests that it is Cordon Portillo (Lobo and Etheridge, personal communication). The only type specimen was collected by Darwin in 1835 in the Andean highlands between Uspallata, Argentina and Santiago de Chile. The two localities referred here as *cf. palluma* are located 5′north and 5′south from Uspallata, close to Cordon Portillo.

Table B1
Anonymous nuclear primers developed and used in this study.

Phy38-F 5′-TTG GTC AAA TTC ATG GAT GC-3′
Phy38-R 5′-ACC AAG GCC TCA GCT AGT CA-3′
Phy41-F 5′-ATC TGG AAG GAC ATG GTT GC-3′
Phy41-R 5′-TGA TAC CAC CCA GGC AAA AG-3′
Phy60-F 5′-TTA TTC CTG GAA CCC CAA CA-3′
Phy60-R 5′-AGT GGC AAG TTT GGA AGT GC-3′
Phy64-F 5′-GCC ATG TCC AGT TTC TTG GT-3′
Phy64-R 5′-GTT TGG ATG GCA CAG GAA GT-3′
Phy84-F 5′-GGC ACT GAA GCA CCA ATA CA-3′
Phy84-R 5′-TGC CTT TCA AAA CCT TCC TG-3′
Phy87-F 5′-ATT CTG ACT CTG CCC CCT TT-3′
Phy87-R 5′-TGC ATT TTT CTT GGC AAC AC-3′
Phy89-F 5′-CCT TGC AGA CGA AGT GAA CA-3′
Phy89-R 5′-AGA GGA CGT GGG GAC TTT TT-3′

group (Fig. 5a) contrasts sharply with its consistent placement as sister to all other clades in all other analyses except in the BEST all genes tree; here the *spurcus* clade is unresolved with respect of the *indistinctus* group (Fig. 6b). There are suitable geographic areas between the distributions of the *indistinctus* and *spurcus* groups west of the *calcogaster* group distribution (Fig. 2); if *Phymaturus* populations occur in this area, then their study should clarify understanding of this strikingly incongruent pattern. We have limited evidence (unpublished data) of hybridization between species of the *spurcus* group, which suggests that this process may have occurred repeatedly in the past.

Third, the placement of *P. tenebrosus* as sister to the *somuncurensis* group + part of the *calcogaster* group in the mtDNA tree (Fig. 5a) is another contrast with nuclear data which place this clade external to the *calcogaster*, *somuncurensis* and *payuniae* groups (Fig. 5b). Thus, similar to the *spurcus* clade, the nuclear data place *P. tenebrosus* in a more basal position, while all genes recover it external to the (*somuncurae* + *calcogaster* [in part]) clade.

Fourth, *P. spp.* 18 and 19 are recovered within the *payuniae* group with mtDNA (Fig. 5a), while with nuclear DNA they are sister to the (*payuniae* + *somuncurensis*) clade. Again, this pattern of incongruence shows the same similarities between the previous two, thus there are three similar examples for the same type of incongruence that require further research.

While some of these incongruences may reflect lack of enough information, others may highlight interesting evolutionary processes. There are two alternative explanations for cases of incongruence at deeper node levels: 1-ancient hybridization (see the case study of Near et al., 2011, for an example); 2-ancient incomplete lineage sorting (Takahashi et al., 2001; McCracken and Sorenson, 2005). Reproductive studies for some species have shown that on average 7–8 years are needed to reach sexual maturity, females reproduce once every 2 years, and give birth to one or two young (Boretto and Ibargüengoytía, 2006; Boretto et al., 2007; Boretto and Ibargüengoytía, 2009). This is a relatively long-generation time for small lizards, which would delay sorting to monophyly. However, the extreme population subdivision due to rock outcrop microhabitat requirements should lead to rapid sorting if there is little gene flow between breeding groups. There are documented examples of incomplete lineage sorting and mitochondrial introgression in the sister genus *Liolaemus* (Camargo et al., 2012b; Morando et al., 2004; Olave et al., 2011) as well as in other groups of lizards (McGuire et al., 2007; Leaché and McGuire, 2006; Leaché, 2009). Analyzing mtDNA and nDNA separately allows us to propose *post hoc* hypotheses about evolutionary processes that may be responsible for these conflicting signals, and contribute to a better understanding of these underlying processes that at least partially explain the diversification of this genus; but more individuals and most probably more genes are needed in order to test these hypotheses.

