INTRODUCTION

Geographic ranges are dynamic properties of species, easily modified in response to biotic and abiotic drivers (Case et al. 2005, Soberon 2007, Linder et al. 2012, Simonov 2014). New locality records, new country records, or new habitat records ought to be documented as they may indicate range extensions resulting from demographic expansions and/or deficiency in species geographical samplings. Cases like these have been observed, for example, in *Loxodontomys micropus* (Waterhouse, 1837) (see Cañon et al. 2010), in *Dromiciops gliroides* Thomas, 1894 (see D’Elia et al. 2016, but also see Suárez-Villota et al. 2018), among many others.

Despite a renewed interest in the taxonomy and systematic of South American sigmodontine rodents, many taxa still lack detailed analyses of morphological, geographical, genetic and ecological variation; thus, the alpha-taxonomy of these groups is only partially resolved. Detailed morphological and genetic studies provide insights into the biology and evolution of species with large anthropogenic impact, as is the case of species of zoonotic or agriculture concern (for example, Yackulic et al. 2010, Pigot and Tobias 2013, Bordes et al. 2015). An example of this is the genus *Calomys* Waterhouse, 1837 where several species share similar morphological and morphometric characteristics, many of which are only recently being duly appreciated (Almeida et al. 2007, Haag et al. 2007, Salazar-Bravo 2015).

A couple of the species in the genus have received recent interest, for example, *Calomys laucha* (Fischer, 1814) (González-Ittig et al. 2014) or *Calomys sorellus* (Thomas, 1900) (Zeballos et al. 2014); in both bases, cryptic species within currently recognized taxonomic units were suggested.

One species of the genus that needs a study of its taxonomic situation is *Calomys tener* (Winge, 1887), the Delicate Vesper Mouse, a small body-sized species, broadly distributed in open vegetative formations in the Cerrado of central Brazil and eastern Bolivia, Caatinga and Pantanal in Brazil (Salazar-Bravo 2015).

Does the widely distributed rodent *Calomys tener* (Cricetidae: Sigmodontinae) constitute a single evolutionary unit?

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ABSTRACT. The nominal species *Calomys tener* (Winge, 1887) ranges broadly in open lands of the Caatinga, Cerrado, Pantanal and Mata Atlântica of Brazil, and was recently reported from the Pampas of southern Brazil, and in the Selva Paranaense of eastern Paraguay and northeastern Argentina. This rodent can be infected with the pathogenic Araraquara hantavirus in Brazil. Given that most epidemiological studies have not taken into account updated taxonomic findings of their rodent hosts, in this study, we obtained sequence data of the Cyt-b and COI genes of specimens of *C. tener* from 22 different geographical localities from throughout the currently known distribution of the species (including individuals from Argentina, Paraguay, Bolivia, and Brazil) to test if it constitutes a single genetic unit or if it presents genetic discontinuities that may represent different evolutionary lineages. Phylogenetic analyses including several species of *Calomys* recovered several clades with strong support. Regarding *C. tener*, it is recovered as sister to the node that cluster *C. laucha* (Fischer, 1814) *sensu lato*, *C. expulsus* (Lund, 1841) and species in the *C. callosus* (Rengger, 1830) species complex. At the intraspecific level there are no genetic gaps among haplotypes of *C. tener* that could suggest more than one species. The recent captures in the Pampas of southern Brazil and in the Selva Paranaense suggest that the species may be colonizing new geographic areas.

KEY WORDS. Cyt-b, phylogenetic relationships, South America.
Several novel reports of occurrence for *C. tener* have been published: for example, the species was recently reported in the Atlantic and Paranaense forests of Argentina and Paraguay and areas in southeastern Brazil (González-Ittig et al. 2014, Quintela et al. 2014), all of which were considered outside of the range of the species (Salazar-Bravo 2015). The ecology of *C. tener* is better known in Brazil, where it occupies a mix of habitat types, including domestic and agricultural areas, and populations appear to be stable all year-long (Umetu and Pardini 2007). Individuals in a handful of populations in São Paulo state are known to be infected with Araraquara hantavirus, currently considered the most pathogenic of all South American hantaviruses (Figueiredo et al. 2010). It has been estimated that more than 50% of total hantavirus pulmonary syndrome (HPS) cases in Brazil are caused by Araraquara hantavirus and that the human fatality rate is over 40% for infected patients (Figueiredo et al. 2009). Although *Necromys lasiurus* (Lund, 1840) is the natural reservoir of this viral genotype, the role of *C. tener* in the circulation of the virus is not completely understood; however, reports of the occurrence of this species in other areas of Brazil and elsewhere in South America should be considered an element of risk for the potential presence of this pathogenic virus in these areas. As suggested by Mills and Childs (1998) and Mills et al. (1999), reservoir studies, including those related to their systematics, taxonomy, and geographic distribution, are necessary for a comprehensive understanding of the biology of emerging zoonotic diseases.

