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Giménez, Analía L.; Giannini, Norberto P.; Almeida, Francisca C.

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Artículo

MITOCHONDRIAL GENETIC DIFFERENTIATION AND PHYLOGENETIC RELATIONSHIPS OF THREE *Eptesicus* (*Histiotus*) SPECIES IN A CONTACT ZONE IN PATAGONIA

Analía L. Giménez[1], Norberto P. Giannini[2] and Francisca C. Almeida[3]

[1]Laboratorio de Investigaciones en Evolución y Biodiversidad, Facultad de Ciencias Naturales y Ciencias de la Salud, Universidad Nacional de la Patagonia San Juan Bosco, Sede Esquel, Esquel, Chubut, Argentina.
[Correspondence: Analía L. Giménez <al_gimenez@yahoo.com.ar>]
[2]Unidad Ejecutora Lillo, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Fundación Miguel Lillo, Facultad de Ciencias Naturales e Instituto Miguel Lillo, Universidad Nacional de Tucumán, San Miguel de Tucumán, Tucumán, Argentina.
[3]Departamento de Ecología, Genética y Evolución, Universidad de Buenos Aires y Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Capital Federal, Argentina.

ABSTRACT. *Eptesicus* (*Histiotus*) *magellanicus* is a relatively rare and poorly known Patagonian endemic. This species is currently recognized as a valid, but until recently some authors treated it as a subspecies of *E.* (*H.*) *montanus*. In fact, no molecular data exist to support the distinctiveness of the former. In Patagonia, the distribution of these two species overlap marginally; they are also sympatric with a third *Histiotus* species, *E.* (*H.*) *macrotus*. In this study, we present for the first time molecular data that corroborate morphological evidence of the separation between *E.* (*H.*) *magellanicus* from other *Eptesicus* (*Histiotus*) species, in special the ones with which it shares its distribution. We sequenced a nuclear intron (*THY*) and the cytochrome b (*Cyt b*) gene from specimens of *E.* (*H.*) *magellanicus*, *E.* (*H.*) *montanus*, and *E.* (*H.*) *macrotus*, collected in the Chubut Province (Argentina), and from an undescribed *Eptesicus* (*Histiotus*) species from Peru. We included these sequences in a phylogenetic analysis together with previously published sequences of four typical *Eptesicus* species. The THY intron showed very little variation, while the *Cyt b* phylogeny recovered three highly supported *Histiotus* clades. A highly supported clade comprising all specimens of *E.* (*H.*) *magellanicus* was the first to split off *Histiotus*, suggesting that the Andean region was important during the early diversification of the genus. Unexpectedly, the clade containing the specimens of *E.* (*H.*) *macrotus* and *E.* (*H.*) *montanus* showed no internal resolution, either questioning their mutual identity as a separate species, or suggesting the occurrence of local hybridization and introgression.

RESUMEN. Diferenciación genética mitocondrial y relaciones filogenéticas entre tres especies de *Eptesicus* (*Histiotus*) en una zona de contacto en la Patagonia. *Eptesicus* (*Histiotus*) *magellanicus* es una especie endémica de Patagonia, relativamente rara y pobremente conocida. Actualmente *Eptesicus* (*Histiotus*) *magellanicus* es reconocida como especie válida, sin embargo recientemente ha sido considerada como subespecie de *Eptesicus* (H.) *montanus* por algunos autores. En Patagonia estas dos especies solapan sus distribuciones marginalmente, donde además son simpátricas con una tercer especie, *Eptesicus* (*H.*) *macrotus*. En este estudio presentamos por primera vez datos moleculares que corroboran la evidencia morfológica que diferencian a *E.* (*H.*) *magellanicus* del resto de las especies de *Histiotus*, en especial con aquellas con las que comparte su distribución. Se secuenciaron un intron del gen de la tirotropina y gen citocromo b para especímenes de *E.* (*H.*) *magellanicus*, *E.* (*H.*) *montanus*, y *E.* (*H.*) *macrotus*, colectados en la provincia del Chubut (Argentina), y dos ejemplares de *Eptesicus* (*H.*) sp. de Perú. Analizamos sus secuencias junto con otras

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previamente publicadas para cuatro especies típicas de *Eptesicus*. El gen tirotropina mostró muy poca variación, mientras que la filogenia obtenida con el gen citocromo b recuperó tres clados de *Histiotus* fuertemente soportados. El clado que incluyó a todos los especímenes de *E.* (*H.*) *magellanicus* fue el primero en separarse dentro de *Histiotus*, sugiriendo que la región andina fue importante durante la diversificación temprana del género. Inesperadamente, el clado que contenía las muestras de *E.* (*H.*) *macrotus* y *E.* (*H.*) *montanus* no mostró resolución interna, sugiriendo dos posibles alternativas, la existencia de una única especie o la ocurrencia de hibridación e introgresión local.