4.3. Morphological and molecular phylogenetic comparison

Lobo et al. (2012d) presented several alternative hypotheses for each of the two main *Phymaturus* groups based on different values for the parameter K used in implied weightening method of Goloboff et al. (2008); they used the K-3 parameter to describe main results. For the *palluma* group, although there are substantial differences between their alternative hypotheses, the four most frequently recovered results are consistent with those recovered from our molecular evidence: 1-the *vociferator* group is recovered as the most basal clade of the *palluma* group; 2-the northernmost distributed species have a nested position in the topology; 3-*P. verdugo* is closely related to *P. cf. palluma*; and 4-the topology of the *mallimaccii* species group is the same between molecular and K-3

Table C1
Markers used, length, number of terminal taxa for each marker and details on missing taxa and model of evolution used. for each marker. Total terminal taxa included: *Phymaturus palluma* group: 21 and *P. patagonicus* group: 32, with one outgroup each.

Locus	Length (bp)	N (21/32)	Missing taxa	Model of evolution (AIC)
Cytb	830/829	21/32	–	nst = 6 rates = invgamma
12S	851/847	21/32	–	nst = 6 rates = invgamma
Cmos	523	18/31	sp4, sp5, sp7/sp26b_6538	TrN: nst = 6 rates = equalpinvar = 0/TrN + G: nst = 6 rates = gamma shape = 0.0110 ncat = 4 pinvar = 0
NT3*	541	21/32	–	TPM1uf: nst = 6 rates = equalpinvar = 0/TPM3uf: nst = 6 rates = equalpinvar = 0
PRLR*	533	21/32	–	TPM2uf: nst = 6 rates = equalpinvar = 0/TVM + I + G: nst = 6 rates = gamma shape = 0.0160 ncat = 4 pinvar = 0.3330
PNN	1004	18/32	Ppun_2699, Proi_4434, sp3/sp15_7975, sp18_3459	TPM3uf: nst = 6 rates = equalpinvar = 0/TIM3: nst = 6 rates = equalpinvar = 0
Phy38	741/735	21/29	–/Pman_5448, Pspe_3600, sp26a_6257	TPM1: nst = 6 rates = equalpinvar = 0/TPM1: nst = 6 rates = equalpinvar = 0
Phy41	582/576	21/31	–/Pman_5448	HKY: nst = 2 tratio = 1.7644 rates = equal pinvar = 0/TrN: nst = 6 rates = equalpinvar = 0
Phy60	926/917	16/32	Proi_4434, sp5, sp7, sp9, sp 12/Pcei_1584	TrN: nst = 6 rates = equalpinvar = 0/TPM1uf + G: nst = 6 rates = gamma shape = 0.1650 ncat = 4 pinvar = 0
Phy64	629/631	13/26	Pant_5857, sp1, sp3, sp6, sp7, sp8, sp10, sp11/Ppat_3205, Pspe_3600, Pspu_3586, sp15_7975, sp16_10367, sp26b_6538	TPM3uf + G:/TPM1uf + G: nst = 6 rates = gamma shape = 0.0150 ncat = 4 pinvar = 0
Phy84	618/616	21/32	–	TIM3: nst = 6 rates = equalpinvar = 0/TIM3 + I + G: : nst = 6 rates = gamma shape = 0.5850 ncat = 4 pinvar = 0.6460
Phy87	–/737	–/30	–/sp19, outgr	–/TPM3uf: nst = 6 rates = equalpinvar = 0
Phy89	632	20/30	sp12/Pzap_8067, sp19	TPM2uf: nst = 6 rates = equalpinvar = 0/HKY: nst = 2 tratio = 1.4486 rates = equal pinvar = 0

morphological hypotheses. The main consistent difference is the (*P. roigorum* + *P. querque*) clade consistently recovered in the morphological analyses and recovered here only in the BEST nuclear analyses (Fig. 4a).