In the case of *C. tener*, different molecular systematic studies have included sequences from few localities (see Table 1). For example, Salazar-Bravo et al. (2001) included sequences of the mitochondrial cytochrome b (Cyt-b) gene from individuals of one locality in Bolivia and one in Brazil. Almeida et al. (2007) used the sequences of Salazar-Bravo et al. (2001) and added Cyt-b data of individuals from five sites in Brazil. Haag et al. (2007) also used the sequences obtained by Salazar-Bravo et al. (2001) and added molecular information of the same gene from individuals from three extra localities in Brazil. González-Ittig et al. (2014) in a study focused on the systematics of *C. laucha*, identified three individuals from Paraguay and Argentina that actually correspond to *C. tener*. The results of all these studies have recovered *C. tener* as monophyletic without any phylogenetic structure or sub-clades, however, these conclusions are partial since none have integrated the whole information available for the species. To detect possible genetic gaps among specimens of this species over a broad geographic area, in the present study we compared sequences of the mitochondrial genes Cyt-b and cytochrome oxidase subunit 1 (COI) of individuals from Argentina, Paraguay, Bolivia, and Brazil, encompassing most of the known distribution of the rodent.

**MATERIAL AND METHODS**

Partial sequences of the Cyt-b (788bp) of 31 individuals identified as *C. tener* were used for this study: from Brazil (n = 19), Bolivia (n = 1), Paraguay (n = 4), and Argentina (n = 7) and five partial sequences of the COI from Brazil, giving a total of 22 sampled localities (Fig. 1, Table 1). The five sequences of the COI gene were published by Müller et al. (2013). Of the Cyt-b sequences, 19 were published in different studies listed in Table 1, whereas 12 were generated at the lab of Jorge Salazar-Bravo (Texas Tech University, Lubbock, Texas, USA) from samples obtained on loan from several institutions (see Acknowledgements).

Tissues were processed for DNA extraction using DNeasy Blood and Tissue kit (Qiagen, Cat# 69505) and the manufacturer's recommendations. DNA was quantified using Nanodrop (NanoDrop™ 1000 Spectrophotometer v3.7) and 1% agarose gels. A fragment of the mitochondrial Cyt-b gene was amplified using one of two primer combinations of Mus14095-F and Mus15398-R or L14415-F and Mus15398-R (González-Ittig et al. 2014). Thermocycler conditions were: initial denaturation at 94 °C for 3 minutes, followed by 30 cycles of denaturation at 94 °C for 45 seconds, annealing at 50 °C for 1 minute, and extension at 72 °C for 1.5 minutes, and final extension at 72 °C for 5 minutes. Reaction mixtures were set at 25μl total volume with 1 μl of DNA (20–30 ng/μl), 0.13 μl of Promega Taq (Cat# M3008, 5U/μl), 0.8 μl of dNTPs mix (Cat#U151B, 10mM), 2 μl of MgCl2 (25 mM), 2.5 μl of 5 × Go Taq Green buffer, and 0.75 μl of each primer (10 μM). Amplified products were visualized in 1% agarose gel; 1Kb ladder was used as standard DNA size. Well amplified products were sequenced in Macrogen, USA (http://www.macrogenusa.com) with both amplifying primers. Raw chromatograms were cleaned, translated to amino-acids to check for premature stop codons and other nonfunctional mutations. Contigs for individual sequences were assembled using SeqManII 5.07 (Lasergene, DNASTAR, Madison, WI, USA). New sequences of *C. tener* were deposited in GenBank (Table 1).

