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DNA Barcoding of *Phymaturus* Lizards Reveals Conflicts in Species Delimitation within the *patagonicus* Clade

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Abstract. —**Under the DNA Barcode initiative, we used the mitochondrial locus cytochrome *c* oxidase I to test if this molecular marker would reliably distinguish among lizard species of the *patagonicus* clade of *Phymaturus*. Using 18 described species and two populations of unidentified species, we calculated intra- and interpopulation genetic distances for all operational taxonomic units and performed phylogenetic reconstructions using maximum parsimony and maximum likelihood. We identified different species that share the same barcode index number (BIN). We recorded only 12 of the 18 previously described species and one candidate species from the new population. By comparing our results with published morphological and molecular phylogenies, as well as with previous debates, we propose possible explanations for this. In some cases (such as the group with the same BIN formed by *Phymaturus spurcus*, *Phymaturus spectabilis*, *Phymaturus excelsus*, and *Phymaturus agilis*), where other authors debated the identity of the species, we suggest that the low genetic distances could be attributable to the presence of one species with high polymorphism. On the other hand, in geographically isolated species such as the group formed by *Phymaturus payuniae* and *Phymaturus nevadoi*, the group formed by *Phymaturus somuncurensis* and *Phymaturus ceii*, and the group formed by *Phymaturus indistinctus* and *Phymaturus videlai*, the topology of the phylogenetic trees indicates that the low genetic distances (also found by other authors analyzing cytochrome *b*) could be attributable to shared ancestral polymorphism resulting from incomplete lineage sorting.**

Resumen.— **Bajo la iniciativa Códigos de barra genéticos, evaluamos si el marcador molecular mitocondrial COI es capaz de distinguir entre las especies de lagartos *Phymaturus* del grupo *patagónico*. Usamos 18 especies descritas y dos poblaciones de especies no identificadas para calcular las distancias genéticas entre las Unidades Taxonómicas Operativas (OTU por sus siglas en inglés) y realizamos reconstrucciones filogenéticas utilizando Máxima Parsimonia y Máxima similitud. Identificamos distintas especies que comparten el mismo número de identificación de código de barras (BIN por sus siglas en inglés). Recuperamos sólo 12 de las 18 especies previamente descritas y una especie candidata de una de las nuevas poblaciones. Comparando nuestros resultados con filogenias morfológicas y moleculares publicadas, así como con debates previos sobre la identidad de especies dentro del género, proponemos posibles explicaciones sobre los resultados obtenidos. En algunos casos (tales como el grupo con el mismo BIN formado por *P. spurcus*, *P. spectabilis*, *P. excelsus*, y *P. agilis*), donde la identidad de las especies fue debatida por otros autores, sugerimos que las cortas distancias genéticas encontradas podrían atribuirse a la presencia de una sola especie con alto grado de polimorfismo. En cambio, en especies aisladas geográficamente tales como el grupo conformado por *P. payuniae* y *P. nevadoi*, el grupo conformado por *P. somuncurensis* y *P. ceii*, y el grupo formado por *P. indistinctus* y *P. videlai*, la topología de los árboles filogenéticos indica que las bajas distancias genéticas (también encontradas por otros autores al analizar citocromo *b*), podrían atribuirse a polimorfismos ancestrales compartidos, resultado de una división de linaje incompleta.**

The DNA Barcoding initiative was developed by Hebert et al. (2003a) as a tool for rapid identification of biological samples. Using the mitochondrial locus cytochrome *c* oxidase I (COI), the primary goal of DNA barcoding is to create DNA-barcode reference libraries for known species. A DNA barcode is very useful for rapid assessment of the diversity of phylogenetic lineages and as a pilot study for further application of genetic or morphological data. The COI is useful both for assigning an unknown specimen to a known species and for discovering new cryptic species (Hebert et al., 2004; Che et al., 2012), or for possible synonymies when the taxonomy based on morphological characters is controversial. The application of this technique has increased recently and now is also used in other fields such as conservation and ecology (Ratnasingham and Hebert, 2007; Valentini et al., 2008; Eaton et al., 2010). The main limitation of barcoding comes from its single-locus identification system (Valentini et al., 2008). As identical mitochondrial sequences can be found in related species, DNA barcoding can fail to identify species in cases of introgression, incomplete lineage sorting, or complex of species (Vences et al., 2005a,b; Smith et al., 2008). Heteroplasmy also can affect the accuracy of the identification system (Valentini et al., 2008). Although trees based on DNA barcoding have yielded results very similar to those using multigenes (Hawlitschek et al., 2013), the use of COI to identify species can be controversial as the COI barcode is itself a species concept.

Ideally, interspecific divergence should be about 10 times higher than intraspecific divergence (Hebert et al., 2004). By evaluating genetic distances between 13,320 species pairs, Hebert et al. (2003b) found that COI divergence ranged from 0.0% to 53.7%. Most pairs (79%) showed a divergence >8%, and >98% of species pairs showed a sequence divergence >2%. For congeneric species pairs of Chordata, the COI mean (±SD) divergence was 9.6 ± 3.8% (Hebert et al., 2003b). Eaton et al. (2010) found similar results (9.8% divergence) for crocodiles. For other genes such as the mitochondrial cytochrome *b*, divergence between vertebrate species is considered to be higher than 2–3% (Johns and Avise, 1998; Avise and Walker, 1999; Hebert et al., 2003b). Between incipient species (ancestral polymorphism), however, intraspecific variation overlaps with interspecific divergence and the marker cannot reliably distinguish between

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species (Meyer and Paulay, 2005). In addition, statistical problems emerge when a small number of individuals per species is analyzed, which compromises species identification (Valentini et al., 2008). Despite this limitation, in an analysis of 36 genera of Chinese amphibians, Che et al. (2012) found that within each genus, intra- and interspecific distances did not overlap (using the Kimura 2-parameter). Here, we applied the COI barcode to the genus *Phymaturus* (Liolaemidae) to test if this method is capable of identifying unique lineages and to assign samples to species based on genetic distance.

Genus *Phymaturus* comprises Andean and Patagonian lizards of Argentina and Chile. Within the genus, two clades are clearly differentiated by morphological characteristics (Cei, 1986; Etheridge, 1995): the *palluma* group in the north (between 25° and 39°S) and the *patagonicus* group in the south (between 36° and 46°S). The nomenclature of this genus has been the focus of debate by several authors for many years (see Cei and Scolaro, 2006, for details) and the taxonomy remains unresolved. Until 1970, only two subspecies of a single species were recognized: *Phymaturus palluma palluma* and *Phymaturus palluma patagonicus* (Peters and Donoso-Barros, 1970). The description of species and subspecies increased in the following years and Etheridge (1995) elevated both subspecies to species and formally described the two groups proposed by Cei (1993) based on squamation and skeletal morphology: the *P. palluma* group and the *P. patagonicus* group. Ten species were known at that time: four in the *palluma* group and six in the *patagonicus* group. Recently, a new species was described (Gonzales Marin et. al., 2016). Currently, 49 species have been described, 23 from the *palluma* group and 26 from the *patagonicus* group; however, the identity of several species is under debate, and there is no consensus on the number of valid species.