Key words: Chiroptera, cytochrome b, Patagonia, phylogeny, southern big-eared brown bat, thyrotropin intron.

Palabras clave: citocromo b, filogenia, murciélago orejón marrón del sur, Patagonia, tirotropina.

INTRODUCTION

Vespertilionidae constitutes the largest chiropteran family and the second family after Muridae within Mammalia (Simmons 2005). With ca. 436 species (Simmons 2005; Amador et al. 2016) of primarily insectivorous bats, vespertilionid bats as a group have a nearly cosmopolitan distribution (Hoofer et al. 2003; Simmons 2005; Van Den Bussche & Lack 2013). As compared to members of other families, vespertilionids exhibit subtle interspecific morphological variation, even among distantly related species, which has posed a challenge for systematists attempting to elucidate their evolutionary history (see Jones et al. 2002; Van Den Bussche & Lack 2013). One example is represented by the complex genus *Eptesicus*. The pioneer work of Hoofer & Van Den Bussche (2003) on the molecular phylogeny of vespertilionids recovered traditional *Histiotus* nested within a clade containing *Eptesicus* species of the Americas (see also Roehrs et al. 2010). This result suggested that New World species of *Eptesicus* (i.e., *E. brasiliensis*, *E. chiriquinus*, *E. diminutus*, *E. furinalis*, and *E. fuscus*) are more closely related to species of *Histiotus* than they are to Old World *Eptesicus* species (e.g., *E. hottentotus* and *E. serotinus*; Hoofer et al. 2003; Roehrs et al. 2010). Recent phylogenies with a wide comprehensive taxonomic coverage have confirmed these results (e.g., Amador et al. 2016). Accordingly, *Histiotus* has since been considered a subgenus of *Eptesicus* (Hoofer & Van Den Bussche 2003; Roehrs et al. 2010; Amador et al. 2016). Species of subgenus *Histiotus* are early differentiated from typical *Eptesicus* by the presence of large tympanic bulla and long pinnae, convergent in size and morphology with those of plecotine bats.

While the distribution of *Eptesicus* sensu lato is nearly cosmopolitan, *Histiotus* is endemic to South America and includes eight species: *E.* (*H.*) *alienus* Thomas 1916; *E.* (*H.*) *humboldti* Handley 1996; *E.* (*H.*) *laephotis* Thomas 1916; *E.* (*H.*) *macrotus* (Poeppig 1835); *E.* (*H.*) *magellanicus* (Philippi 1866); *E.* (*H.*) *montanus* (Philippi & Landbeck, 1861); *E.* (*H.*) *velatus* (I. Geoffroy 1824) (Simmons 2005), and the recently described *E.* (*H.*) *diaphanopterus* (Feijó et al. 2015). Controversies exist about the status of some of these species and their phylogenetic relationships remain unresolved (e.g. Handley & Gardner 2008). One problematic case involves the southern big-eared brown bat, *E.* (H.) *magellanicus*, a Patagonian endemic species with type locality in the Magellan Strait in southern Chile (Philippi 1866). Some authors have placed *E.* (H.) *magellanicus* under synonymy with *E.* (H.) *montanus* (Koopman 1993) or as subspecies of the latter (Osgood 1943; Cabrera 1958; Handley & Gardner 2008); currently, it is recognized as a valid species based on distinctive morphological characters (e.g., Barquez et al. 1999; Simmons 2005; Giménez et al. 2012). In comparison with *E.* (*H.*) *montanus*, *E.* (*H.*) *magellanicus* has darker hair, wing membranes, and pinnae, as well as ears that are smaller on average (Barquez et al. 1993; 1999; Giménez et al. 2012). The species is distributed exclusively in Southern Chile and Argentina (Barquez et al. 1999; Simmons 2005; Giménez et al. 2012), inhabiting mainly the Valdivian Temperate Forests and Magellanic Subpolar Forests eco-regions sensu Olson et al. (2001) (see also Giménez et al. 2012). Interestingly, *E.* (*H.*) *magellanicus* coexists in sympatry in the northern extreme of its range with *E.* (*H.*) *macrotus* and marginally with *E.* (*H.*) *montanus*.