All sequences, including those available online, were aligned using default parameters with ClustaW in MEGAS 5.0 (Tamura et al. 2011). Identical sequences of *C. tener* were identified and collapsed into haplotypes using PopART v1.7 (Leigh and Bryant 2015). Haplotypes were used for all further phylogenetic analyses; in the matrix we included sequences published by Almeida et al. (2007), Haag et al. (2007) and González-Ittig et al. (2014) corresponding to the following species: *Calomys expulsus* (Lund, 1841), *C. fecundus* (Thomas, 1926), *C. callous* (Rengger, 1830), *C. hummelincki* (Husson, 1960), *C. lepidus* (Thomas, 1884), *C. venustus* (Thomas, 1894), *C. muculinus* (Thomas, 1913), *C. tocanitinsi* Bonvicino, Lima & Almeida, 2003, *C. sorellus* and *C. laucha* (both clades A and B). Sequences of *Eligmodontia typus* Cuvier, 1837, *Auliscomys sublimis* (Thomas, 1900), *Phyllotis xanthopygus* (Waterhouse, 1837), *Andalgalomys pearsoni* (Myers, 1977) and *Salinomys delicatus* Braun & Mares, 1995 were used as outgroups. Maximum Parsimony (MP) analysis was performed with PAUP 4.0.b10 (Swofford 1998), where characters were unordered and equally weighted. We performed a heuristic search of 1000 iterations of random taxon addition using the TBR (tree bisection-reconnection) branch swapping algorithm. Maximum Parsimony
analyses resulting in more than one most parsimonious tree were summarized in a strict consensus tree. Nonparametric bootstrap support values were calculated using 1000 replicate searches. The best-fitting model of sequence evolution was selected using jModeltest 2 (Darriba et al. 2012) in which likelihood scores for 88 different models were computed. The HKY+I+G model was selected using the Bayesian information criterion and the following starting parameters were used subsequently in Bayesian inferences (BI) and Maximum Likelihood (ML) analyses: a base frequency of A = 0.3030, C = 0.3096, G = 0.0877, T = 0.2997; a transition/transversion = 4.233; a proportion of invariable sites of 0.4860; a gamma distribution with alpha = 1.0360. The BI analyses were performed using MrBayes 3.2 (Ronquist et al. 2012) with two independent Markov chain-Monte Carlo (MCMC) runs, with one cold and three heated chains each. Runs were performed for five million generations and trees were sampled every 1000 generations. We discarded the first 25% of the samples as "burn in" and the two runs converged on similar posterior estimates with an average standard deviation of split frequencies of 0.006. We assessed convergence and mixing visually using Tracer v1.5 to plot likelihood scores for all parameters by generation time and by calculating effective sample sizes (Rambaut and Drummond 2007). ML trees were constructed using the online version of program PhyML ver 3.0 (http://www.atgc-montpellier.fr/phyml) (Guindon et al. 2010). In PhyML we used the same substitution model and the parameters described above; 1000 bootstrap replicates were performed.

In addition, a median-joining network was constructed using PopART v1.7 with 1,000 permutations: the method estimates the relative abundance of each haplotype and the genealogical relationships among them. Kimura-2 parameter genetic distances (K2p) among haplotypes of *C. tener* were calculated with MEGA5.0.

**RESULTS**

A total of 28 distinct haplotypes were identified from the 31 *C. tener* individuals analyzed. In the alignment with other species of *Calomys* and outgroups of other genera, from the 794 sites, 342 characters were variable of which 275 were parsimony-informative. In the phylogenetic trees obtained with different phylogenetic methods (ML, BI and MP) for species of...
Calomys, several clades are identified with strong support. For example, the one composed by the species C. lepidus/C. sorelus/C. musculus. The phylogenetic position of C. hummelincki is not resolved, since the node has low support values. Another clade strongly supported is the one composed by the species C. expulsus and species of the C. callosus complex (C. tocanensis/C. callosus/C. fecundus/C. venustus) (Fig. 2). Sister to this clade is the one composed by clades A and B of C. callosus.