Within the *palluma* group, the most debated species is *P. palluma* (Molina, 1782). The holotype was collected by Darwin during his trip to Chile and Argentina, but its location is not precise (see Cei and Scolaro, 2006; Etheridge and Savage, 2006). By comparing morphometric traits of the neotype of *P. palluma* and other populations, Scolaro (2010) reasonably concluded that the type locality of *P. palluma* was Uspallata–Paramillos (northern Mendoza Province). On the contrary, Lobo and Etheridge (2013) suggest the type locality was, in fact, the Cordón del Portillo (130 km south of Uspallata, 2800 m above sea level [m asl]) because Darwin notes a "viviparous lizard" from there; however, this supposition ignores that, at those altitudes, all lizards are viviparous (both *Liolaemus* and *Phymaturus*), making this is a speculative argument. Hence, we follow Scolaro (2010) and argue that Uspallata–Paramillos is the type locality of *P. palluma*. If this is correct, then Lobo and Etheridge's (2013) proposed synonymy of *Phymaturus gynechlomus* (Corbalán et al. 2009) and *P. palluma*, based on the proximity of the type localities, also should be reevaluated. Another debate is on the identity of *Phymaturus dorsimaculatus* Lobo and Quinteros (2005) and *P. vociferator* Pincheira-Donoso (2004). Pincheira-Donoso et al. (2008) synonymized these species, but Lobo et al. (2010a) rejected this proposal. Within the *patagonicus* group, the debate is centered on the identity of *Phymaturus agilis* Scolaro et al. (2008), which Lobo et al. (2012a) synonymized with *Phymaturus spectabilis* Lobo and Quinteros (2005) based on the birth of different morphs from a pregnant female. Avila et al. (2014) remarked that *P. spectabilis*, *Phymaturus excelsus* Lobo and Quinteros (2005), and *Phymaturus tenebrosus* Lobo and Quinteros (2005) show a brown pattern similar to *Phymaturus spurcus* Barbour (1921), questioning the identity of these species and suggesting the existence of a very polymorphic population. Therefore, this group of species is especially interesting for evaluating the performance of COI barcodes in identify them as unique lineages.

The first phylogeny for the genus *Phymaturus* was based on morphology and included 15 of the 49 currently described species (Lobo and Quinteros, 2005). This phylogeny was updated by Lobo et al. (2012b) based on morphological characters of 17 *patagonicus* species and 10 described *palluma* species, in addition to other undetermined species, two of which were recently described formally. These authors added different mitochondrial genes for five described species (three in the *patagonicus* group and two in the *palluma* groups) and two undescribed species from GenBank. At the same time, Morando et al. (2013) published a molecular phylogeny using 2 mitochondrial genes (cytochrome *b* and 12S), 4 protein-coding nuclear genes, and 7 anonymous nuclear loci for 27 described species (17 for the *patagonicus* group and 10 for the *palluma* group) and 22 candidate species. Published data on genetic divergence between *Phymaturus* species pairs are scarce, but Morando et al. (2013) highlighted that cytochrome *b* genetic distances between some pairs of species from the *patagonicus* group clade are low (*Phymaturus nevadoi* vs. *Phymaturus payuniae*: 0.91%; *Phymaturus somuncurensis* vs. *Phymaturus ceii*: 1.3%; *Phymaturus manuelae* vs. *P. spurcus*: 1.69%).

The main objective of this study is to contribute to the library of the Barcode of Life Data System and test whether the mitochondrial gene cytochrome *c* oxidase subunit I (COI) distinguishes 18 lizard species of the *patagonicus* clade of *Phymaturus*, 5 of which were not included in the molecular phylogeny of Morando et al. (2013). When COI is not able to recognize these species as different entities, we compare our results to published phylogenies and discuss hypotheses based on the classical biological species concept (Mayr, 1942). Because we could not test reproductive isolation, we inferred it based on geographical isolation.

Materials and Methods

Sampling Techniques.—From 2009 to 2013 we collected at least four individuals of each *Phymaturus* species by noosing. *Phymaturus* lizards occupy rocky promontories principally of volcanic origin. Most species are considered endemic because their distributions are confined to their type localities (Fig. 1). Therefore, to obtain individuals from representative populations, all lizards of each species were collected at their type localities (and surrounding areas when possible; Appendix 1). Most collected specimens were euthanized and tissue samples (muscle or liver) were preserved in 96% ethanol and stored in a freezer. Vouchers specimens were deposited in the IADIZA Herpetological Collection (Instituto Argentino de Investigaciones de las Zonas Áridas). For 15 specimens, tissues were obtained from the tail, and the individuals were photographed and released at the site of capture. Tissue samples were analyzed following protocols of the Barcode of Life Data Systems (http://www.boldsystems.org/).

We analyzed a total of 101 specimens, 93 of which belonged to 18 known species of *Phymaturus* of the *patagonicus* group and individuals from two localities, Sierra del Chacay (three individuals) and Los Adobes (two individuals), to evaluate if these populations share the same barcode index numbers with known species. We also included one individual of *Phymaturus punae* (*palluma* group) as well as two species from the same

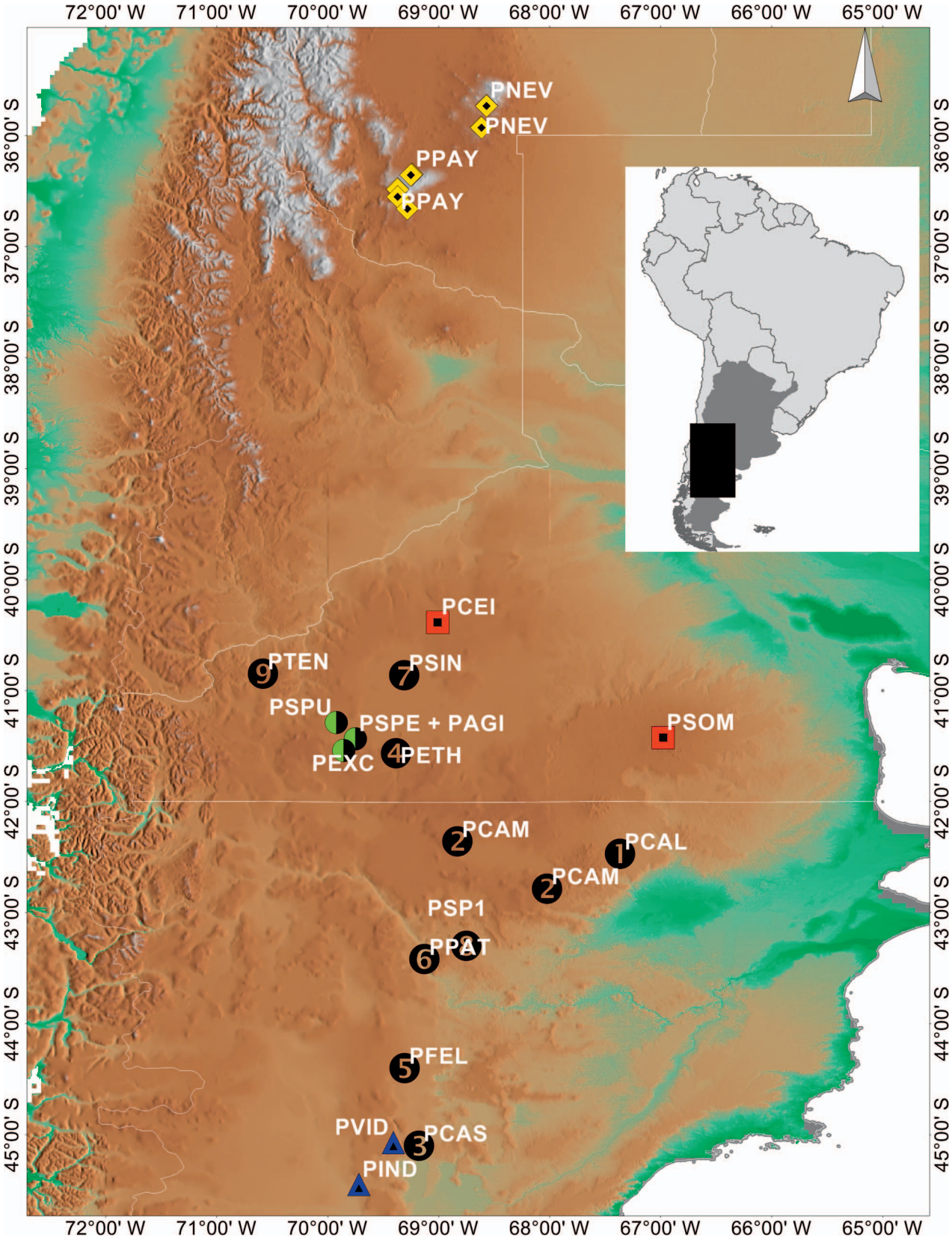

FIG. 1. Sampling localities of *Phymaturus* of the *patagonicus* clade in Argentina. Green/black circles, yellow rhombus, blue triangles, and red squares indicate localities of OTUs with similar BINs. PSPU: *P. spurcus*; PSPE: *P. spectabilis*; PEXC: *P. excelsus*; PAGI: *P. agilis*; PPAY: *P. payuniae*; PNEV: *P. nevadoi*; PIND: *P. indistinctus*; PCAS: *P. castillensis*; PSOM: *P. somuncurensis*; PCEI: *P. ceii*. Numbers indicate localities of well-delimited species: 1-PCAL: *P. calcogaster*; 2-PCAM: *P. camilae*; 3-PCAS: *P. castillensis*; 4-PETH: *P. etheridgei*; 5-PFEL: *P. felixi*; 6-PPAT: *P. patagonicus*; 7-PSIN: *P. sinervoi*; 8-PSP1: *P. sp.* 1; 9-PTEN: *P. tenebrosus*.

family (Liolaemidae), *Liolaemus buergeri* (one individual) and *Liolaemus petrophilus* (one individual), used as outgroups. Each species and unidentified populations were considered operational taxonomic units (OTUs).

