MOLECULAR SYATEMATICS OF BONNETED BATS (MOLOSSIDAE: *EUMOPS*) BASED ON MITOCHONDRIAL AND NUCLEAR DNA SEQUENCES

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ABSTRACT

Previous understanding of the relationships among the species of bats in the genus *Eumops* has been based on phenetic and cladistic analyses of morphological data. The objective of this study was to construct a phylogeny of the bats within the genus *Eumops* using DNA sequence data from 2 mitochondrial genes (cytochrome b and nicotinamide adenine dinucleotide dehydrogenase subunit 1) and 1 nuclear locus (β-fibrinogen intron 7) for members of *Eumops* and outgroups from the family Molossidae. Data for each locus were analyzed separately using maximum-likelihood and Bayesian methods then were combined for complete data analyses using Bayesian Inference and Bayesian concordance analysis on a total of 2715 base pairs. Our results conflicted with some of the relationships proposed in previous morphological studies. Minor disagreements existed between the individual mitochondrial and nuclear data sets. However, the monophyly of the genus was significantly supported in all of the analyses.

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INTRODUCTION

The genus *Eumops* consists of the mastiff and bonneted bats in the family Molossidae. Bats in the family Molossidae, 16 genera of approximately 100 species (Simmons 2005), are characterized by a free tail that extends past the tip of their uropatagium. *Eumops*, a new world genus, currently consists of 15 species that can be found in Texas, Arizona, Mexico, Central and South America, and the Caribbean (Eger 2007; Gregorin 2009; McDonough et al. 2008). The genus is defined by a combination of characteristics such as joined ears, moderate to deep basisphenoid pits, closed anterior palate, and fine wrinkles on the lips (Freeman 1981). However, there are few well defined synapomorphies that delimit the relationships of species within the genus *Eumops*. Bats in the genus *Eumops* are morphologically highly variable with forearm size ranging from 37-82 mm (Eger 1977). The relative thickness of jaw bones in *Eumops* has been found to be variable with *E. perotis* and *E. auripendulus* being at opposite ends of the spectrum (Freeman 1981). *Eumops* also shows high levels of karyotypic polymorphism with diploid numbers ranging from 38-40 and fundamental numbers ranging from 54-64 (Genoways et al. 2005; McDonough et al. 2008; Warner et al. 1974). Individuals within the *E. glaucinus* complex also differ in the placement of the X-chromosome centromere (Genoways et al. 2005; Warner et al. 1974).

Historically, there have been multiple *Eumops* species with recognized subspecies (Eger 1977; Gregorin 2009; Sanborn 1932; Simmons 2005; Timm and Genoways 2004). Worthy of particular note is the *E. bonariensis* complex which has contained as many as 4

subspecies including *E. b. beckerii/patagonicus, E. b. bonariensis, E. b. delticus*, and *E. b. nanus* (Eger 1977). Barquez et al. (1999) recognized *E. bonariensis* and *E. patagonicus* as separate species based on prolonged sympatry in two parts of Argentina. Size differences between these species are also pronounced with *E. patagonicus* having smaller forearms, narrower ears, and a shorter skull with less separation between basisphenoid pits. Evidence of sympatry was also used as justification to elevate *E. nanus* and *E. delticus* to specific status (Eger 2007).

Analysis of 32 morphometric characters for many of the current *Eumops* species (Eger 1977) provided much of the current framework for the relationships of the species within *Eumops*. Eger's (1977) use of overall similarities produced a phenogram that did not agree with proposed relationships based on genetic similarities (Dolan and Honeycutt 1978). Dolan and Honeycutt (1978) suggested high similarity between *E. dabbenei* and *E. underwoodi* and a more distant relationship between *E. glaucinus* and *E. auripendulus* than was portrayed in the phenetic morphological analysis by Eger (1977). By using overall similarities instead of shared, derived characters species relationships can be difficult to define because overall similarities can represent retained ancestral traits (Wiley et al. 1991).

A more recent cladistic morphological study by Gregorin (2009) using 39 characters provided an updated review of the relationships within the genus *Eumops*. Gregorin proposed a separation of the genus into two divergent clades and the recognition of 5 distinct lineages based on morphological synapomorphies at the specific level (Fig. 1). While this more recent work provided additional support for the relationships within *Eumops*, it also left a large unresolved polytomy within the *E. bonariensis* clade and weak support for the position of *E.*

glaucinus (the *E. bonariensis* clade will be referred to as the *E. nanus* clade in this report since we did not include *E. bonariensis* in our analysis).

McDonough et al. (2008) provided support for the elevation of 2 species within the *Eumops glaucinus* complex using gene sequence, karyotypic, morphological, and AFLP data. Their analysis supported three distinctive clades within the *Eumops glaucinus* complex: *E. glaucinus* from Paraguay and Venezuela, *E. ferox* and *E. floridanus* from Cuba, Jamaica, Mexico, and the United States, and a new species from Ecuador. The distinctive Ecuador clade proposed by McDonough et al. (2008) was later given the name *Eumops wilsoni* (Baker et al. 2009).

Although McDonough et al. (2008) resolved some of the uncertainty within the *Eumops glaucinus* complex, the entire genus has yet to be studied using a molecular approach. The objective of our study was to use multiple molecular data sets (both nuclear and mitochondrial) to test the hypothesis of relationship among *Eumops* species that have been proposed by cladistic analysis of morphological data (Gregorin 2009). We aimed to determine if there was support for monophyly of the genus and to determine the sister relationships among species within the genus *Eumops*.

MATERIALS AND METHODS

Taxonomic sampling for molecular analysis.-Individuals representative of 12 of the 15 species in the genus *Eumops* were included in our analysis. Samples from Ecuador, Costa Rica, Jamaica, Venezuela, Cuba, Mexico, United States, Paraguay, Guyana, Panama, and Nicaragua were loaned from various institutions (Appendix I). Other taxa within the family Molossidae were used as outgroups; *Nyctinomops macrotis*, *N. femorosaccus, Molossus ater,*

Promops centralis, Tadarida brasiliensis. These taxa shared a common ancestor with *Eumops* 24.4-28.7 million years ago and represent many of the genera that are most closely related to *Eumops* (Ammerman et al. 2012). Close outgroups were chosen in order to decrease the chance of long branch attraction by a distant lineage (Bergsten 2005).

Gene selection.-Mitochondrial sequences from both cytochrome-b (Cytb) and nicotinamide adenine dinucleotide dehydrogenase subunit 1 (ND1) and nuclear sequence data from β-fibrinogen intron 7 (βFib) were used to test relationships among *Eumops* species. Previous studies successfully recovered relationships at the generic and specific levels of classification using these genes (Baker and Bradley 2006; Johnson and Clayton 2000; Lerner et al. 2008; Mayer et al. 2007; McAliley et al. 2007; Spinks and Shaffer 2007; Tagliaro et al. 2005). Although nuclear genes usually have fewer parsimony-informative sites, βFib has been shown to have a higher percentage of parsimony-informative sites than other nuclear genes, which is probably because it is an intron and is not under the same selective pressure as coding nuclear genes (Ammerman et al. 2012; Fujita et al. 2004; Mathee and Davis 2001). By collecting mitochondrial sequences in conjunction with nuclear sequences, an independent test of proposed phylogenetic relationships is generated, thus allowing for comparison between the relationships determined by the two genomes (Moore 1994; Teeling et al. 2000).