At the intraspecific level there is no phylogenetic structure among haplotypes of C. tener that would suggest more than one species, even when samples have been collected throughout a large part of its geographical distribution (encompassing individuals from Argentina, Paraguay, Bolivia, and Brazil). In Fig. 2, haplotypes from localities 1, 4, 5, 12 and 13 situated in Brazil are the first to split off, while those from localities 15, 16 and 17 located in Argentina are among the last. The median-joining network (Fig. 3) showing the frequencies and the relationships of the 28 haplotypes does not corroborate these ancestral-derived positions, since none of the haplotypes 11, 17, 18, 20, 21, 22, 24 and 25 are sister to the node that clusters C. tener sensu lato, C. expulsus and C. callosus sensu lato.
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The order of the support values in the nodes is as follows: Bayesian posterior probabilities/maximum likelihood bootstrap/maximum parsimony bootstrap. Sequences from GenBank are indicated with their accession number and the species names. In the clade corresponding to C. tener the haplotype number, the specimen having each haplotype and the sampling locality is indicated. The vertical bars represent associations also observed in the network of Fig. 3, although with no statistical support. The intraspecific relationships recovered with the different phylogenetic methods are indicated.

23, 24 or 25 are in the center of the network. No obvious pattern was observed between haplotype identity and geographic locations, except for a tendency that haplotypes unique to Argentina (haplotypes 1, 2, 3, 4, 5 and 6) group together on one side of the network and the Brazilian haplotypes mentioned above are in the other side of the network (Fig. 3). Although with low support, some relationships among haplotypes in Fig. 3 are also recovered with the three or with two different phylogenetic methods.
phylogenetic methods (indicated in Fig. 2 with a vertical bar). The K2p genetic distances among \textit{C. tener} haplotypes averaged 1.29\% ± 1.05, also compatible with intraspecific genetic variation (Bradley and Baker 2001). In the network, haplotypes 8 and 9 from locality 11 are separated from the remaining haplotypes by more than 12 mutations, moving away from the general trend of diversification within \textit{C. tener}.

Four CO1 haplotypes were identified among the five individuals of \textit{C. tener} sequences. In the alignment with other species of \textit{Calomys} and outgroups, from the 640 sites, 173 characters were variable of which 100 were parsimony-informative. The phylogenetic trees obtained ML, BI and MP have the same topology with varying levels of support for each node. The clade of \textit{C. tener} is strongly supported and has a sister relationship with a clade formed by \textit{Calomys cerqueiral} Bonvicino, Oliveira & Gentile, 2010 and \textit{C. expulsus} (Fig. 4). As in the Cyt-b dataset, there are no large genetic gaps among haplotypes of \textit{C. tener} that could suggest more than one species, even though samples were collected in distantly locations from Brazil (locations 4, 10 and 14 in Fig. 1). In addition, the K2p genetic distances among \textit{C. tener} haplotypes averaged 1.31 ± 1.01%.

**DISCUSSION**

In this study, we used the Cyt-b gene from 31 individuals and the COI gene from five individuals collected throughout the range of the Delicate Vesper Mouse. Our sampling was broad and includes the known distribution range of the species as currently understood (Salazar-Bravo 2015). The mean genetic distance detected was 1.29\% and 1.31\% with the Cyt-b and COI genes, respectively, which are in line with the estimations...
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for intraspecific comparisons according to Bradley and Baker (2001). These estimates are also in accordance with intraspecific variation reported for other species of Calomys (e.g., Salazar-Bravo et al. 2001, Almeida et al. 2007, González-Ittig et al. 2007, Bonvicino et al. 2010, Nascimento et al. 2011). It is important to highlight that the monophyletic nature of C. tener was supported with two markers (Figs 2, 4). Notwithstanding, this study was performed only with mitochondrial DNA which does not undergo recombination, has uniparental inheritance, and reflects only the female evolutionary history. The shortcomings of this approach are evident when males and females have different dispersal patterns or when closely related species hybridize, so the resulting genetic structure could potentially be different for both sexes. Therefore, to confirm that C. tener has low levels of intraspecific divergence and to reflect the diversification processes of both sexes, nuclear genes should be included in further studies (Dupuis et al. 2012).