DNA Extraction, PCR Amplification, and Sequencing.—We recovered 659 base pairs of the COI (DNA barcode region) from the 101 specimens using standard high-throughput barcoding protocols. Whole genomic DNA from small (approximately 1–2 mm^3) pieces of ethanol-preserved muscle or liver were extracted on the Biomek FX© liquid handling station using 1.0 μm PALL glass fiber media filter plates following the protocol of Ivanova et al. (2006). Polymerase chain reaction (PCR) amplification using C_VF1LFt1 – C_VR1LRt1 (Mammal cocktail) was done as described by Ivanova et al. (2007). Products were labeled with the BigDye© Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, California, USA) as described in Hajibabaei et al. (2005) and sequenced bidirectionally using an ABI 3730XL capillary sequencer following the manufacturer's instructions. Bidirectional reads were assembled, manually edited in CodonCode Aligner software v.3.5.2 (CodonCode Corp., Centerville, Massachusetts, USA), and aligned using the default parameters of CLUSTAL X (Thompson et al., 1997). The Barcode of Life Data System (Ratnasingham and Hebert, 2007) stores DNA barcode data (COI sequences, chromatogram trace files, and collateral specimen information) at www.barcodinglife.org in the Completed Projects section in the project (Pat: Patagonia-outgroup). Sequences were also deposited in NCBI GenBank (see Appendix 1).

Data Analyses.—We considered well-delimited species when OTUs had different barcode index numbers (BIN). The BINs are assigned by the refined single linkage (RESL) algorithm (Ratnasingham and Hebert, 2013). The implementation of single-linkage clustering requires the selection of a threshold parameter (t) that represents the level of sequence divergence for the designation of OTUs and in most cases is near 2.2% (Ratnasingham and Hebert, 2013). Using the Markov clustering (MCL) algorithm, clusters with members showing high sequence variation but lacking discontinuity remain a single OTU whereas those with clear internal partitions in their sequences are assigned to different OTUs, even if their separation is less than 2.2% (Ratnasingham and Hebert, 2013).

The genetic distance between individuals was calculated using the Kimura 2-parameter (K2p) model (Kimura, 1980), one of the simplest and most-widely used metrics in barcoding studies (Che et al., 2012). This allowed better comparisons with other studies. The model estimates evolutionary distances in terms of the number of nucleotide substitutions (Kimura, 1980). Next, we calculated intra- and interspecific genetic divergences by averaging the values of all possible combinations. Neighbor-joining trees (NJ) based on K2p distances were constructed using MEGA 4 (Tamura et al., 2007). Electropherograms were scored using PROSEQ 2.91 (Filatov, 2002).

The best-fit substitution model of sequence evolution was identified with jModelTest 2.1.4 (Darriba et al., 2012). The selected model under the Akaike information criterion (Akaike, 1974) was GTR+I+G with base frequencies A = 0.3118, C = 0.2650, G = 0.1363, and T = 0.2869. The proportion of invariable sites (I) was 0.5820 and the gamma distribution shape parameter (G) was 1.4670. Phylogenetic reconstructions were carried out using maximum parsimony (MP) in TNT (Goloboff et al., 2008) and maximum likelihood (ML). For MP, we employed a heuristic search with 250 random addition sequences, saving five trees per replicate with the TBR branch-swapping algorithm. We summarized the trees of MP in a strict consensus tree. The node supports were calculated with standard bootstrap support based on 1,000 repetitions. The ML was conducted under the best-fit model of evolution obtained with jModelTest in 'Phyml' (Guindon et al., 2010) with 1,000 bootstrap repetitions. Descriptive statistics are expressed as mean ± SD.

Results and Discussion

Of the 20 OTUs in the *patagonicus* clade (18 formally described species and two populations that we could not assign to known species), only 13 were recovered as unique lineages (12 described and 1 candidate species) based on BINs assigned by the Barcode of Life Data (BOLD) System.

Genetic distances calculated by Kimura 2-parameters indicated that intraspecific divergence ranged from 0–1.29% for all OTUs. The divergence between species belonging to different groups of the genus *Phymaturus* ranged from 17.99 ± 0.15% (*P. punae–P. patagonicus*, number of comparisons [n_c] = 12) to 20.48 ± 0.24% (*P. punae–P. agilis*, n_c = 12 or *P. punae–P. spectabilis*, n_c = 12). These values are greater than those found between the two *Liolaemus* species (10.72%, *L. petrophilus–L. buergeri*, n_c = 1) and the average expected values for congeneric Chordata species pairs reported by other authors (9.60%, Hebert et al., 2003b). Within the *patagonicus* clade, we found a mean divergence of 6.07 ± 2.64% between all OTUs; however, if we consider the divergence between only well-delimited species (i.e., those with different BINs), this value increases to 6.66 ± 2.1%. For these comparisons, when two or more OTUs shared the same BIN, we used the former described species. The highest value was from the comparison between *P. patagonicus* and *P. tenebrosus* (9.69%, Table 1).

The COI allowed us to confirm that individuals from Sierra del Chacay correspond to the recently described species *Phymaturus camilae* from Sacanana with a similar dorsal pattern (Scolaro et al., 2013). Both OTUs have the same BIN, the genetic distance between them is 0 ± 0% (n_c = 12), and they form a monophyletic clade with high bootstrap support (>90%) (Figs. 2, 3, 4). This result expands the range of distribution of this species 80 km to the southeast. On the other hand, individuals from Los Adobes (*sp*. 1 in figures) are more related to *P. patagonicus* (2.63 ± 0%, n_c = 6) and to the clade formed by *P. agilis*, *P. spectabilis*, *P. spurcus*, and *P excelsus*. Genetic distances between *sp*. 1 and these OTUs range from 2.12 to 2.17%. Moreover, the BIN assigned to this population differs from known species. Hence this population from Los Adobes should be studied in more detail, incorporating additional locus and morphological characters to confirm its specific status.

Even though 68% of comparisons between OTU pairs in the *patagonicus* clade diverged by more than 6%, we identified four groups of formally described species with low genetic distances (below 1%) and identical BINs (Figs. 2, 3, 4). The first group comprises *P. spurcus*, *P. spectabilis*, *P. excelsus*, and *P. agilis* (divergence: 0.32 ± 0.12%). The second group comprises *P. payuniae* Cei and Castro (1973) and *P. nevadoi* Cei and Roig (1975) (divergence: 0.63 ± 0.36%). The third group comprises *P. somuncurensis* Cei and Castro (1973) and *P. ceii* Scolaro and Ibargüengoytía (2007) (divergence: 0.92%), and one individual of *Phymaturus sinervoi* Scolaro et al. (2012), which showed a 0.92% genetic distance from *P. somuncurensis* and 0.00% from *P. ceii*. The fourth group comprises *P. indistinctus* Cei and Castro

TABLE 1. Intra- and interspecific distances among well-delimited species (those showing different barcode index numbers [BINs]) of *Phymaturus* according to Kimura 2p. When BINs are shared, only the former described species were used for interspecific comparisons. Mean ± SD of total comparisons.