For the *patagonicus* group, there are also substantial differences between alternative topologies presented by Lobo et al. (2012d), but again some of their more consistent results are, in general terms, congruent with hypotheses presented here: 1-basal position of the *indistictus* and *spurcus* groups (their clades A and B); 2-more nested position of the *payuniae* clade (part of their clade D); and 3-*P. calcogaster* closely related to *P. patagonicus*. The most consistent incongruence between morphological and molecular hypotheses is the placement of *P. tenebrosus* as sister to *P. ceii* with four different groups of *K*-values; this relationship is never recovered with molecular data.

4.4. Biogeographical and conservation implications

Although there is no fossil record for this genus, we can extrapolate estimated dates, inferred from calibrated evolutionary rates from the sister genus *Liolaemus* (Fontanella et al., 2012b), to place the origin of this genus sometime around the uplift of the Andes (~22 m.a.; Gregory-Wodzicki, 2002). Multiple subsequent geological and climatic processes affected the physiognomy and thermal environments of Patagonia, thus promoting population distributional shifts and divergence events (Sérsic et al., 2011). Cei (1986) and Pereyra (1992) proposed Patagonia as a center of origin for *Phymaturus*, and Scolaro et al. (2003) hypothetized that the basaltic plateaus of Patagonia were refugia and speciation centers. Congruent with these proposals, Díaz Gómez (2009) used a Fitch optimization algorithm for historical biogeographic analyses to hypothesize a Central Patagonia (an area corresponding to almost all the current distribution of the *patagonicus* group) origin for this genus, whereas DIVA analyses suggest that the Cordillera Andina and Valle Central area (corresponding to the area encompassed between localities 5 and 11 in Fig. 2) is ancestral. Within the *palluma* group, the geographically most proximal clade to the Cordillera and Valle Central area is the *vociferator* group, which is recovered as the basal clade in our trees, congruent with this ancestral area inference. From this area, the group seems to have radiated towards the south (*payuniae* group) and towards the north (*mallimaccii* group). The geographical ranges of the *vociferator* and *palluma* clades encompass geologically complex areas, and several phylogeographic breaks as well as past fragmentation events, based on other lizards as well as plants, have been proposed for these regions (reviewed in Sérsic et al. (2011)). The different seemingly recently radiated entities we identified as *P. spp.* 1–10 within these two clades could have originated as a consequence of these same breaks and fragmentation events, but the different life-history attributes of *Phymaturus* (long generation time, etc.) may "set" molecular divergence for this genus at a different rate. More dense sampling and the use of hABC methods (Beaumont, 2010; Hickerson et al., 2006; Huang et al., 2011) will be needed to critically test alternative co-diversification hypotheses among different taxa for this region.

At the northern limit of the southernmost distributed taxa of the *palluma* group (*P. querque*, Fig. 2, loc. 15), which also separates the northernmost distributed *patagonicus* clade (*payuniae* clade), another sharp phylogeographic break has been proposed for several terrestrial taxa (Fig. 2, break 3 in Sérsic et al. (2011)). The geographical and topological congruence of the trees presented here with this break suggests that the same process affected vicariance histories in similar ways for a variety of taxa in this region.

The *patagonicus* group seems to have radiated from its southernmost distribution area in Central Patagonia (*indistinctus* group), and/or from an area close to western Patagonia in southwestern Rio Negro province (*spurcus* group), towards the Somuncurá and adjacent plateaus (*somuncurensis* and *calcogaster* groups), and towards the north (*payuniae* group), where species from these two clades have come into contact in five localities in northern Neuquén and southern Mendoza provinces (Fig. 2). Thus, our results for this group are also congruent with Patagonia Central being