DNA Sequencing.-We extracted total genomic DNA from frozen liver, heart, or kidney tissue using the DNeasy Tissue Kit (QIAGEN Inc., Valencia, California) following manufacturer's protocol. Sequences from both mitochondrial (Cytb and ND1) and nuclear (βFib) genes were amplified using conserved vertebrate primers (Table 1). We amplified

DNA templates using either Eppendorf Taq polymerase (5U/ul; Eppendorf, Westbury, New York) or AmpliTaq 360 DNA polymerase (5U/ul; Applied Biosystems, Foster City, California). Reactions were carried out in 12.5ul volume following a standard polymerase chain reaction protocol (Palumbi 1996). Each reaction consisted of 200-500 ng of DNA, 3 U of *Taq* polymerase, 0.16 uM of forward and reverse primer, 2mM $MgCl_2$, 0.16 mM deoxynucleoside triphosphates and 1X reaction buffer.

The same thermal profile was used for each fragment except for varying annealing temperatures: initial denaturation at 94°C for 3 min; 39 cycles at 94°C for 1 min, annealing for 1 min (Cytb 48-50°C, βFib 48-57°C, and ND1 56°C), and 72°C for 1 min, with a final extension of 72°C for 3 min. Differences in annealing temperatures within each locus were due to varying primer sets being used to amplify the locus and to individual sample variation (some samples produced multiple bands unless the annealing temperature was increased).

We quantified products from the PCR using a Qubit fluorometer (Invitrogen, Carlsbad, California). The PCR products were then purified using ExoSAP-IT (USB-Affymetrix, Cleveland, Ohio) and subjected to DNA sequencing using GenomeLab DTCS-Quick Start Mix in a Beckman Coulter CEQ 8000 automated sequencer following manufacturer's protocol except reaction volumes were quartered (Beckman Coulter Inc., Fullerton, California). We sequenced each sample with the same primers used in PCR as well as additional internal primers to create overlapping sequences in order to increase the accuracy of the sequence data (Table 1).

Phylogenetic analysis of individual data sets.-We used Sequencher version 5.0 (Gene Codes Corporation, Ann Arbor, Michigan) and MEGA5 (Tamura et al. 2011) to align the

sequences and check for amino acid translation of the mitochondrial genes. The Model Selection function in MEGA5 was used to determine which of 24 possible evolutionary models best fit each individual gene for both maximum likelihood (ML) and Bayesian analyses. We considered models with the lowest Bayesian Information Criterion (BIC) to describe the substitution pattern that best fit the data set (Tamura et al. 2011). We calculated average overall and pairwise genetic distances to estimate evolutionary divergence between lineages using the Kimura 2-parameter model without gamma correction in order to allow for comparison with Bradley and Baker's (2001) test of the genetic species concept. Bootstrap analysis (Felsenstein 1985) for 1000 pseudoreplicates using ML criteria was performed in MEGA5. Gaps were treated with partial deletion with site coverage cutoff set at 75% in βFib in order to keep as many phylogenetically informative characters as possible. We considered nodes with ML bootstrap (BS) values >70 as significantly supported (Hillis and Bull 1993).

Bayesian Inference (BI) analysis of each individual data set was completed using MrBayes version 3.2.1 (Ronquist and Huelsenbeck 2003). The analyses consisted of 2 simultaneous runs each with four Markov Chain Monte Carlo (MCMC) chains (one heated and three cold) run for 5 million generations on each of our individual data sets. Trees were sampled every 100 generations for a total of 50,000 trees sampled. We used a burn-in of 12,500 to discard the first 25% of sampled trees. Bayesian posterior probabilities (BPP) >0.95 were considered significant node support (Suzuki et al. 2002).

Phylogenetic analysis of combined data matrix.-The individual data sets (ND1, Cytb, and βFib) were placed into a concatenated data set for further analysis using Bayesian methods to provide a better estimate of the relationships between species (Heulsenbeck and

Ronquist 2005). We placed the individual alignments into one alignment that contained all three genes. Individuals with missing data for some of the genes were retained in the concatenated data set because according to Weins and Morrill (2011) missing data are less important than missing taxa in a phylogenetic analysis. Individual evolutionary models, which we determined to best fit our data sets previously, were applied to each gene partition in the concatenated dataset and then MrBayes was run as previously described for the individual BI analyses.

Concatenation of loci is known to be powerful, but it is also known to cause inflated support values (Kubatko and Degnan 2007). Because of this, Bayesian concordance analysis (BCA) was performed using the program BUCKy (Bayesian Untangling of Concordance Knots) (Larget et al. 2010). BCA allows for discordance among loci, but makes no assumption as to what is the underlying cause of the discordance (e. g. incomplete lineage sorting). Additional independent Bayesian analyses were run on the three individual datasets for all 36 taxa for 2 million generations with trees sampled every 1000 generations. We used the best-fit evolutionary model for each gene. We then summarized the two independently inferred sets of gene trees for each gene, combining the 2 MCMC runs resulting from the Bayesian analysis (files ending with *.runx.t) using the *mbsum* command. A burnin of 25% was set since all saved trees from the BI were included in the *.t files (Larget et al. 2010). Finally, BCA was performed on the 3 summarized files using the BUCKy command line (Ane et al. 2006). The primary concordance tree was built from clades that are supported by a majority of the sampled genes, giving a useful summary of the dominant phylogenetic history. Concordance factors (CF) represent the proportion of sampled trees across all genes

that supported a particular clade.

RESULTS

Phylogenetic analysis of cytochrome b.-A total of 715 base pairs was sequenced from 32 taxa (including outgroups; Appendix 1) for Cytb resulting in 216 parsimony informative characters. Model Selection analysis in MEGA5 determined that the Tamura-Nei with gamma distribution (TN93+G, α=0.24) was the best-fit evolutionary model for this data set. ML and BI analyses recovered significant support for the monophyly of the genus and three deeply divergent lineages within *Eumops* with no disagreement between ML and BI topologies (Fig 2).

Significant support was recovered using both ML and BI for the placement of *Eumops perotis* in the same clade (clade 2) as the *E. glaucinus* complex (Fig. 2). Most individuals of the same species grouped together, except for *E. ferox*. The specific relationships of *E. perotis* and *E. maurus* + *E. auripendulus* within clade 2 remain unclear. *E. ferox* did not represent a monophyletic group. The position of *Eumops hansae* also remains unclear. While the topology seems to suggest that *E. hansae* is outside of other members of the genus, this placement did not have significant support.