DNA data have provided phylogenetic resolution among and within families of bats at different levels, particularly the morphologically difficult vespertilionids (Hoofer & Van Den Bussche 2003; Roehrs et al. 2010; Van Den Bussche & Lack 2013; Ruedi et al. 2013; Shi & Rabosky 2015; Amador et al. 2016). Major contributions of these studies includes the detection of a surprising number of cryptic species (e.g., Juste 2004; Mayer et al. 2007) and the taxonomic resolution

of many species complexes (e.g., Bickham et al. 2004; Lim et al. 2004; Hoofer et al. 2006). However, subgenus *Histiotus* has been poorly sampled from a molecular perspective. *Eptesicus* (*H.*) *magellanicus* was included in some of the studies (Roehrs et al. 2010; 2011), but not together with *E.* (*H.*) *montanus* and *E.* (*H.*) *macrotus* in order to test its genetic differentiation and how it is related to these species. The goal of the present study was to assess the phylogenetic relationships of Patagonian long-eared brown bats, subgenus *Histiotus*, thereby testing the validity of *E.* (*H.*) *magellanicus* using molecular data.

MATERIAL AND METHODS

Samples

Specimens of *E.* (*H.*) *magellanicus*, *E.* (*H.*) *macrotus*, and *E.* (*H.*) *montanus* were collected in the Chubut province of Argentina and five specimens of each species were selected for sequencing (APPENDIX 1). We also sequenced for this study two specimens of a Peruvian form of *Histiotus* that likely represent an undescribed species (tissues kindly provided by Paul Velazco, APPENDIX 1). **Fig.** 1 shows a map with the collection localities for these samples (localities are listed in APPENDIX 2). We also included in the analysis sequences obtained from GenBank of *Eptesicus serotinus*, from Eurasia, and three South American species of typical *Eptesicus*: *E. furinalis*, *E. diminutus*, and *E. fuscus*. GenBank sequences of *Scotomanes ornatus* were used as outgroup. Accession numbers of the sequences used in this study are listed in APPENDIX 1. DNA was extracted from ethanol-preserved tissues using the DNeasy QIAGEN kit and quantified with a NanoDrop spectrophotometer.

Molecular data

Two loci were selected for this study. The first locus was a fragment (487 bp) containing an intron of the thyrotropin gene (*THY*), previously used in vespertilionid phylogenies (Lack et al. 2010; Roehrs et al. 2010) and amplified with the primers BatThy(F) and BatThybI (Eick et al. 2005). The second was the mitochondrial gene cytochrome b (*Cyt b*), which has been extensively used in species level phylogenies in mammals (e.g. Almeida et al. 2007; Almeida et al. 2014) and completely sequenced for this study (1140 bp). The fragment containing *Cyt b* was amplified and sequenced in two parts, using the external universal primers L14724 and H15915 (Irwin et al. 1991) and internal primers designed for the present study Hist2F (GCCTTYCATTTYCTACTYCC) and Hist1R (AGTGGRTTGGCTGGTATRTA). PCR was carried out using standard protocols (30 μl of final reaction volume, included 2 μl of total genomic DNA extract, 1.2 μl of each primer [10 μM], 1 μl of $MgCl_2$ [25 mM], 2.4 μl of a dNTP-Mix [10 mM] and 1 unit of Invitrogen Taq DNA polymerase) except that $MgCl_2$ was added to a final concentration of 3.3 mM for the amplification of *Cyt b*. Annealing temperatures in the thermal cycling procedure for the amplification of the *THY* fragment gene were 50°C for the first five cycles and 55°C for the remaining 25 cycles, while for the *Cyt b* fragment we used 48°C for the first five cycles and 50°C for the remaining 25 cycles. Amplification was checked by agarose gel electrophoresis and PCR products were sent to Macrogen Inc. (South Korea) for purification and sequencing of both forward and reverse strands. Raw sequences were edited with Geneious 4.8.5 and aligned with MAFFT v. 7.299b (Katoh & Standley 2013). Kimura 2 parameter (K2p) and uncorrected p-distances were estimated with the program MEGA 7 (Kumar et al. 2015).

Phylogenetic analysis

The two sequenced loci were analyzed separately and concatenated in a single matrix. Maximum likelihood searches were performed with RAxML v. 8.2.4 (Stamatakis 2014), using partitioning of the sequence according to codon positions (*Cyt b* only) and the GTRGAMMA (general time reversible model under the GAMMA model of rate heterogeneity) substitution model. The best tree was obtained over 100 independent runs and statistical support for nodes was obtained with 1000 bootstrap replicates. Parsimony trees were obtained with TNT (Goloboff et al. 2008) using 200 heuristic searches with random sequence addition replicates plus branch swapping by TBR (tree bisection and reconnection). Bayesian searches were conducted with MrBayes v. 3.2.6 (Ronquist & Huelsenbeck 2003), with optimal partition scheme and substitution models according to PartitionFinder v.1.1.1 (Lanfear et al. 2012). Searches were done for five million generations (2 runs, 4 chains each), sampling trees every 2000 generations (the first 25% of trees were discarded as burn-in). Convergence of searches was checked based on PSRF and ESS values, in the latter case with Tracer (Rambaut & Drummond 2003). Trees were visualized and edited with FigTree (Rambaut 2009).