	P. calcogaster	*P. camilae*	*P. castillensis*	*P. etheridgei*	*P. felixi*	*P. indistinctus*	*P. patagonicus*	*P. payuniae*	*P. sinervoi*	*P. somuncurensis*	*P. spurcus*	*P. tenebrosus*
P. calcogaster	0.05 ± 0.07											
P. camilae	1.41 ± 0.06	0 ± 0										
P. castillensis	7.12 ± 0.07	6.58 ± 0.02	0 ± 0									
P. etheridgei	2.69 ± 0.07	3.15 ± 0.03	7.46 ± 0.08	0 ± 0								
P. felixi	6.64 ± 0.11	6.79 ± 0.08	1.49 ± 0.08	7.33 ± 0.12	0.31 ± 0.26							
P. indistinctus	7.62 ± 0.26	7.42 ± 0.25	1.96 ± 0.16	8.03 ± 0.20	1.77 ± 0.16	1.03 ± 0.89						
P. patagonicus	8.31 ± 0.07	8.46 ± 0.02	6.41 ± 0.01	8.96 ± 0.01	6.17 ± 0.08	7.20 ± 0.17	0 ± 0					
P. payuniae	7.63 ± 0.25	7.55 ± 0.16	5.95 ± 0.2	7.95 ± 0.16	6.19 ± 0.27	7.05 ± 0.29	4.58 ± 0.27	0.36 ± 0.42				
P. sinervoi	2.69 ± 0.54	3.07 ± 0.67	7.39 ± 0.36	1.89 ± 0.32	7.31 ± 0.40	7.91 ± 0.38	8.61 ± 0.02	7.53 ± 0.15	1.29 ± 1.41			
P. somuncurensis	1.73 ± 0.06	2.17 ± 0.01	6.74 ± 0.01	2.34 ± 0.03	6.61 ± 0.09	7.24 ± 0.27	8.27 ± 0.00	7.42 ± 0.24	1.98 ± 0.64	0 ± 0		
P. spurcus	7.98 ± 0.07	8.31 ± 0.02	6.41 ± 0.01	8.87 ± 0.10	6.34 ± 0.08	7.54 ± 0.17	2.48 ± 0.00	4.08 ± 0.24	8.76 ± 0.09	8.30 ± 0.00	0 ± 0	
P. tenebrosus	3.31 ± 0.06	3.29 ± 0.01	8.11 ± 0.01	3.95 ± 0.04	8.69 ± 0.09	9.33 ± 0.26	9.69 ± 0.00	9.68 ± 0.19	4.65 ± 0.45	3.76 ± 0.00	9.01 ± 0.00	0 ± 0

(1973) and *P. videlai* Scolaro and Pincheira-Donoso (2010) (divergence: 0.87 ± 0.15%). The following section discusses this study's findings in relation to published phylogenies.

Group 1) Phymaturus spurcus, P. spectabilis, P. excelsus, *and* P. agilis.—These four species are distributed in a small part of the Río Negro Province, with a maximum distance of 40 km between populations (Fig. 1). All four species (or OTUs) have the same BIN, and phylogenetic analyses recovered them as a monophyletic clade with high bootstrap support (>85%) (Figs. 2, 3, 4; green clade). Despite the similarities in the pattern of spots in some species (*P. spectabilis* and *P. excelsus*, Fig. 5), only the identity of *P. agilis* has been debated in the literature. Lobo et al. (2012a) collected a *P. spectabilis* pregnant female that gave birth to two individuals in captivity. One of them had the *P. agilis* spot pattern and the other had the *P. spectabilis* pattern. Although the color pattern of neonates can change throughout life, these authors pointed out that there is a chance the two offspring with different morphs could be the product of hybridization between the two closely related species, and they concluded that *P. agilis* is a junior synonym of *P. spectabilis*. Moreover, from summer breeding seasons between December 2011 to March 2013, one of us (JAS) recorded several females of various species giving birth to different morphs, such as a female of *P. spurcus* giving birth to morphs of *P. excelsus* and females of *P. excelsus* giving birth to *P. excelsus* and *P. spectabilis* individuals. Lobo et al. (2012a) also recognized different morphs in *P. excelsus* adults (bold and brown). Despite the fact that these brown morphs look very similar to *P. spurcus* (see Lobo et al. 2012a:fig. 2) and that these two species live together (Lobo and Quinteros, 2005), they attributed the "brown morphs" of *P. excelsus* to intraspecific dimorphism. Avila et al. (2014) suggested that *P. spurcus, P. spectabilis*, *P. excelsus*, and *P. tenebrosus* form a very polymorphic population.

Unfortunately, Lobo et al. (2012a) compared morphological and meristic characters between only *P. agilis* and other species (*P. spectabilis*, *P. excelsus*, and *P. spurcus*), but not between other pairs. Experiments in laboratory and genetic studies should be carried out to identify the causes of morphological variability and the potential existence of hybrids. For these reasons we took a conservative approach, and we analyzed sequences of each morph and described species as different OTUs. Based on COI results, we suggest the possibility the area has only one unique species (*P. spurcus*, according to the principle of priority) with strong morphological variation and includes at least three morphs. Following the terminology of Lobo et al. (2012a), who described the morphs of *P. excelsus* as bold and brown, we named these morphs "full-brown" (the *P. spurcus* pattern), "spotted brown" (the *P. agilis* pattern), and "bold" (the *P. spectabilis–excelsus* pattern, which can vary between black and white or dark and light brown). *Phymaturus tenebrosus* appears as a different species in our phylogenetic trees. Morando et al. (2013) also found a low divergence between *P. spurcus* and *P. manuelae* Scolaro and Ibargüengoytía (2008) (1.69%) based on cytochrome *b*. Similarly, Lobo et al. (2012b) recovered a clade composed of *P. spurcus*, *P. manuelae*, *P. spectabilis*, and *P. excelsus* supported by five synapomorphies. Therefore, we conclude that *P. manuelae* (a polychromatic species, Scolaro and Ibargüengoytía, 2008) belongs to this group, and this may be a step toward resolving relationships among these species.

Group 2) Phymaturus payuniae *and* P. nevadoi.—These two species are restricted to the Payunia region. Whereas *P. nevadoi* can be found within only a few kilometers of its type locality (Agua de la India Muerta, Sierra del Nevado; Cei and Roig,

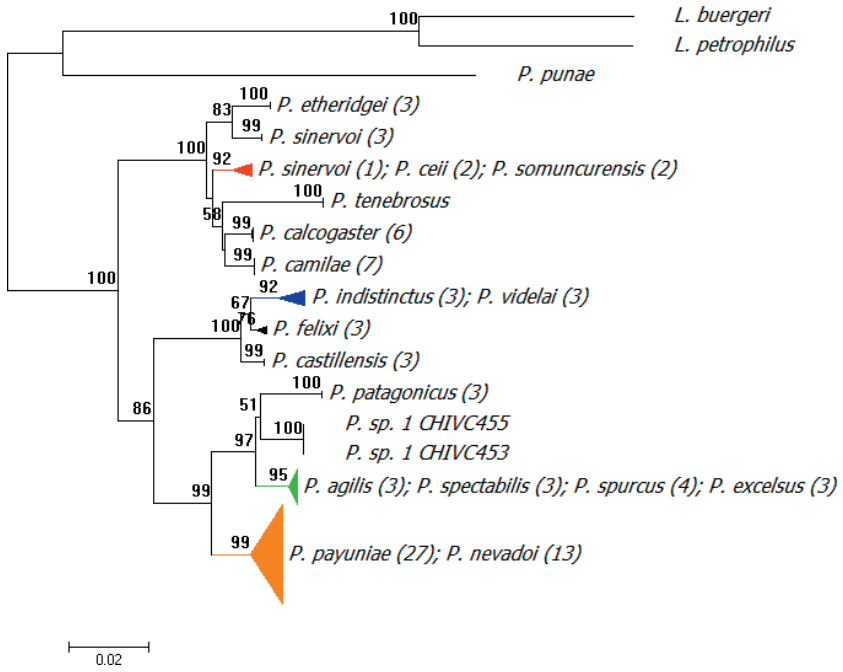

FIG. 2. Neighbor-joining tree of Kimura 2-parameter distances based on cytochrome *c* oxidase I (COI) for *Phymaturus* operational taxonomic units (OTUs) of the *patagonicus* clade. Bootstrap values >50% are given above the nodes. Different colors in clades indicate species (or OTUs) sharing the same barcode index number (BIN).