The taxonomic uniformity of the species was already suggested by cytogenetic studies performed by other authors over the last 30 years. For example, Mattevi et al. (2005) reported 2n = 66 (FN = 66–68) for specimens from Minaçú (Goiás). A 2n = 66 (FN = 66) was reported by Bonvicino et al. (2010) for individuals from Santa Tereza (Espírito Santo), Jaborandi (Bahia), Mimosa de Goiás (Goiás) (specifically the Cyt-b of individual MN36437, karyotyped in that study, was included in the present study, Table 1). This chromosome number was also reported for specimens from Distrito Federal, Cocos (Bahia), Campinas, Itirapina, Itapetininga, Pedreira and Rio Claro, all in

Figure 4. Phylogram of the Bayesian consensus tree obtained from COI data set for species of Calomys with emphasis in Calomys tener. The order of the support values in the nodes is as follows: Bayesian posterior probabilities/maximum likelihood bootstrap/maximum parsimony bootstrap. Sequences from GenBank are indicated with their accession number and the species names. In the clade corresponding to C. tener the haplotype letter, the specimen having each haplotype and the sampling locality is indicated.
São Paulo state and Gaúcha do Norte (Mato Grosso) (Yonenaga 1975, Bonvicino and Almeida 2000, Fagundes et al. 2001). In addition, Haag et al. (2007) detected the chromosomal number 2n = 66 (FN = 66–70) for specimens from Serra da Mesa (Goiás), Aliança do Tocantins (Tocantins), Quintão (Rio Grande do Sul), Tupi Paulista (São Paulo) all in Brazil and one specimen from Santa Rosa de la Roca (Santa Cruz) in Bolivia. Haag et al. (2007) also sequenced the Cyt-b of all the karyotyped specimens which were also included in the present study (Table 1).

In this study, the only discordant result that alters genetic continuity, is that involving haplotypes 8 and 9 from locality 11, which are very divergent. Given the central geographic position of this locality (Fig. 1) and the general level of genetic differentiation within the species, these haplotypes could be the result of sequencing errors as was already advocated by Almeida et al. (2007). Therefore, the molecular results obtained here and the substantial cytogenetic evidence, support the inference that C. tener constitutes only one taxonomic unit. The type locality of the species is Lagoa Santa in the state of Minas Gerais in Central Brazil corresponding to the Cerrado ecoregion. Notwithstanding, it should be noted that Mattevi et al. (2005) reported the chromosomal complement for two specimens (LF4974 and LF5020) from Rondônia state, Brazil, with 2n = 64 (FN = 64) and suggested they could correspond to C. tener. However, the authors, with caution, denominated these individuals as Calomys sp. Given the chromosomal uniformity detected by many authors for C. tener, it is unlikely that the karyotype from Rondônia corresponds to this species because it lacks one autosomal pair and presents differences in the size and shape of the X chromosome. Unfortunately, no molecular data is available for the two specimens from Rondônia, therefore we cannot explicitly test the hypothesis that either of these correspond to C. tener.

The taxonomic status of C. tener and C. laucha are historically intertwined; originally described in the late 19th century, C. tener maintained its specific status until Philip Hershkovitz included it in his polytypic concept of C. laucha (Hershkovitz 1962). The status of C. tener, with respect to C. expulsus and C. callosus was forcefully established by Bonvicino and Almeida (2000), but it was not until Salazar-Bravo et al. (2001) that a C. laucha and C. tener were included in a phylogenetic analysis of the genus. In this work, Salazar-Bravo et al. (2001) resolved these species as sister taxa, with little or no support. Further work by Almeida et al. (2007) showed –with a larger sampling of taxa and individuals – that C. tener was in fact the sister taxon to all lowland forms of Calomys. Similar results were reported by Bonvicino et al. (2010). A great part of the current taxonomic confusion stems from the similar morphological and morphometric characteristics of C. tener and C. laucha. In fact, they are so similar morphologically that even the last reviser of the genus confused individuals of C. tener from Mato Grosso for C. laucha (Olds 1988). Although C. tener is more or less uniform across its geographic range (upper parts of body yellowish to dark brown, with a reddish hue in most specimens; ventral region grayish to whitish with base of hairs gray) the geographical variation in C. laucha is extensive (as described in Salazar-Bravo 2015). In fact, in areas of potential geographical overlap between these species (Misiones region of Argentina or Paraguay) morphological patterns of geographic variation would make it difficult to separate these forms on body coloration alone. Morphometrically, these forms also overlap in multivariate space (Olds 1988, Bonvicino et al. 2003, Teta et al. 2017). Calomys tener is however, on average, larger (TL = 143.18 vs 132.4) and has a relatively longer tail than C. laucha (T/HB = 80.4 in C. tener, but 72.7 in C. laucha). Calomys tener has a longer maxillary toothrow than C. laucha (3.45 vs 3.2 mm); finally, C. tener can be distinguished from C. laucha by the presence of ridges along the sides of the supraorbital region (not present in C. laucha).