Within species, pairwise genetic distances (K2P) ranged from 0.0018 (*E. wilsoni*) to 0.0899 (*E. patagonicus*). The average overall genetic distance between ingroup species was 0.1191 with values ranging from 0.0084 between *E. ferox* and *E. floridanus* to 0.1804 between *E. dabbenei* and *E. hansae* (Table 2).

Phylogenetic analysis of ND1.-A total of 957 base pairs was sequenced from 34 taxa (including outgroups; Appendix 1) for ND1 resulting in 295 parsimony informative

characters. Analysis using Model Selection in MEGA5 determined that the Tamura-Nei with gamma distribution (TN93+G, α=0.32) was the best-fit evolutionary model for this data set. No significantly supported clades conflicted in the ML and BI analyses (Fig. 3). We recovered a very similar topology as recovered for Cytb. A noteworthy difference between the clades recovered in the Cytb analysis and the ND1 analysis was the placement of *Eumops hansae* within clade 1 (*E. nanus* clade; Fig. 3). Significant support was found for the placement of *E. hansae* in this clade based on both BPP and BS values.

There was significant support for the monophyly of the genus and many monophyletic species groups, but *E. ferox* and *E. nanus* represent exceptions to this. *E. nanus* from Panama (TK12526) clustered with *E. patagonicus* rather than with *E. nanus* from Mexico. This specimen also had a smaller genetic distance from *E. patagonicus* (0.033) than from the *E. nanus* from Mexico (0.056). Similarly, *E. ferox* individuals do not form a monophyletic lineage with respect to *E. floridanus.*

Within species, pairwise genetic differences (K2P) ranged from 0.0013 (*E. perotis*) to 0.0345 (*E. nanus*). The average overall distance between species (with outgroups removed) was 0.1019 with values ranging from 0.013 between *E. floridanus* and *E. ferox* and 0.188 between *E. auripenduluss* and *E. hansae.*

Phylogenetic analysis of β-Fibrinogen intron 7.-A total of 1043 base pairs was sequenced from 28 taxa (including outgroups) for βFib resulting in 95 parsimony informative characters. When aligning the sequences in MEGA5, *Eumops hansae* was found to have a 262 base pair insert that was shared exclusively by both *E. hansae* individuals that were sequenced. This fragment was compared to GenBank (www.ncbi.gov) using BLAST and a

match was found to a previously described SINE insert (Borodulina and Kramerov 1999). Other smaller deletions also were present in the βFib data set including a 9-bp deletion in all *Eumops patagonicus* and a 2-bp deletion from both *Eumops hansae* specimens. Partial deletion (75% coverage cutoff) resulted in the exclusion of the large SINE insert from *E. hansae* from the ML analysis and the inclusion of each of the small deletions found in the alignment.

Model selection analysis in MEGA5 determined that the Tamura 3-parameter without gamma correction (T3P) was the best-fit evolutionary model for this data set. ML and BI analyses recovered significant support for the monophyly of the genus (Fig. 4). Closely related species showed some lack of separation into individual species clades. This lack of separation was seen in the *E. glaucinus* complex and within the uniquely recovered *E. auripendulus* + *E. nanus* clade. The placement of *E. hansae* as basal to the rest of the genus was not significantly supported by either BI or ML analyses. Significant support was found for the placement of *E. perotis* in the same clade with the *E. glaucinus* complex (Clade 2) which also was seen in the Cytb and ND1 analyses (Fig. 2, 3) but the sister relationships within Clade 2 remain an unresolved polytomy. The placement of *E. nanus* differed significantly from the mitochondrial gene trees. In the βFib analysis, *E. nanus* grouped with *E. auripendulus* rather than sister to *E. patagonicus* (Clade 1).

Within species, pairwise genetic differences (K2P) ranged from 0.0023 (*E. perotis*) to 0.0159 (*E. wilsoni*). The average overall distance between species (with outgroups removed) was 0.0375. The smallest interspecific distance was between *Eumops dabbenei* and *E. underwoodi* (D=0.0034) and the largest distance was between *E. hansae* and *E. nanus*

(D=0.0758).

Concatenated Bayesian Analysis.-A total of 2715 base pairs from 36 taxa were included in the Bayesian analysis of our concatenated data set. Analysis of the partitioned concatenated data set including both mitochondrial genes (ND1 and Cytb) and the nuclear gene (βFib) recovered a topology similar to that recovered from the analyses of mitochondrial genes (Fig. 5). Significant support was not found for the placement of *E. hansae* as a basal lineage in the genus. Significant support was found using BI for individual species as monophyletic, except within *E. ferox, E. floridanus*, and *E. nanus*. The placement of *E. perotis* in the same clade as the *E. glaucinus* complex (Clade 2) also was significantly supported, but the sister relationships of *E. perotis* and *E. maurus* + *E. auripendulus* with other members of Clade 2 remain unresolved (Fig. 5).

Bayesian Concordance Analysis.-The primary concordance tree generated with BCA using the independent posterior probabilities of the individual gene trees is the same topology as the Bayesian tree generated based on our concatenated data set (Fig. 5). Identical results were generated when using α priors of 0.1, 1, and 10. Generally, CF values were lower on the branches representing earlier divergences within the genus.

DISCUSSION

Morphological comparison.-The evolutionary history of the *Eumops* genus recovered in our study based on DNA sequence data is not consistent with many of the existing morphological predictions (Eger 1977; Freeman 1981; Gregorin 2009). Our phylogeny generated from each mitochondrial gene recovered some of the same relationships recovered in Gregorin's (2009) cladogram such as the close relationship of *Eumops maurus* + *E.*

auripendulus, E. dabbenei + *E. underwoodi*, and *E. nanus* + *E. patagonicus*. Some of the characters, such as large body size and a high sagittal crest, used by Gregorin (2009) can be considered to have evolved convergently as is evidenced by their appearance in multiple distantly related clades. The groupings based on these homoplastic characteristics therefore do not represent a true phylogenetic history (Wiley and Lieberman 2011). We found support that two (slightly domed skull and warts on upper boarder of ears) of the five possible convergent characters mentioned by Gregorin (2009) do represent true convergences based on our analyses of mitochondrial DNA sequence data. The other three characters with alternative groupings proposed by Gregorin (2009) were high sagittal crest, broad braincase, and large body size. Our analyses recovered clades that were consistent with these morphological synapomorphies.

Position of Eumops glaucinus *complex*.-McDonough et al. (2008) presented evidence that the *Eumops glaucinus* complex contained multiple species, but the sister relationships of this complex with the other species within the genus was not evaluated. Gregorin's (2009) strict consensus cladogram based on ordered characters (Fig. 4 in Gregorin 2009) could not resolve the relationship between *E. glaucinus* and the other species. His unordered analysis supported (only with decay index, no bootstrap support was recovered) grouping *E. glaucinus* with the *E. bonariensis* group although he noted *E. glaucinus* shared a similar size with *E. auripendulus*. He also noted that *E. glaucinus* shared the characteristics of overall skull shape and pelage color with *E. dabbenei* + *E. underwoodi*, a grouping we find more consistent with our molecular data (Fig. 2-5). We found significant support for *E. glaucinus* as a member of clade 2 (Fig. 5).