Divergence times

We estimated the divergence times among the *Eptesicus* lineages sampled in the current study using previously estimated *Cyt b* substitution rates in vespertilionids. The *Cyt b* matrix was trimmed, leaving only one sequence per nominal taxa. Then we used the program BEAUti to generate a file with data and priors for the dating analysis in BEAST v 1.8.4 (Drummond et al. 2012). The three codon positions of the *Cyt b* gene were unlinked so that the substitution model parameters were estimated independently. We assumed a log-normal relaxed clock and a "tree prior" following a birth-and-death speciation model with incomplete sampling. The prior of the mean substitution rate was set as 0.023 subs/site/million years (my) with an exponential standard deviation prior with mean equal to 0.4, as estimated based on fossil data (Ruedi & Mayer 2001; Hulva et al. 2004). For the root height prior, we used a normal distribution with mean 23.2 my and standard deviation of 2.0 to match the age of the split between *Scotomanus* and *Eptesicus* estimated in Amador et al. (2016). We performed two separate runs of 2 million generations, sampling every 1000 generations. Convergence of the two runs was checked with TRACER (Rambaut et al. 2014), in which the effective sample size of all parameters were > 200 in each run. After discarding the first 20% of trees as burn-in the trees obtained in the two threes were combined and summarized.

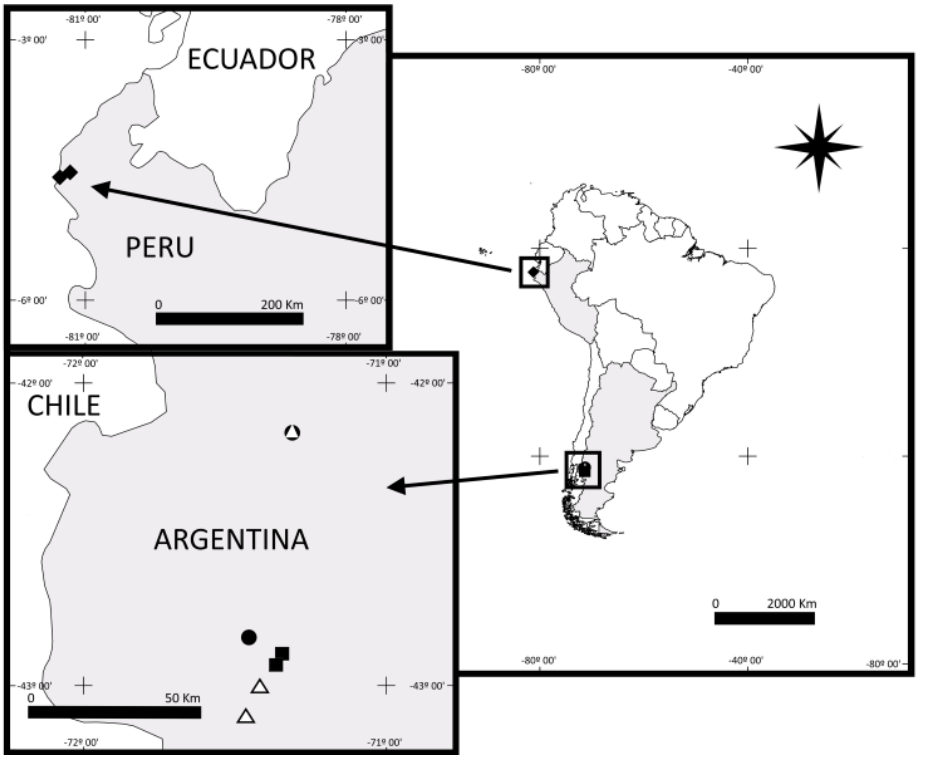

Fig. 1. Map of recorded localities for sequenced specimens of *Eptesicus* (*Histiotus*) *magellanicus* (●), *E.* (*H.*) *macrotus* (△), *E.* (*H.*) *montanus* (■) and *E.* (*H.*) sp. (◆).

RESULTS

The thyrotropin gene fragment containing intron 3 showed very little variation (33 variable sites, 9 parsimony informative sites) in our matrix and no resolution of phylogenetic relationships within *Eptesicus*. By contrast, the *Cyt b* exhibited highly relevant systematics variation (378 variable sites, 275 parsimony informative sites) and so the phylogeny based on this gene recovered several clades of *Histiotus* specimens with varying statistical support in all analyses. Overall, the trees obtained with the three different search strategies were mostly congruent (**Fig.** 2). The exception was due to the weakly supported *Histiotus* clade that was monophyletic in MP and BI, but included the clade containing *E. diminutus* and *E. furinalis* in the ML analysis. Among *Histiotus* species, a clade comprising all species but *E.* (*H.*) *magellanicus* was recovered with 95-100% statistical support in all analyses, a result that attests to a clear genetic separation of that species from the other *Histiotus* lineages. The five specimens of *E.* (*H.*) *magellanicus* clustered tightly with minimum branch lengths within the clade (**Fig.** 2). The next *Histiotus* clade to diverge was also highly supported and comprised the Peruvian specimens (**Fig.** 2), which is in agreement with the hypothesis that this form may indeed represent a new species. The third *Histiotus* clade, also highly supported, included all sequences from both *E.* (*H.*) *macrotus* and *E.* (*H.*) *montanus*. Surprisingly, this clade had no internal resolution and minimum branch lengths. The analysis of the concatenated matrix revealed, as expected, the same topologies obtained with the *Cyt b* gene, but with lower support values (as a consequence of a large number of invariable characters from the *THY* partition – **Fig.** S1).