In a recent study, González-Ittig et al. (2014) also confirmed the two species; three specimens corresponding to C. tener (one from Argentina and two from Paraguay) were wrongly classified as C. laucha according to external characters. In the present study, the same occurred with specimens from localities 16- Estancia Santa Inés (Misiones) and 17- RN 12 y Arroyo Itaembé Mini (Misiones) both in Argentina. Considering body size and cranial measurements, Salazar-Bravo et al. (2001) and Bonvicino et al. (2010) separated two groups of Calomys, a larger-bodied species group, including C. callosus, C. expulsus, C. tocantinsi, C. callidus and C. cercueinai, and a smaller-bodied species group, including C. tener and C. laucha. However, in the phylogenetic tree obtained here (Fig. 2) and in those obtained by Almeida et al. (2007) and by Haag et al. (2007), C. tener is not the sister species of C. laucha. It is important to highlight that phylogenetic trees reported by those authors and that of Fig. 2 show the same global topology with the following relationships: (C. tener (C. laucha and the big clade including all the large-bodied species of Calomys)).

Quintela et al. (2014) recorded for the first time the sympatry between C. tener and C. laucha and warned that the distribution limits in the Rio Grande do Sul state in Brazil should be investigated. Considering the two clades found for C. laucha by González-Ittig et al. (2014), it is highly probable that what Quintela et al. (2014) found corresponds to our clade B. Thus, there is no evidence of sympatry with C. laucha sensu stricto (or clade A) (see a discussion on the geographic distribution of the species in Teta et al. 2017). In Rio Grande do Sul both clade B of C. laucha and C. tener could be getting in contact occasionally; it is known the two species have very low densities, because dozens of studies (and thousands of trap-nights) have been conducted throughout the state and until recently none reported captures of the two species (Cademartori et al. 2004, Badzinski et al. 2012, Quintela et al. 2012, 2014). González-Ittig et al. (2014) suggested that clade B of C. laucha may be experiencing a process of population expansion and dispersal into this region. The same could be occurring in C. tener. In the present study, many of the basal haplotypes of the Cyt-b gene are from localities of...
north-central Brazil (localities 1, 4 and 5), however, the two most basal haplotypes, H_11 and H_25, are from localities 12 and 13 in Rio Grande do Sul. In the COI gene, the most basal haplotype is H_D from locality 14 also in Rio Grande do Sul; thus the results of both genes are challenging the hypothesis of a north-south colonization of *C. tener* (Figs 2, 4). Contradicting the phylogenetic trees, in the network of the Cyt-b gene, both H_11 and H_25 are in a peripheral position which is more compatible with new haplotypes (Fig. 3). Almeida et al. (2007) described an intraspecific geographic structure given that they found a clade containing samples from São Paulo state, which was clearly separated from the remaining specimens. In the present study we did not find this pattern. We found instead that some haplotype-relationships in Fig. 2 were also detected in the network (Fig. 3), but without a clear geographic pattern. Nevertheless, it is important to consider that the sample size is low and it is not possible to make too many interpretations about the demographic history of the species, what should be performed in a future phylogeographic study.

In summary, *C. tener* constitutes only one evolutionary unit corroborated by the molecular data presented here using individuals encompassing most of the known geographical distribution of the species. In the present study we tried to survey the overall genetic differentiation of the species not deepening in the genetic structure itself. Because *C. tener* is a potential human health risk by Araraquara hantavirus in South America, additional surveys in areas not represented in our sampling, coupled with a broader phylogeographic study is needed to expand our understanding of the ecological and biogeographical processes experienced by the species.

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Calomys tener constitutes a single evolutionary unit


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