Each of the mitochondrial trees supports the position of *E. wilsoni* as basally divergent in the *E. glaucinus* complex as hypothesized by McDonough et al. (2008). The lack of complete separation of *E. ferox* and *E. floridanus* into monophyletic groups may be due to incomplete lineage sorting and was also recovered by McDonough et al (2008). The separation of the *E. ferox* samples, which was recovered in each of the mitochondrial trees and the combined analyses, is consistent with geographic location; TK13585 and TK13589 were collected in Mexico while TK32052 and TK32033 were collected in Cuba. *E. floridanus* is distinguished from other members in this complex based on larger overall size and body mass, a narrower palate, and proportionally shorter condylobasal length (Timm and Genoways 2004). In the cytb data set, *E. floridanus* had less sequence divergence from the *E. ferox* from Cuba (0.0066) than the two geographically distinct *E. ferox* populations had from each other (0.0081). According to Bradley and Baker (2001) this low level of sequence divergence is expected at the subspecific level. The evidence based on sequence divergence among and between these two species combined with incomplete separation of the two species suggests reevaluation of *E. floridanus* as a valid species.

Position of Eumops perotis.-Our analysis offered increased resolution of the relationships within this genus with significant support for the placement of *E. perotis* within the same clade as the *E. glaucinus* complex. However, this placement does not fully resolve the relationship of *E. perotis* to other species in the clade. Gregorin (2009) found that unequivocal synapomorphies of quadrish basisphenoid pits and a narrowed crest between the basisphenoid pits joined *E. perotis* with the previously described *E. bonariensis* group, but this relationship was not supported in any of the gene trees we generated, suggesting

convergence of these characters in multiple lineages. We can refute the proposed placement of *E. perotis* with the *E. bonariensis* group by Gregorin (2009). We believe that the characters grouping *E. perotis* with the other large bodied bats in clade 2 recovered in our analyses, such as the high sagittal crest shared with *E. auripendulus*, may be more phylogenetically informative in the morphological classification of this genus. Our recovery of *E. perotis* as more closely related to *E. dabbenei* + *E. underwoodi* leaves the *E. bonariensis* complex + *E. hansae* as a distinct, small bodied (FA < 50mm) clade.

The specific relationships of the species within this clade (which also contains *E. underwoodi* + *E. dabbenei* and *E. maurus* + *E. auripendulus* in our total data analyses) remain relatively uncertain. Although *E. trumbulli* was not included in our molecular analysis we can postulate on its relationship to the other members in *Eumops*. It seems more likely that *E. trumbulli* would group with *E. perotis* in future analyses rather than remaining sister to the *E. nanus* clade as was recovered by Gregorin (2009). Additionally, *E. perotis* and *E. trumbulli* share five morphological synapomorphies that group them. Together, these two species also share large body size and similar skull shapes with the *E. dabbenei* + *E. underwoodi* clade (Gregorin 2009).

Position of Eumops hansae *and the* Eumops nanus *clade*.-Dolan and Honeycutt (1978) suspected that the large amount of diversity seen in *Eumops hansae* both morphologically and genetically warranted consideration of its possible placement in a different genus. However our analyses support the monophyly of the genus and thus, the inclusion of *E. hansae* within the genus (BPP > 0.99, Fig. 2, 3). Additionally, although there was some uncertainty in the position of *E. hansae*, the ND1 analysis placed *E. hansae* as

sister to the *E. nanus* group (Fig. 3). Sanborn (1932) first suspected the close relationship of *E. bonariensis* and *E. hansae* based on overall similarities in external morphology and skull shape. Gregorin's (2009) morphometric analysis confirmed this grouping with 7 morphological synapomorphies defining the unresolved polytomy in which these species previously recognized as subspecies of *E. bonariensis* appear.

The incomplete separation of *E. patagonicus* and *E. nanus* into monophyletic species groups remains problematic (Fig. 3; Fig. 5). According to current species ranges, *E. nanus* extends from Central Mexico to southern Brazil (including Columbia, Ecuador, Venezuela, and parts of Guyana and Peru) (Eger 2007). The range of *E. patagonicus* seems to be more restricted, extending from the east coast of Argentina through Paraguay and up into central Bolivia (Eger 2007). Thus these 2 species' distributions do not overlap. More likely we would expect this pattern of non-monophyly to exist between closely-related species with overlapping distributions, such as *E. patagonicus* and *E. bonariensis* or *E. nanus* and *E. delticus*, which would allow for possible interspecific breeding between these individuals (Nesi et al. 2011). All of the *E. nanus* that did form a monophyletic group were from Mexico, while the *E. nanus* (TK12526) that grouped with *E. patagonicus* was collected from Panama. Our data suggest this specimen may actually be *E. patagonicus,* although sequence data was only available for ND1 and significant support for this result was only recovered in our concatenated analysis (Fig. 5). The lower sequence divergence we recovered between the *E. nanus* from Panama and our *E. patagonicus* specimens (0.033) than between the two geographically distinct *E. nanus* groups from Panama and the Yucatan (0.056) suggests that the distributions and elevation of these species (or subspecies) need to be reevaluated.

Available data regarding the elevation of species previously recognized as subspecies within the *E. bonariensis* complex is rather nonspecific; the addition of the other members of the *E. bonariensis* complex (*E. delticus* and *E. bonariensis*) from across their range will be essential in determining the cause of the uncertainty in position of these species.

Analysis of β-Fibrinogen.-Lack of parsimony informative characters for βFib may be the reason why some species, such as *E. ferox* and *E. floridanus* in the *E. glaucinus* complex, do not fall out into fully demarcated clades. The average overall genetic divergence within the mitochondrial genes was approximately four times greater than that of βFib. Individuals within this complex are recently diverged, causing us to expect very little difference in their highly conserved nuclear genes (Ammerman et al. 2012, Steppan et al. 2004). The grouping of *E. nanus* and *E. auripendulus* in this nuclear gene tree conflicts with the placement of these species recovered in the mitochondrial trees. Contamination during DNA extraction, PCR, or even sequencing could be an explanation, but each of the *E. nanus* and *E. auripendulus* samples were processed multiple times. If contamination were the cause of this unique grouping, we would not expect for samples processed at different times to have independently acquired the same contaminant. Additionally, this unique grouping has not been supported or proposed as a possible explanation of the relationships within *Eumops* in any of the morphological data that we reviewed. Eger (1977) placed *E. auripendulus* as more closely related to *E. maurus* than *E. nanus* in her phenogram of average taxonomic difference (*E. nanus* was presented as *E. bonariensis* in her phenogram because *E. nanus* was recognized as a subspecies of *E. bonariensis* at the time). The morphologically based cladogram also does not support this grouping (Gregorin 2009). Curiously, the two characters

from his analysis that we showed to be homoplastic in our analyses of our mitochondrial trees (slightly domed head and warts on upper boarder of ears) are concordant with this otherwise unique recovery (Gregorin 2009). Even so, there is much morphological and molecular support for the close relationship of *Eumops nanus* and *E. patagonicus*. We need to increase the number of nuclear genes in order to get a better understanding of the evolution of the nuclear genome of *Eumops* species. Addition of microsatellite or AFLP data would also be useful in delineating these closely related species due to their rapid rates of evolution (Larsen et al. 2010, McDonough et al. 2008).