Considering only the species with complete sequences, mean K2p genetic distances in *Cyt b* sequence between *Eptesicus* species varied from 0.3% [*E.* (*H.*) *macrotus* x *E.* (*H.*) *montanus*] to 18.0% [*E. serotinus* x *E.* (*H.*) *macrotus* + *E.* (*H.*) *montanus*]. Between *E.* (*H.*) *magellanicus* and *E.* (*H.*) *montanus* the average distance was 13.4%, ranging from 13.1% to 13.8% (11.7% - 12.1% p-distance). The shortest distance of *E.* (*H.*) *magellanicus* to another species

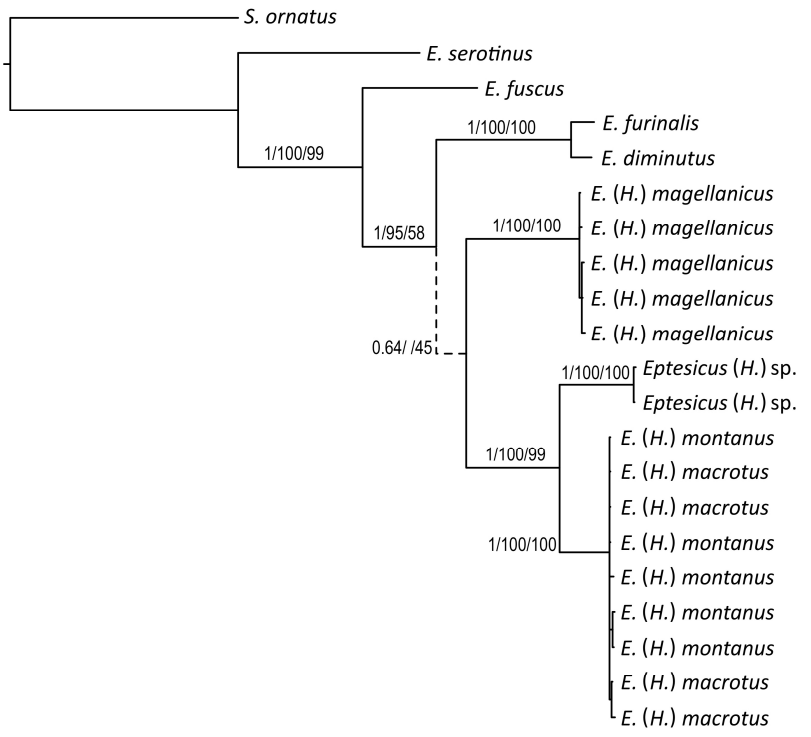

Fig. 2. Bayesian tree obtained with sequences of the *Cyt b* gene, showing the Bayesian posterior probabilities and bootstrap values of the maximum likelihood and parsimony trees, in this order, for each clade. The dashed branch was recovered only in the Bayesian and maximum parsimony analyses. Clades within the main *E.* (*Histiotus*) subclasses did not received significant support values (<0.90 posterior probability and <70 bootstrap).

was to *Eptesicus* (*H.*) sp., which averaged 12.6%. As a comparison, Baker & Bradley (2006) found, in a review, that intrageneric distances in bats ranged from 3.3% to 14.7%, while intraspecific distances ranged from 0.6% to 2.3%.

The dated *Cyt b* tree is shown in **Fig.** 3. Divergence date estimates placed the origin of *Histiotus* and its first split, separating the lineage of *E.* (*H.*) *magellanicus* from the remaining lineages, in the Pliocene. The other splits within the subgenus happened most likely in the Pleistocene.

DISCUSSION

In this study, we report molecular evidence showing that *Eptesicus* (*H.*) *magellanicus* represents an independent mitochondrial lineage, which correlates with the morphological separation previously reported. The monophyly of *E.* (*H.*) *magellanicus* samples was highly supported in our phylogenetic analysis of the *Cyt b* locus and the genetic distances between specimens of *E.* (*H.*) *magellanicus* and other *Eptesicus* species were in the upper range observed between congeneric bats species. This result taken together with the morphological distinctiveness of *E.* (*H.*) *magellanicus*, complies with the species delimitation criterion based on the agreement of multiple lines of evidence strongly supporting the recognition of a separate species (De Queiroz 2007).