the ancestral area for this group (Díaz Gómez, 2009), from which *palluma* and *patagonicus* groups followed south-to-north diversification routes. Within each of the *patagonicus* main clades, more recent radiations appear to be confined to more restricted geographic areas, with divergence proceeding to the point that almost every geographically isolated population of *Phymaturus* has diverged to the level of a candidate species or DCL; a result congruent with recent morphological studies describing several new geographically isolated species (Avila et al., 2011; Lobo et al., 2012a,b,). As noted above, several phylogeographic breaks have been proposed for central Patagonia, some of which can be related to some of the *patagonicus* group clades. For example, phylgeographic break 7 clearly separates the *indistinctus* group from the others, while break 5 separates the *payuniae* clade from the others (breaks in Fig. 2, from Sérsic et al., 2011). It is also possible that break 6 (Sérsic et al., 2011) is related to the separation of *P. tenebrosus* (for which phylogenetic relationships are not clear) from the *somuncurensis* and *calcogaster* clades. Some stable areas have been hypothetized to exist in the Somuncurá and adjacent plateaus, where *P. spp*. 11, 14, 15, 17, 20, 21 and 22 (from the *somuncurensis* and *calcogaster* clades), could have been isolated and differentiated during more recent times, possibly during the end of the Pliocene/Pleistocene. During glacial and interglacial periods, river basins were considerably larger than today, which fragmented the Patagonian landscape (Martinez and Coronato, 2008; Martinez and Kutscher, 2011), and may have also contributed to the successive vicariant events that affected populations of the *patagonicus* groups. In agreement with this *in situ* differentitation hypothesis, a phylogeographic study based on the endemic plant *Anarthrophyllum desideratum* (Fabacea) (Cosacov et al., in press), which is eaten by *Phymaturus*, revealed that this species survived *in situ* in a highly fragmented area in northern Central Patagonia. A recent niche modeling study (Debandi et al., 2011), comparing the two main groups of *Phymaturus* did not find evidence for differences in habitat preference between the two groups, and some detected variation may only represent differences in habitat availability in their respective regions; this implies that conserved niche preferences throughout the evolutionary history of the genus, and most probably diversification events were the results of vicariance processes. However, our evidence suggests very dynamic demographic histories for many of these clades, and future studies will need to be framed so that multiple temporal components of this history can be tested (McCormack et al., 2011).

All described species of *Phymaturus* were identified as "vulnerable" in the most recent conservation workshop for the Argentinian herpetofauna (Buenos Aires, 2010; Abdala et al., 2012). Based on our results, it is possible that due to a combination of habitat requirements and life history characteristics, almost every isolated population of *Phymaturus* may constitute a different species or distinct population, thus this work provides evidence to support the conservation assessment that *Phymaturus* species be considered "vulnerable". We also hightlight that in some areas interesting evolutionary processes are being uncovered, and these should also be taken into account when making conservation decisions.

4.5. Taxonomic implications and future perspectives

In 1970, *Phymaturus* was interpreted as a monotypic genus and 40 years later it includes 38 described species. In this work we present evidence for 22 candidate species (10 and 12 in the *palluma* and *patagonicus* groups, respectively), some of which are being described based on morphological differences (Avila et al., in preparation). There is also morphological evidence for nine candidate species from the *palluma* group that also require further study (Lobo et al., 2012d). If sufficient evidence supports species status for the candidate species, the total number of *Phymaturus* species may increase to 69. The results we present, based on cytb sequences, show several cases of non-monophyly or unresolved potentially non-monophyletic taxa, especially within the *palluma* group, suggesting that species boundaries are still poorly understood and considerable work is needed to achieve an integrative taxonomic understanding on the diversity of this interesting group (Padial et al., 2010). We collected nuclear sequence data for one individual per species, which precludes statistical tests for alternative explanations for the observed non-monophyletic patterns. But geographic distributions and conflicting mtDNA and nuDNA topologies suggest that in several of these cases, past hybridization events could have played an important role in the history of this genus. A multilocus approach based on increased sample sizes and additional nuclear loci, in combination with new methods for inferring species trees that incorporate the coalescent (reviewed in Edwards, 2009; Knowles and Kubatko, 2010), will provide the resolution to distinguish among these alternative process, and the roles they may have played in the diversification of this genus.

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Appendix A

See Table A1.

Appendix B

See Table B1.

Appendix C

See Table C1.

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