1975), the distribution of *P. payuniae* includes the Payún Matrú and the Paýun Liso volcanoes as well as the Payún highland plain (Fig. 1) (Corbalán et al., 2013).

The brief description of *P. nevadoi* was based on only three specimens (a female and two males) that were not collected by the authors of the species (Cei and Roig, 1975). The main differences between this species and *P. payuniae* is the absence of sexual dichromatism (present in *P. payuniae*) and the size of ventral scales (larger in *P. nevadoi*, Cei and Roig, 1975). Males of the two species are indistinguishable.

During field trips to the Sierra del Nevado to collect individuals for this study, we found some females with spots similar to those seen on males and other females with a dorsal pattern more similar to *P. payuniae* females (Fig. 5). For this reason, we sequenced a large number of samples for these species ($n = 13$ for *P. nevadoi* and $n = 27$ for *P. payuniae*) and covered as many sites as possible, including the population called *P. sp.* 12 (sensu Morando et al., 2013). We did not find divergences greater than 2% in COI sequences among individuals of the two species, among different populations, nor among different female morphs of Sierra del Nevado. The topology of the phylogenetic trees shows two species (Fig. 3; orange clade) with the same BIN. Both species are grouped together in the same clade with high bootstrap support (>85%) (Figs. 2, 3, 4; orange clade). The allopatric distribution of the species as well as the paraphyletic topology of the phylogenetic trees (Fig. 3) could indicate a recent speciation and that species are showing incomplete lineage sorting. The existence of one species with morphological intraspecific variation, however, should not be discarded based on genetic distances. Morando et al. (2013) also highlighted the low pairwise cytochrome *b* genetic distances between these two species (0.91%) and grouped them with their *payuniae* group that also includes *Phymaturus delheyi* and *Phymaturus sitesi* Avila et al. (2011), *Phymaturus zapalensis* Cei and Castro (1973), and three candidate species (*P. sp.* 12, *P. sp.* 16, and *P. sp.* 17). Lobo et al. (2012b) did not use *P. delheyi* and *P. sitesi* in their cladogram, but they found that *P. zapalensis* formed a monophyletic group with *P. payuniae* and *P. nevadoi*. To clarify the identity of these five species, we suggest a revision of the Morando et al. (2013) *payuniae* group, as the descriptions of *P. sitesi* and *P. delheyi* also are based on sexual dichromatism, ventral scales, and number of midbody scales (Avila et al., 2011).

Group 3) Phymaturus somuncurensis *and* P. ceii (+P. sinervoi).—*Phymaturus somuncurensis* (Fig. 5) has long been considered an endemic species of the Somuncurá plateau. *Phymaturus ceii* (Fig. 5) was described for outcrops near Chasicó, south of El Cuy Plateau, Río Negro Province, 210 km from the Somuncurá plateau. Males show a variable dorsal color pattern and are distinguished from *P. somuncurensis* by having larger hind limbs and greater axilla–groin distance (Scolaro and Ibargüengoytía, 2007). Morando et al. (2013) reported a low genetic distance using cytochrome *b* between these two species (1.3%) and called them the *somuncurensis* group, which included these two species, *Phymaturus etheridgei*, the candidate species *P. sp.* 20 (now *P. sinervoi*), and another candidate species (*P. sp.* 22 sensu Morando et al., 2013). In contrast, using morphological characters Lobo et al. (2012b) found that *P. etheridgei* and *P. somuncurensis* belonged to one clade (clade C) whereas *P. ceii* belonged to another (clade D), suggesting *P. somuncurensis* and *P. ceii* had distinctive morphology. Using COI, we found a low genetic distance between them (0.92%). They shared the same BIN and were grouped in a monophyletic clade (Figs. 2, 3, 4; red clade). On the other hand, *P. etheridgei* is clearly separated from the other species, with genetic distance over 2% for *P. ceii* and *P. somuncurensis* (2.50 ± 0.03 and 2.34 ± 0.03, respectively). Moreover, *P. etheridgei* has a different BIN and individuals are grouped in a different but related clade. Taking into account the allopatric distribution of species and morphological differentiation, we think they have recently undergone speciation and that the low genetic distances could be because of incomplete lineage sorting; however, sample size is too low to draw conclusions. More-detailed studies are needed to solve this

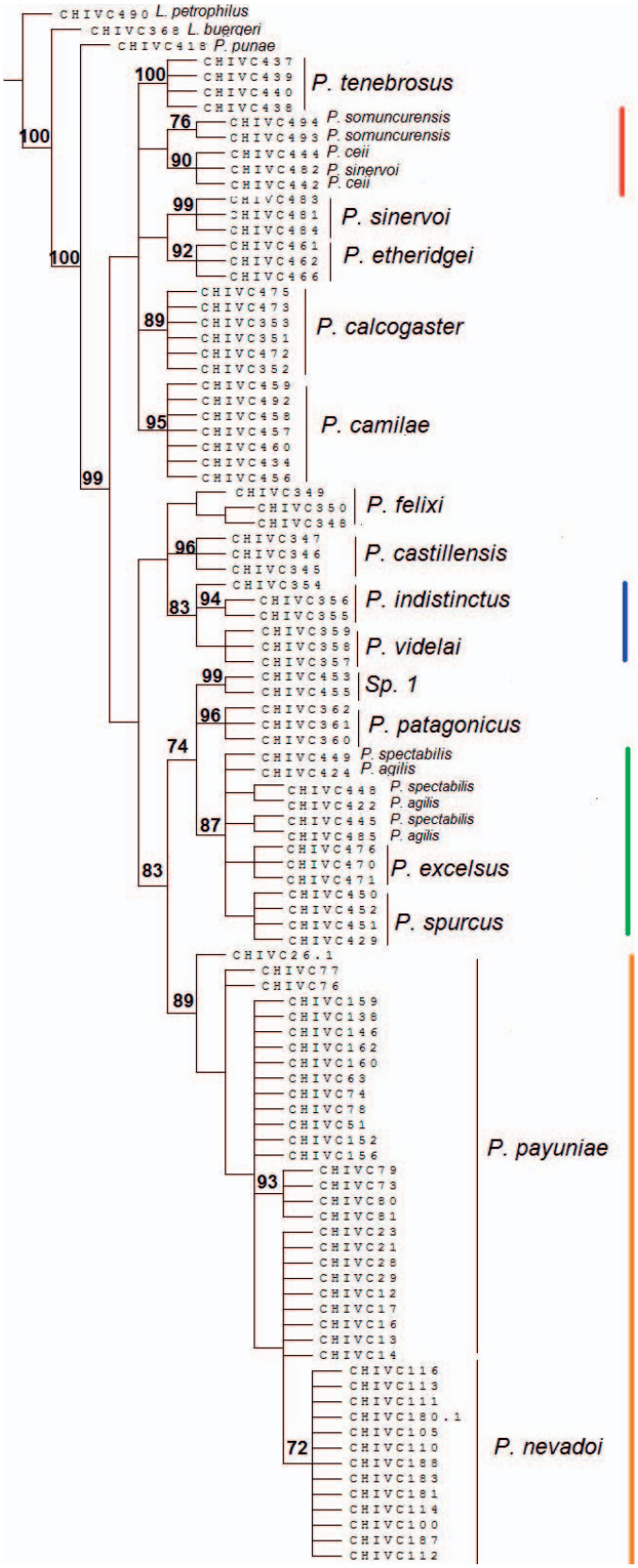

FIG. 3. Maximum parsimony analysis of *Phymaturus* of the *patagonicus* clade based on cytochrome *c* oxidase I (COI) sequences. Bootstrap supporting >70% are included. Different colors in clades indicate species (or operational taxonomic units [OTUs]) sharing the same barcode index number (BIN).

species delimitation problem. We found three of the four sequenced individuals of the recently described species *P. sinervoi* showed 2.65% divergence with *P. ceii* and 2.32% with *P. somuncurensis*; however, no genetic distance was found between one individual from the same locality and *P. ceii*. Increasing sample size should be the next step to confirm the identity and relationships of this species.