Handley & Gardner (2008) questioned the validity of the characters used for the diagnosis of *E.* (*H.*) *magellanicus* remarking its distinctiveness from *E.* (*H.*) *montanus*, such as the dark coloration. According to these authors, the dark coloration that *E.* (*H.*) *magellanicus* presents is likely a local adaptation to the humid forest habitats that characterize its distribution in southern Chile and adjacent Argentina. Nevertheless, specimens from both species plus *E.* (*H.*) *macrotus* have been found in this region with well-marked differences in coloration (and other traits). Moreover, the specific fur color of each species seems to be constant all over their distributions (Barquez et al. 1999; Giménez et al. 2012). In fact, *E.* (*H.*) *magellanicus* is the most readily identified species from the three found in sympatry

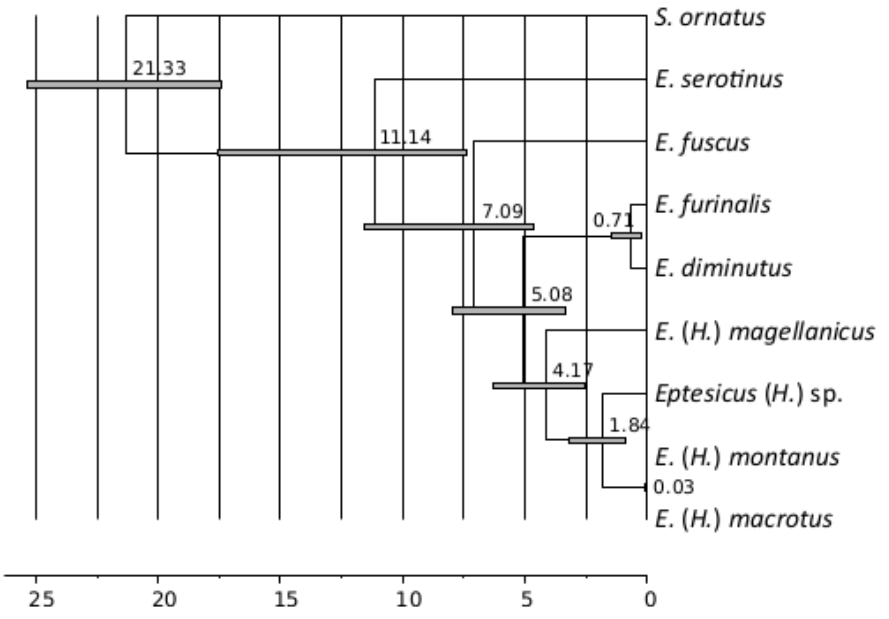

Fig. 3. Dated *Cyt b* tree. Numbers on nodes represent the divergence date estimates in million years ago.

in the Patagonian region where collections were made for this study.

The diagnostic characters of *E.* (*H.*) *magellanicus* are a dark general coloration, wing membranes and ears almost black, dorsal hairs dark brown at the base and light brown at the tips, ventral hairs black at the base and yellow at the tips; and the ear length that does not exceed 25mm (Barquez et al. 1993; 1999; 2013; Giménez 2010; Giménez et al. 2012; Giménez & Giannini 2017). These characters allow distinguishing *E.* (*H.*) *magellanicus* from species with complete or partial sympatric distributions, *E.* (*H.*) *macrotus* and *E.* (*H.*) *montanus*, respectively. Likewise, our recent study on Patagonian bats (Giménez & Giannini 2017) found that ear length along with tail length were important variables separating the three forms of *Histiotus* that inhabit the region. Tail length and uropatagium extension influence the way bats catch insects either using aerial hawking or gleaning (Norberg 1994). In turn, ear size and shape are important adaptive characters because they directly affect reception of echolocation calls (Obrist et al. 1993; Fuzessery 1996; Fenton & Bogdanowicz 2002) and flight efficiency (through an increase in parasitic drag; Speakman & Thomas 2003; Canals et al. 2005). Smaller ears may be of physiological importance (i.e. thermoregulation as smaller ears decrease heat loss; Soriano et al. 2002) in *E.* (*H.*) *magellanicus* as compared with *E.* (*H.*) *macrotus* or *E.* (*H.*) *montanus* given that the former is endemic to the Valdivian Temperate Forests and Magellanic Subpolar Forests eco-regions (sensu Olson et al. 2001), while the other two species have a much wider distribution in South America.