Large Insert in BFib of Eumops hansae.-The large, 262 base pair, insert found in the βFib intron of *E. hansae* represents a SINE, short interspersed element, which was originally discovered in bats of the families Vespertilionidae and Molossidae (Borodulina and Kramerov 1999). SINEs are 'jumping genes' that belong to the retrotransposon class of mobile elements that can propagate in their host genomes through retrotransposition (Fantaccione et al. 2008). A BLAST search from GenBank also returned high similarity matches for records of this SINE in *Artibeus* (family Phyllostomidae), *Tadarida*, *Otomops* (family Molossidae), *Myotis, Rhogeessa,* and *Neoromicia* (family Vespertilionidae). Further investigations into the evolutionary mechanisms underlying this SINE repeat and possible implications of this insert for *E. hansae* are justified.

Complete data analyses (BCA and BI).-Our concatenated analysis recovered support for basically the same relationships as both of the mitochondrial gene trees, giving us additional support for the monophyly of this genus and many of the species within it (Fig. 5). This complete data analysis also recovered the relationships seen in the nuclear gene, less the

unique grouping of *E. auripendulus* + *E. nanus* recovered in βFib. Our study is the most comprehensive molecular study on the entire *Eumops* genus thus far and so we consider our tree resulting from the analysis of our concatenated data set the most representative species tree. There is still some lack of resolution, in particular the relationship of *E. perotis* with other bats in Clade 2 and the position of *E. hansae*.

The BCA allowed us to integrate information from multiple individual datasets and clarify the natural groups within *Eumops* (Baum 2007). We recovered additional evidence for the possible placement of *E. nanus* with *E. patagonicus*, which differed significantly from the nuclear gene tree that placed *E. nanus* sister to *E. auripendulus*. The low CF values from the BCA for the individuals with missing sequence data for ND1 (Appendix I) are likely due to the loss of informative characters found in the ND1 gene. The overall lower CF support values (when compared to BS and BPP) illustrate the difference between these computational procedures used in these measures of support (Weisrock et al. 2012). Using a BCA may better reflect the level of incongruence between gene trees, in particular, between the mitochondrial trees and the nuclear gene tree because the genetic histories are directly estimated from the individual gene genealogies, which are then used to estimate the proportion of the sampled genes with different histories (Baum 2007; Cranston et al. 2009). The resulting concordance tree provides an estimate of the discordance and divergence at various points in the history of the genus *Eumops.* By taking into account the incongruence between gene trees using BCA, we do not change our view of the phylogeny of this group generated based on our other molecular analyses, but instead offer increased evidence where disagreement existed and a more comprehensive view of the support across the tree

(Cranston et al. 2009).

Although some relationships remain unresolved within the genus and some species within the genus *Eumops* were not included in this study, we were able to offer increased resolution and significant support for many of the proposed relationships, including the monophyly of the genus. We reject Gregorin's (2009) hypothesis of the relationships within *Eumops* and put forth a new hypothesis of these relationships. Additional taxa and additional genes, especially from the nuclear genome, will undoubtedly clarify the hypotheses of relationships within *Eumops*.

Table 1.--Primers used in PCR and DNA sequencing for each of the genes utilized to analyze relationships among *Eumops* species. An asterisk (*) indicates a primer used only in sequencing. All other primers were used in both PCR and sequencing.

Gene	Name	Sequence (5'-3')	Reference
Cytb	14841	AAAAAGCTTCCATCCACCATCTCAGCATGAAA	Irwin et al. 1991
	15547	GGCAAATAGGAAATATCATTC	Edwards et al. 1991
	Gludg	TGACTTGAARAACCATCGTTG	Palumbi 1996
βFib	B17L	GGAGAAAACAGGACAATGACAATTCAC	Prychitko and Moore 1997
	B17U	TCCCCAGTAGTATCTGCCATTAGGGTT	Prychitko and Moore 1997
	FGB-FelF	CACAACGGCATGTTCTTCAGCACG	Yu and Zhang 2005
	FGB-FelR	TACCACCATCCACCACCATCTTCTT	Yu and Zhang 2005
ND1	ER65	CCTCGATGTTGGATCAGGACATCC	Petit et al. 1999
	ER66	GTATGGGCCCGATAGCTTAATTAGC	Petit et al. 1999
	*ER70	CAGACCGGCGTAATCCAGGTGGGTT	Petit et al. 1999
	*ER89	CTCTATCAAAGTAACTCTTTTATCAGA	Petit et al. 1999
	*ER340	AGGTTCAAYTCCTCTCTCTAACA	Dolman 2009

Table 2.—Average Kimura 2-parameter cytochrome b distances between species of *Eumops* bats based on 715 base pairs of the cytochrome b gene for 29 taxa. Outgroups were not included.

		1	2	3	4	5	6	7	8	9	10	11
1	*E. wilsoni*											
2	*E. perotis*	0.1105										
3	*E. dabbenei*	0.1159	0.1201									
4	*E. underwoodi*	0.0966	0.1114	0.0498								
5	*E. ferox*	0.0674	0.1184	0.0952	0.0910							
6	*E. floridanus*	0.0679	0.1200	0.1000	0.0936	0.0084						
7	*E. hansae*	0.1535	0.1666	0.1804	0.1796	0.1692	0.1704					
8	*E. glaucinus*	0.0747	0.1319	0.0903	0.0987	0.0349	0.0372	0.1631				
9	*E. maurus*	0.1148	0.1151	0.1201	0.1223	0.1178	0.1249	0.1653	0.1161			
10	*E. auripendulus*	0.1148	0.1215	0.1129	0.1267	0.1178	0.1249	0.1707	0.1117	0.0191		
11	*E. nanus*	0.1416	0.1604	0.1481	0.1595	0.1473	0.1501	0.1676	0.1421	0.1615	0.1523	
12	*E. patagonicus*	0.1394	0.1485	0.1525	0.1525	0.1378	0.1388	0.1518	0.1525	0.1477	0.1477	0.0619