Group 4) Phymaturus indistinctus *and* P. videlai.—These species (Fig. 5) are geographically separated by about 70 km (Fig. 1). *Phymaturus videlai* can be distinguished from *P. indistinctus* based on several morphological characteristics (Scolaro and Pincheira-Donoso, 2010); however, we found a low genetic divergence between the two species (0.87 ± 0.15%). They share the same BIN and form a monophyletic clade with moderate to high bootstrap support (>78%) (Figs. 2, 3, 4; blue clade). Lobo et al. (2012b) reported the clade formed by *P. indistinctus* and *P. videlai* was supported by 11 synapomorphies and recovered a clade supported by 5 characters comprising *P. indistinctus*, *P. videlai*, *Phymaturus castillensis* Scolaro and Pincheira-Donoso (2010), and *Phymaturus felixi* Lobo et al. (2010b). Although *P. castillensis* and *P. felixi* showed some individuals with very similar external morphology, and genetic distances between them are below 2.2%, they appear to be well-defined species based on their BINs. Phylogenetic analyses recovered them as two closely-related clades, one comprising *P. indistinctus* and *P. videlai* as its sister group (Figs. 2, 3, 4). Because of the physiographic conditions in the distributional area and the monophyly of both species, reproductive isolation may be possible and *P. indistinctus* and *P. videlai* may have experienced recent speciation. In that case, incomplete lineage sorting is the more plausible hypothesis to be tested in this pair of species that are very similar genetically. Further studies with larger samples and nuclear genes should be completed for a better understanding of the identity of the group of species recovered by Lobo et al. (2012b).

Final Considerations.—Species delimitation ideally requires data from many different sources such as morphology, behavior, and multiple molecular markers (Funk and Omland, 2003; Hajibabaei et al., 2007). When obtaining data from all these sources is not possible, the COI barcode can be a useful tool. Several studies have demonstrated its effectiveness for identifying unique lineages in different animal groups, including reptiles (Hajibabaei et al., 2007; Eaton et al., 2010; Nagy et al., 2012; Murphy et al., 2013). In addition, cryptic species can be discovered when there are high levels of intraspecific divergence in COI barcodes (Funk and Omland, 2003; Hebert et al., 2004). In this study, we found that the sample from Los Adobes showed high COI divergence from other species, suggesting it is a candidate species. We also were able to assign an undetermined population (from Sierra del Chacay) to a species (*P. camilae*).

In contrast, DNA sequences of recent lineages may not be distinct. This pattern may be explained not only by introgression of haplotypes but also by rapid speciation after the colonization of new areas or by past extinction and recolonization events that mask the original pattern of divergence (Hawlitschek et al., 2013). Therefore, species boundaries are blurred when hybridization or introgression occurs (Hebert et al., 2003a). Mitochondrial introgression is well documented in amphibians and reptiles, causing divergent mitochondrial genomes to coexist within species (Murphy et al., 2013). Moreover, intergeneric hybrids are well documented in turtles, causing taxonomic confusion (Stuart and Parham, 2007; Murphy et al., 2013). Therefore, DNA barcoding can fail to

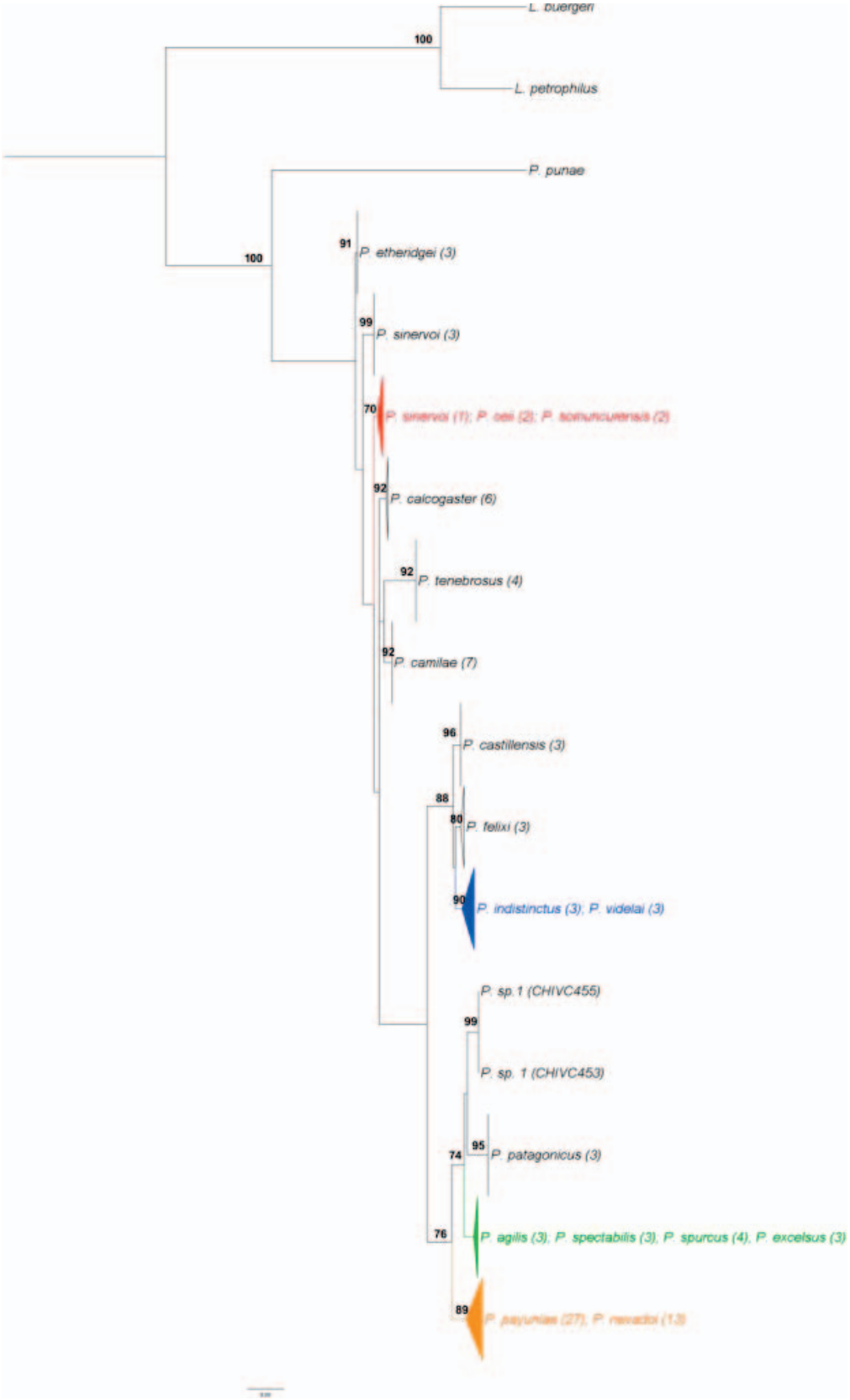

FIG. 4. Maximum likelihood analysis of *Phymaturus* of the *patagonicus* clade based on cytochrome *c* oxidase I (COI) sequences. Sample sizes are in parenthesis. Bootstrap supporting >70% are included. Different colors in clades indicate species (or operational taxonomic units [OTUs]) sharing the same barcode index number (BIN).