Independently of the reconstruction method, the *Cyt b* phylogenies recovered *E.* (*H.*) *magellanicus* as sister to all other *E.* (*Histiotus*) species included in the analyses. *E.* (*H.*) *montanus*, *E.* (*H.*) *macrotus*, and *Eptesicus* (*H.*) sp. clustered together with high support, suggesting that the *E.* (*H.*) *magellanicus* lineage was the first to split off the group ca. 4.17 my ago (Pliocene) with a subsequent diversification of the subgenus likely in the Early Pleistocene (1.84 my). In this scenario, *Eptesicus* (*H.*) *magellanicus* would have originated by isolation in the remote and climatically extreme Patagonia early in the evolution of the big-eared brown bats. During the Pliocene in Patagonia, a decrease in temperature was recorded due to glacial periods. Also, a gradient of rainfall appeared, restricting the temperate forests to its current position in the Antarctic domain and leading to aridization of eastern areas (Iglesias et al. 2011). It is possible that a discontinuity in the forest covers isolated an ancestral *Histiotus* population in southern

Patagonia that evolved into *E.* (*H.*) *magellanicus*. In this case, the presence of *E.* (*H.*) *macrotus* and *E.* (*H.*) *montanus* in the region would be explained by secondary contact following southward migration.

The Peruvian specimens were also distinguished from all other *Histiotus*, which agrees with the hypothesis that they represent an undescribed species. The thyrotropin intron analyzed herein suggests it may be related to *E.* (*H.*) *velatus*, although this result lacks statistical support (see **Fig.** S1). The clade of *Eptesicus* (*H.*) sp. was the second branch to split off among the *Histiotus* lineages, suggesting that the Andean region was important during the early evolution of *Histiotus*. A broader taxonomic sampling is required to determine the identity and the relationships of those Peruvian specimens, as well as to test the biogeographic hypothesis raised by our results.

One unexpected result of our study was the lack of mitochondrial molecular differentiation between *E.* (*H.*) *macrotus* and *E.* (*H.*) *montanus* samples. Reciprocal monophyly is expected in separate species, but its absence alone, particularly in an analysis involving few loci, does not constitute evidence of lack of lineage separation (De Queiroz 2007). Thus, the conclusion that one of these species may not be valid must be taken with caution, for several reasons. First, both species can be readily distinguished using morphological characters (Barquez et al. 1993; 1999; Giménez 2010). Second, unlike *E.* (*H.*) *magellanicus*, both *E.* (*H.*) *macrotus* and *E.* (*H.*) *montanus* have much wider distributions that were not sampled for this study. Because our focus here was on *E.* (*H.*) *magellanicus* and no doubts had been raised in relation of the taxonomic status of *E.* (*H.*) *macrotus* and *E.* (*H.*) *montanus*, we only included samples found in sympatry with *E.* (*H.*) *magellanicus* for these species. Third, the observed pattern of lack of genetic divergence in morphologically distinct taxa has been observed in other valid bat species (e.g. Almeida et al. 2014).

One explanation for non-monophyly of morphologically differentiated species is incomplete lineage sorting which is a very common cause of poly/paraphyly between closely related species (Pamilo & Nei 1988). Stochastic sorting, however, progresses more rapidly in mitochondrial as compared to nuclear loci due to differences in effective population sizes (Pamilo & Nei 1988; Birky et al. 1989). Therefore, given the phylogenetic results and the low levels of sequence variation observed within the [*E.* (*H.*) *montanus* + *E.* (*H.*) *macrotus*] clade, it is unlikely that these species would show reciprocal monophyly in other loci, unless it is a locus directly associated with speciation (Wu & Ting 2004; Nosil & Schluter 2011).

An alternative explanation for disagreements between DNA sequences and morphology in species assignment is DNA introgression. Introgression happens when part of the DNA of a species is incorporated into another species gene pool following hybridization (Funk & Omland 2003). Introgression will cause distinctive species to appear polyphyletic at the involved loci. Mitochondrial DNA (mtDNA) introgression is particularly prone to last and, therefore, to influence phylogenetic results (Funk & Omland 2003). In fact, the pattern expected in cases of mtDNA introgression has been observed in several bat families (Berthier et al. 2006; Nesi et al. 2011; Mao et al. 2013; Almeida et al. 2014; Dool et al. 2016), and even in the *Eptesicus* genus (Artyushin et al. 2009). One way to further evaluate the alternative explanations for the polyphyly of *E.* (*H.*) *macrotus* and *E.* (*H.*) *montanus* is to compare the mitochondrial gene tree with gene trees obtained with nuclear loci (Funk & Omland 2003). The inclusion of additional, more variable nuclear loci is fundamental to tackling this question (e.g. Dool et al. 2016).