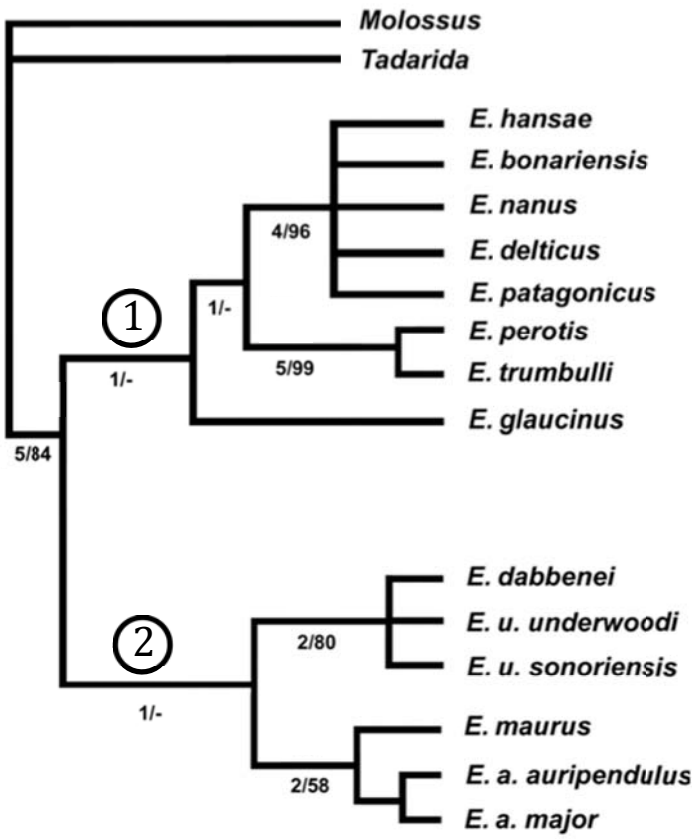

Fig. 1.--Most parsimonious cladogram of *Eumops* species resulting from analysis using unordered characters equally weighted. Small numbers below the branches represent decay index and bootstrap. Large numbers represent clades. (From Gregorin 2009).

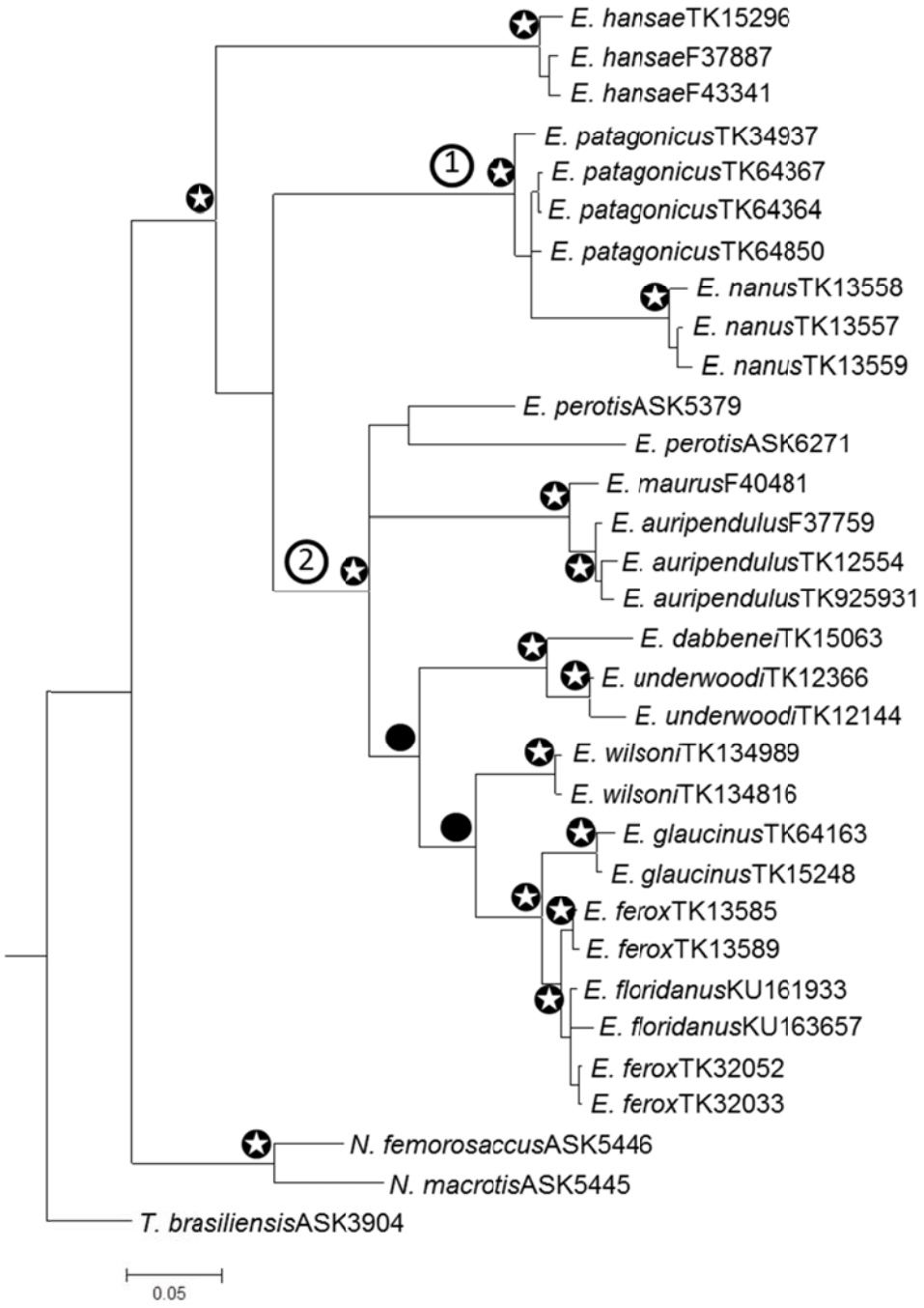

Fig 2.--Bayesian tree of *Eumops* species generated with 715 base pairs of the Cytb gene from a total of 32 taxa including outgroups. A black circle represents a node with significant BPP (BPP > 95) support only. A black circle with a star represents significant support with BPP and BS (BS > 70; generated from 1000 MLBS replicates). Numbers represent nodes (see results and discussion). Refer to Appendix for acronyms and collection site.