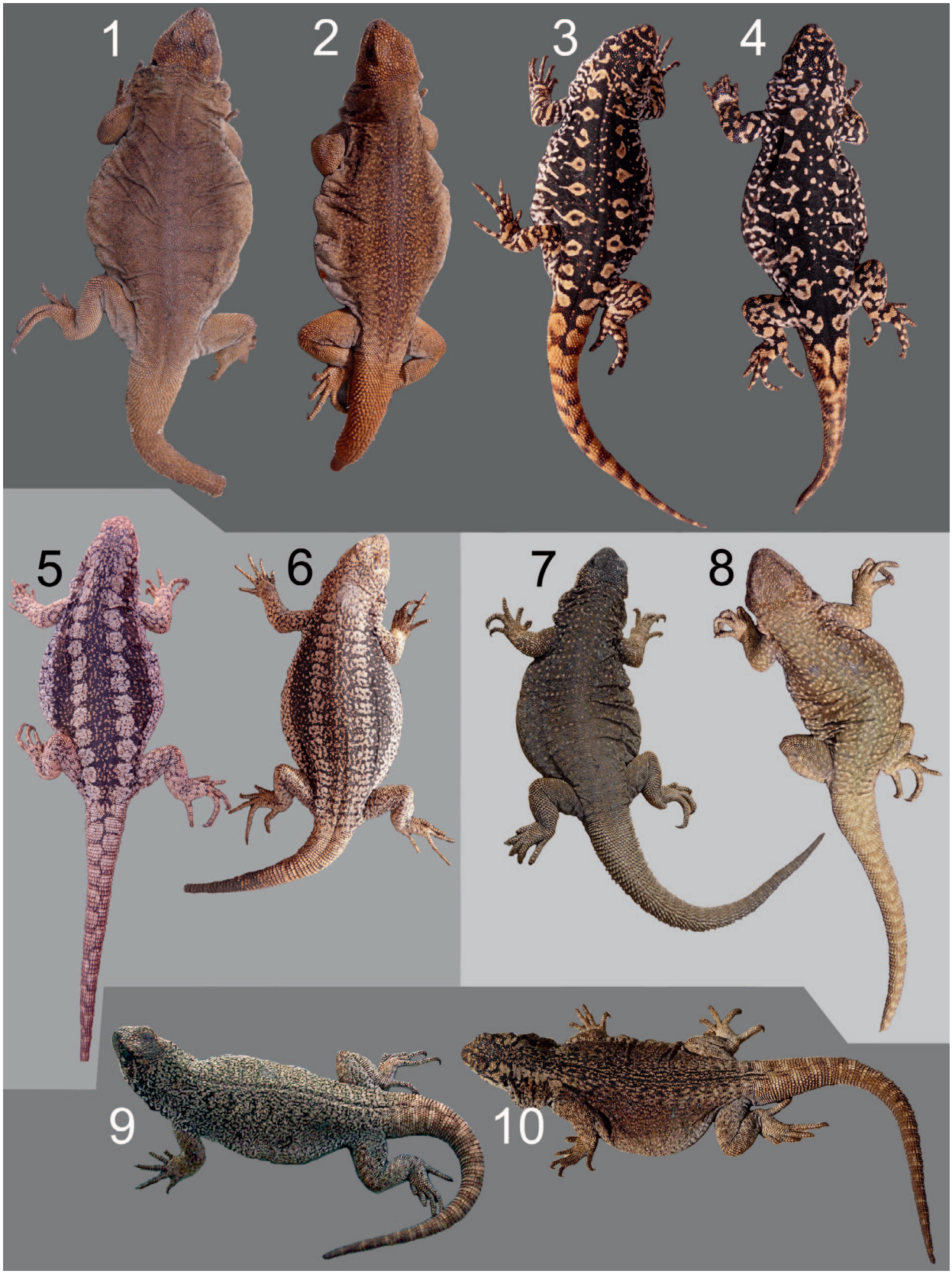

FIG. 5. Species of *Phymaturus* that show conflict in delimitation based on cytochrome *c* oxidase I (COI) marker: First group: (1) *P. spurcus*, (2) *P. agilis*, (3) *P. spectabilis*, (4) *P. excelsus*; Second group: (5) *P. payuniae*, (6) *P. nevadoi*; Third group: (7) *P. somuncurensis*, (8) *P. ceii*; Fourth group: (9) *P. indistinctus*, (10) *P. videlai*.

identify species when introgression, incomplete lineage sorting, or complex species are involved (Vences et al., 2005a,b; Smith et al., 2008). In such cases, nuclear loci are necessary to reliably identify species (Hebert et al., 2003a; Murphy et al., 2013).

We demonstrated that COI barcodes were useful tools for identifying unique lineages into the *Phymaturus* genus when genetic distances between species pairs are >2%. Whereas intraspecific distance in the clade is low (0.36 ± 0.49%, n_c = 518), the mean interspecific genetic distances in most cases is >6%. In cases of low distance (<2%), we could not clearly distinguish different species.

Most *Phymaturus* species of Patagonia are microendemics with isolated distributions in basaltic plateaus. These distributions seem to follow the paleo-geological hypothesis proposed by Coira (1979). The current landscape is composed by very old volcanic tablelands (Tertiary), separated by deep and narrow valleys, and eroded by Paleocene sea ingressions (Coira, 1979), suggesting speciation via vicariance.

Low genetic distances found in nearby species (even sympatric), where gene flow is possible, could be interpreted as a single species with high morphological variation. For the group formed by *P. spurcus*, *P. spectabilis*, *P. agilis*, and *P. excelsus*, the existence of polymorphism has been suggested by Avila et al. (2014), and the existence of dimorphism (brown vs. bold morphs) was reported for *P. excelsus* by Lobo et al. (2012a). The related *P. manuelae* also was described having a noticeable polychromatism (Scolaro and Ibargüengoytía, 2008). Color polymorphism is quite common in lizards (Pérez i de Lanuza et al., 2012) and can be the result of social signaling, stress, or active camouflaging (Stuart-Fox and Moussalli, 2008). *Phymaturus agilis* was previously synonymized with *P. spectabilis* based on females giving birth to morphs of both species (Lobo et al., 2012a). Following the same criteria of those authors, *P. spurcus*, *P. spectabilis*, and *P. excelsus* also should be synonymized because all of these species give birth to different morphs, with the formerly described *P. spurcus* being the valid species epithet for this group. In contrast, another equally probable explanation could be a process of hybridization, or reticulation, resulting from past speciation followed by secondary contact. Hybridization can be tested with multigenerational fertility experiments, but they are difficult to complete. Ibargüengoytía (2004) demonstrated that females of the *patagonicus* group can have biannual reproductive cycles, and juveniles of these viviparous species take 2 yr to reach sexual maturity. Also, future studies might consider the use of nuclear loci to examine speciation and gene flow and test the hybridization or alternative hypotheses.

Low genetic distances in more geographically isolated species (and probably isolated reproductively) could be attributed to recent speciation because evidence (the paraphyletic topology of the phylogenetic trees) suggests that incomplete lineage sorting is present in the group formed by *P. payuniae* and *P. nevadoi*. This process also is probably occurring in the groups formed by *P. somuncurensis* and *P. ceii*, and by *P. indistinctus* and *P. videlai*, but a larger sample is needed to make robust conclusions. Low genetic distances were found both with the COI locus and with cytochrome *b* (Morando et al., 2013), although using single nucleotide polymorphisms (SNPs) and nuclear genes would be preferable.

This is the first study where barcodes were used for lizard species from Argentina. It has been helpful in suggesting candidate species, expanding known geographic ranges, and detecting conflicts in species delimitation. It represents an initial step toward more-focused research. In the future, studies of population genetics may cast light on what is happening in the species complexes identified in this study.

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APPENDIX 1. We provide collection data of individuals used to obtain COI sequences. The Barcode sample ID for each individual is indicated with the acronym CHIVC. Vouchers stored in IADIZA collection (Instituto Argentino de Investigaciones de las Zonas Áridas, Mendoza, Argentina) are indicated with the acronym CH-IADIZA. TT = Terra Typica.