CONCLUSION

In this study, we present molecular evidence from the mitochondrial DNA (*Cyt b*) that supports the validity of the species status of *E.* (*H.*) *magellanicus*, as previously indicated by fixed morphological differences observed between this and other *Eptesicus* (*Histiotus*) species distributed in southern South America. The phylogenetic position of Western montane species preliminarily suggests that Patagonia and more generally the Andes were important biogeographic regions during the early evolution of *Histiotus*. In addition, our study showed unexpected lack of phylogenetic resolution between morphologically distinct specimens of *E.* (*H.*) *montanus* and *E.* (*H.*) *macrotus*, suggesting either their synonymy or, most likely, the occurrence of processes such as local hybridization and introgression. The systematics of *Histiotus* is only beginning to be understood and an integral revision of the subgenus is needed.

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APPENDIX 1

Sequenced specimens of *Eptesicus* (*Histiotus*). Acronyms for mammal collections: Colección de Mamíferos del Laboratorio de Investigaciones en Evolución y Biodiversidad (LIEB-M; Universidad Nacional de la Patagonia San Juan Bosco, Esquel, Chubut, Argentina); Mammal Collection of American Museum of Natural History (AMNH-M; New York, E.E.U.U.).

Species	Voucher ID	*Cyt b*	*Thy*
Eptesicus (*Histiotus*) *macrotus*	LIEB-M 762	MK429700	MK429718
Eptesicus (*Histiotus*) *macrotus*	LIEB-M 848		MK429713
Eptesicus (*Histiotus*) *macrotus*	LIEB-M 852	MK429698	MK429717
Eptesicus (*Histiotus*) *macrotus*	LIEB-M 853	MK429697	MK429714
Eptesicus (*Histiotus*) *macrotus*	LIEB-M 1100	MK429695	MK429711
Eptesicus (*Histiotus*) *magellanicus*	LIEB-M 855	MK429709	MK429715
Eptesicus (*Histiotus*) *magellanicus*	LIEB-M 859	MK429708	MK429716
Eptesicus (*Histiotus*) *magellanicus*	LIEB-M 860	MK429710	
Eptesicus (*Histiotus*) *magellanicus*	LIEB-M 1105	MK429706	MK429721
Eptesicus (*Histiotus*) *magellanicus*	LIEB-M 1109	MK429707	MK429712
Eptesicus (*Histiotus*) *montanus*	LIEB-M 1113	MK429699	MK429719
Eptesicus (*Histiotus*) *montanus*	LIEB-M 1115	MK429703	MK429720
Eptesicus (*Histiotus*) *montanus*	LIEB-M 1116	MK429701	MK429726
Eptesicus (*Histiotus*) *montanus*	LIEB-M 1117	MK429702	MK429725
Eptesicus (*Histiotus*) *montanus*	LIEB-M 1118	MK429696	
Eptesicus (*Histiotus*) sp.	AMNH M-278521	MK429704	MK429722
Eptesicus (*Histiotus*) sp.	AMNH M-278524	MK429705	MK429723
Eptesicus serotinus	GenBank	AF376837	HM593075
Eptesicus furinalis	GenBank	EU786865	GU328440
Eptesicus diminutus	GenBank	AF376833	GU328438
Eptesicus fuscus	GenBank	AF376835	JX902561

APPENDIX 2

List of the localities of sequenced specimens of *Eptesicus* (*Histiotus*).

Eptesicus (*Histiotus*) *macrotus*. Argentina: Chubut Province, Ea. El Principio, 10 km of Esquel (LIEB-M 852, ♀); Chubut Province, Trevelin, Wales School (LIEB-M 762, ♂; LIEB-M 1100, ♀); Chubut Province, El Coihue Reserve (LIEB-M 853, ♀).

E. (*H.*) *magellanicus*. Argentina: Chubut Province, Arroyo La Camioneta, Cerro La Torta (LIEB-M 855, ♀; LIEB-M 1105, ♀; LIEB-M 1109, ♀); Chubut Province, El Coihue Reserve (LIEB-M 859, ♀; LIEB-M 860, ♀).

E. (*H.*) *montanus*. Argentina: Chubut Province, Laguna La Zeta, 4 km of Esquel (LIEB-M 1113, ♂; LIEB-M 1115, ♂; LIEB-M 1116, ♂; LIEB-M 1117, ♀); Chubut Province, Esquel, National University of the Patagonia San Juan Bosco, 4 km of Esquel on road N° 259 (LIEB-M 1118, ♀).

Eptesicus (*Histiotus*) sp. Perú: Piura, Talara, Quebrada Parinas, 9.6 km Northeast of Talara (AMNH M-278521, ♀; AMNH M-278524, ♂).

SUPPLEMENTARY ONLINE MATERIAL

Supplement 1: **Fig.** S1. Maximum likelihood tree based on the concatenated sequences of the cytochrome b gene and the thyrotropin gene intron. Bootstrap values are shown above branches.