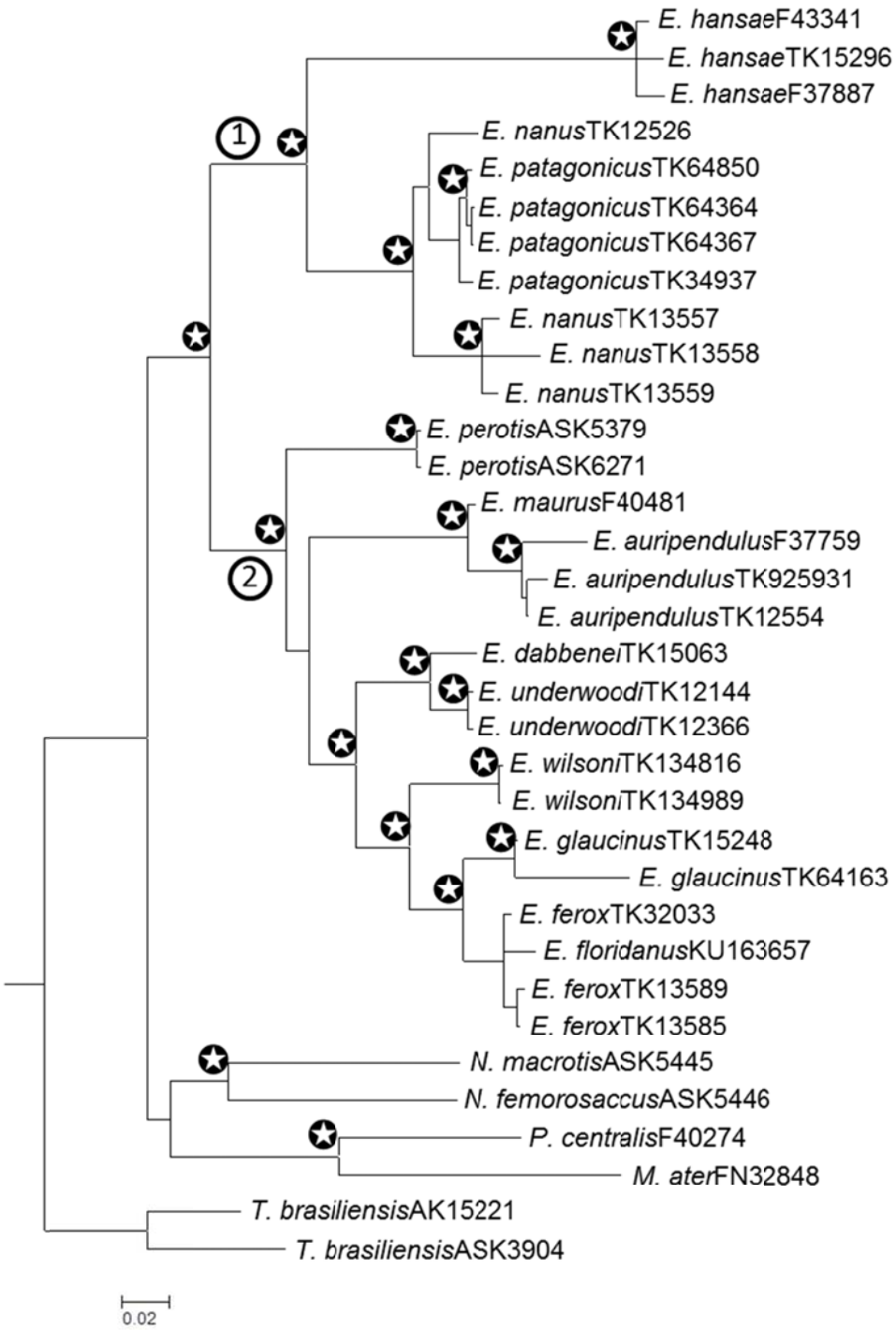

Fig 3.--Bayesian tree of *Eumops* species generated with 947 base pairs of the ND1 gene from a total of 34 taxa including outgroups. A black circle with a star represents significant support with BPP (BPP > 95) and BS (BS > 70; generated from 1000 MLBS replicates). Numbers represent nodes (see results and discussion). Refer to Appendix for acronyms and collection site.

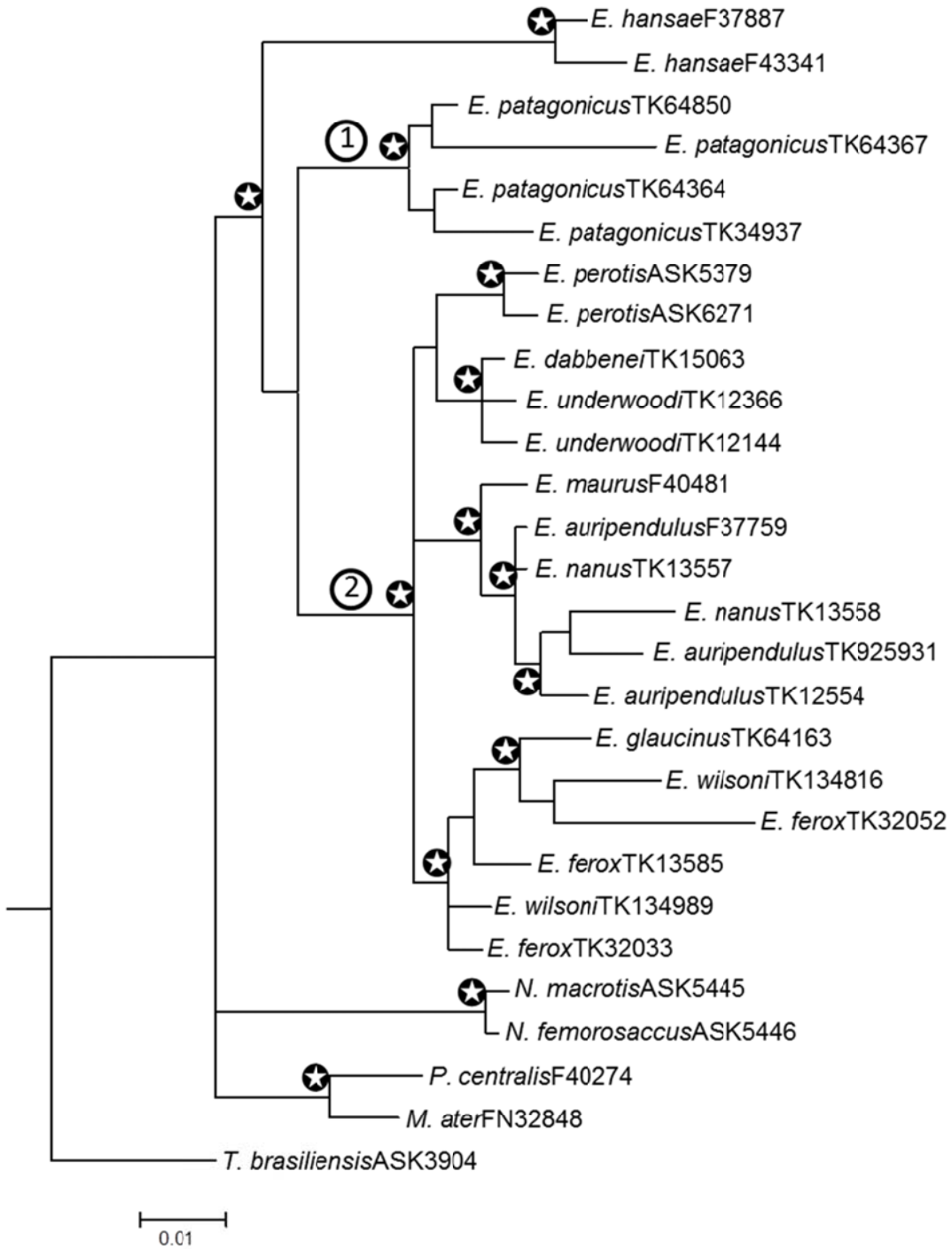

Fig 4.--Bayesian tree of *Eumops* species generated from 1043 base pairs of the βFib gene of a total of 28 taxa including outgroups. A partial deletion was used on gaps with 75% site coverage cutoff. A black circle with a star represents significant support with BPP (BPP > 95) and BS (BS > 70; generated from 1000 MLBS replicates). Numbers represent nodes (see results and discussion). Refer to Appendix for acronyms and collection site.