Species	Locality	Coordinates	Barcode sample ID	Collection number	GenBank accession no.
patagonicus group					
Phymaturus agilis	Yuquiche hill, Provincial Road 6, 22 km Southwest of Ingeniero Jacobacci, Río Negro Province (TT)	41°25′48′′S, 69°45′0′′W	CHIVC422; CHIVC424; CHIVC485	CH-IADIZA 674	KU565036 KU565037 KU565035
P. calcogaster	Laguna de la Vaca, Telsen, Chubut (TT)	42°30′0′′S, 67°21′36′′W	CHIVC351; CHIVC352; CHIVC353; CHIVC472; CHIVC473; CHIVC475	CH-IADIZA601: CH-IADIZA602; CH-IADIZA603; CH-IADIZA707; CH-IADIZA708; CH-IADIZA710	KU565038 KU565041 KU565039 KU565043 KU565040 KU565042
P. camilae	Sacanana, Chubut (TT)	42°23′24′′S, 68°49′12′′W	CHIVC457; CHIVC458; CHIVC459; CHIVC460	CH-IADIZA693; CH-IADIZA694; CH-IADIZA695; CH-IADIZA696	KU565049 KU565046 KU565047 KU565045
	Sierra del Chacay, Chubut	42°48′36′′S, 68°1′12′′W	CHIVC492; CHIVC434; CHIVC456	CH-IADIZA691; CH-IADIZA694; CH-IADIZA692	KU565048 KU565050 KU565044
P. castillensis	Est. La Juanita, Sa. del Castillo, Sarmiento, Chubut (TT)	45°08′S, 69°10′W	CHIVC345; CHIVC346; CHIVC347	CH-IADIZA604; CH-IADIZA605; CH-IADIZA606	KU565051 KU565052 KU565053
P. ceii	Near Chasicó (south of El Cuy hill), Río Negro (TT)	40°22′48′′S, 69°0′36′′W	CHIVC442; CHIVC444	CH-IADIZA680; CH-IADIZA682	KU565054 KU565055
P. etheridgei	Quetrequile, Río Negro (TT)	41°35′24′′S, 69°22′48′′W	CHIVC461; CHIVC462; CHIVC466	CH-IADIZA697; CH-IADIZA698; CH-IADIZA702	KU565058 KU565057 KU565056
P. excelsus	Ojo de Agua, Río Negro (TT)	41°32′24′′S, 69°50′59′′W	CHIVC470; CHIVC471; CHIVC476	CH-IADIZA705; CH-IADIZA706; CH-IADIZA711	KU565060 KU565059 KU565061
P. felixi	102 km South of Paso de Indios, Chubut (TT)	44°25′48′′S, 69°17′59′′W	CHIVC348; CHIVC349; CHIVC350	CH-IADIZA607; CH-IADIZA608; CH-IADIZA609	KU565062 KU565063 KU565064
P. indistinctus	Las Pulgas, Chubut (TT)	45°27′0′′S, 69°43′12′′W	CHIVC354; CHIVC355; CHIVC356	CH-IADIZA610; CH-IADIZA611; CH-IADIZA612	KU565067 KU565065 KU565066
P. nevadoi	Sierra del Nevado, near Agua de la India Muerta, Mendoza (TT)	35°43′48′′S, 68°34′12′′W	CHIVC100; CHIVC105; CHIVC110; CHIVC181; CHIVC183; CHIVC187; CHIVC188; CHIVC180.1	CH-IADIZA498; CH-IADIZA506; CH-IADIZA507; CH-IADIZA512; CH-IADIZA511; CH-IADIZA519; CH-IADIZA513; CH-IADIZA518	KU565079 KU565075 KU565080 KU565078 KU565076 KU565071 KU565074 KU565070
	Sierra del Nevado, Mendoza	35°55′48′′S, 68°36′36′′W	CHIVC111; CHIVC112; CHIVC113; CHIVC114; CHIVC116;	CH-IADIZA508; CH-IADIZA497; CH-IADIZA499; CH-IADIZA500; CH-IADIZA505;	KU565077 KU565068 KU565072 KU565069 KU565073
P. patagonicus	60 km Northwest Dolavon, Chubut (TT)	43°27′0′′S, 69°7′12′′W	CHIVC360; CHIVC361; CHIVC362	CH-IADIZA613; CH-IADIZA614; CH-IADIZA615	KU565081 KU565082 KU565083
P. payuniae	Payún Matrú, La Payunia Reserve, Mendoza	36°21′36′′S, 69°15′0′′W	CHIVC21	CH-IADIZA452	KU565102
	Payún Matrú, La Payunia Reserve, Mendoza	36°21′0′′S, 69°14′24′′W	CHIVC23; CHIVC28; CHIVC26.1; CHIVC29 CHIVC12; CHIVC13; CHIVC14; CHIVC16; CHIVC17	CH-IADIZA454; CH-IADIZA449; CH-IADIZA458	KU565097 KU565104 KU565109 KU565106 KU565094 KU565099 KU565098 KU565101 KU565084
	Yardangs, La Payunia Reserve, Mendoza	36°28′48′′S, 69°22′12′′W; 36°29′24′′S, 69°22′12′′W	CHIVC160; CHIVC152; CHIVC146; CHIVC159 CHIVC156; CHIVC138; CHIVC162	CH-IADIZA439; CH-IADIZA445; CH-IADIZA438; CH-IADIZA444 CH-IADIZA446; CH-IADIZA443; CH-IADIZA437	KU565086 KU565093 KU565095 KU565103 KU565087 KU565088 KU565108

APPENDIX 1. Continued.

Species	Locality	Coordinates	Barcode sample ID	Collection number	GenBank accession no.
	Escorial, La Payunia Reserve, Mendoza	36°32′59′′S, 69°22′12′′W	CHIVC73; CHIVC74		KU565092 KU565096
	Payún Highland, Mendoza	36°39′36′′S, 69°16′48′′W	CHIVC81; CHIVC76; CHIVC78; CHIVC80; CHIVC79; CHIVC77	CH-IADIZA456; CH-IADIZA448; CH-IADIZA453; CH-IADIZA450	KU565091 KU565085 KU565110 KU565089 KU565107 KU565105
	La Payunia Reserve, Mendoza	36°28′48′′S, 69°22′12′′W	CHIVC63; CHIVC51	CH-IADIZA451; CH-IADIZA457	KU565090 KU565100
P. sinervoi	Cari Laufquen basaltic tableland, Provincial Road 6, 61 km north of Ingeniero Jacobacci, Río Negro (TT)	40°53′24′′S, 69°17′59′′W	CHIVC481; CHIVC482; CHIVC483; CHIVC484	CH-IADIZA714; CH-IADIZA715; CH-IADIZA716; CH-IADIZA717	KU565114 KU565113 KU565115 KU565112
P. somuncurensis	Somuncurá Plateau, Río Negro (TT)	41°25′12′′S, 66°58′48′′W	CHIVC493; CHIVC494	CH-IADIZA728; CH-IADIZA729	KU565116 KU565117
P. spectabilis	Yuquiche hill, Provincial Road 6, 22 km Southwest of Ingeniero Jacobacci, Río Negro (TT)	41°25′48′′S, 69°45′0′′W	CHIVC445; CHIVC448; CHIVC449	CH-IADIZA689; CH-IADIZA684; CH-IADIZA685	KU565118 KU565119 KU565120
P. spurcus	Huanuluan, Río Negro (TT)	41°17′24′′S, 69°55′12′′W	CHIVC429; CHIVC450; CHIVC451; CHIVC452	CH-IADIZA689; CH-IADIZA686; CH-IADIZA687; CH-IADIZA688	KU565121 KU565124 KU565122 KU565123
P. tenebrosus	Cerro Alto, Río Negro (TT)	40°52′48′′S, 70°34′12′′W	CHIVC437; CHIVC438; CHIVC439; CHIVC440	CH-IADIZA675; CH-IADIZA676; CH-IADIZA677; CH-IADIZA678	KU565125 KU565127 KU565126 KU565128
P. videlai	Buen Pasto, Chubut (TT)	42°4′12′′S, 69°24′36′′W	CHIVC357; CHIVC358; CHIVC359	CH-IADIZA616; CH-IADIZA617; CH-IADIZA618	KU565129 KU565130 KU565131
P. sp.1	Los Adobes, Chubut	43°19′48′′S, 68°44′24′′W	CHIVC453; CHIVC455	CH-IADIZA697; CH-IADIZA699	KU565034 KU565033
palluma group					
P. punae	Caserones, San Guillermo Reserve, San Juan	29°15′0′′S, 69°23′24′′W	CHIVC418	CH-IADIZA671	KU565111
Liolaemus					
L. buergeri	Route N°145, Pehuenche's valley, Mendoza	35°58′12′′S, 70°18′36′′W	CHIVC368	CH-IADIZA628	KU565031
L. petrophilus	Sacanana, Chubut	42°23′24′′S, 68°49′12′′W	CHIVC490	CH-IADIZA722	KU565032