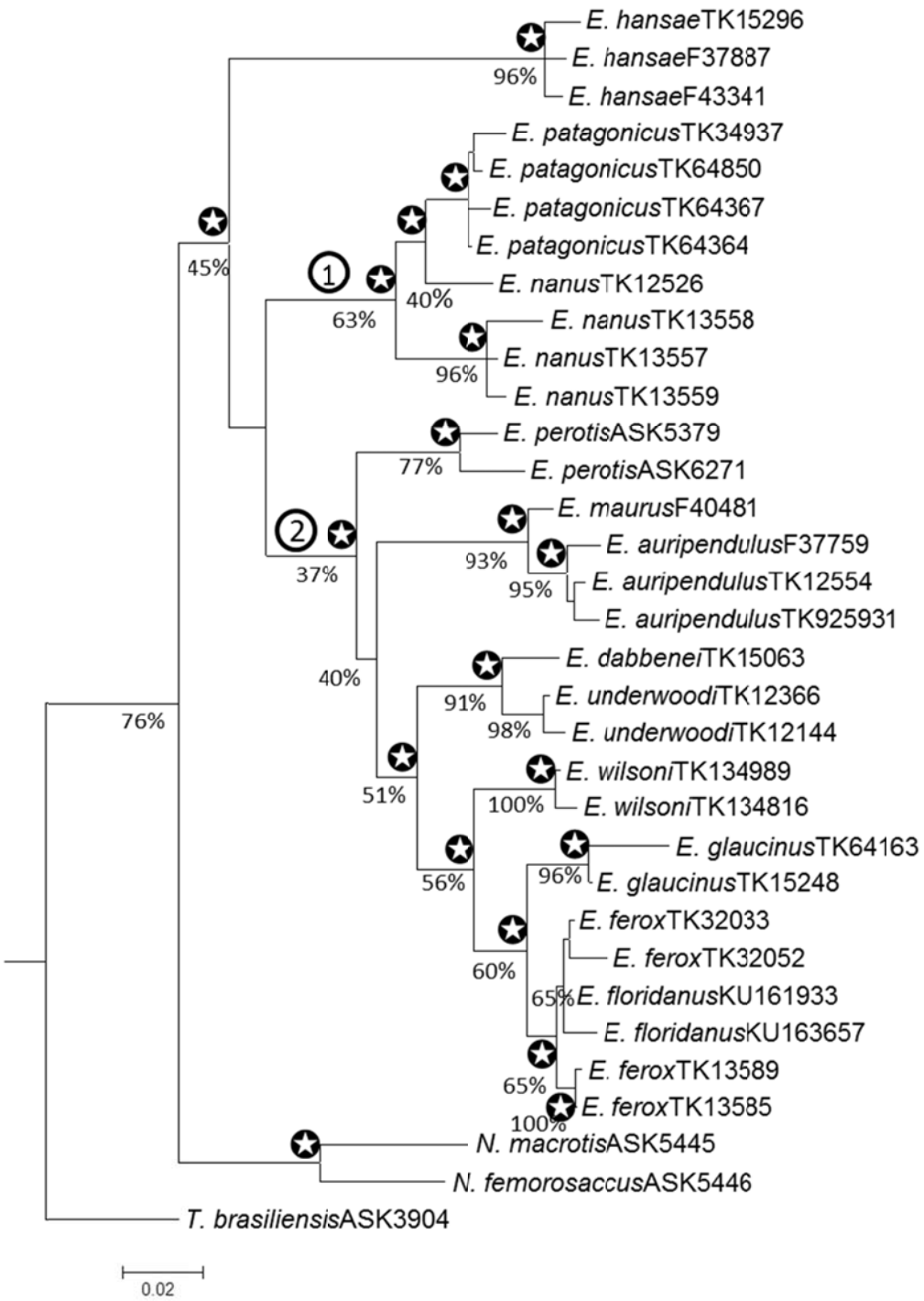

Fig 5.--Bayesian tree of *Eumops* species generated with 2715 base pairs of the concatenated data set from 36 taxa including outgroups. A black circle with a star represents significant support with BPP (BPP > 95) and BS (BS > 70; generated from 1000 MLBS replicates). Numbers represent nodes (see results and discussion). Percentages below branches represent CF values generated from the BCA. Refer to Appendix for acronyms and collection sites.

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APPENDIX I

List of specimens sequenced in this study.

Species	Catalog no./Tissue no.[a]	Locality	GenBank accession nos.		
			Cytb	ND1	βFib
Eumops auripendulus	ROM105526/F37759	Ecuador, Napo	JQ731823	HQ671531	HQ671608
Eumops auripendulus	TTU29815/TK12554	Costa Rica, Alajuela	JQ731822	JQ765469	JQ765483
Eumops auripendulus	CM46610/TK9384	Jamaica, St. Ann Parish	JQ731824	JQ765470	JQ765482
Eumops dabbenei	TTU33406/TK15063	Venezuela, Guarico	JQ731806	JQ765456	HQ671609
Eumops ferox	TTU52617/TK32052	Cuba, Guantanamo	JQ731816	–	JQ765488
Eumops ferox	TTU47519/TK13585	Mexico, Yucatan	JQ731820	JQ765463	JQ765487
Eumops ferox	TTU47521/TK13589	Mexico, Yucatan	JQ731809	JQ765462	–
Eumops ferox	TTU52613/TK32033	Cuba, Guantanamo	JQ731818	HQ671532	HQ671610
Eumops floridanus	KU161933/RMT4618	USA, Florida	JQ731811	–	–
Eumops floridanus	KU163657/RMT4611	USA, Florida	JQ731810	HQ671533	–
Eumops glaucinus	TTU33408/TK15248	Venezuela, Guarico	JQ731819	JQ765459	–
Eumops glaucinus	TTU80255/TK64163	Paraguay, Concepción	JQ731817	JQ765464	JQ765484
Eumops hansae	ROM108361/F43341	Guyana, Potaro-siparuni	JQ731814	HQ671534	JQ765493
Eumops hansae	ROM105642/F37887	Ecuador, Napo	JQ731815	JQ765460	JQ765492
Eumops hansae	TTU33409/TK15296	Venezuela, Miranda	JQ731813	JQ765461	–
Eumops maurus	ROM106326/F40481	Ecuador, Napo	JQ731821	JQ765468	HQ671611
Eumops nanus	TTU29308/TK12526	Panama, Chiriqui	–	JQ765477	–
Eumops nanus	TTU47518/TK13559	Mexico, Yucatan	JQ731832	JQ765476	–
Eumops nanus	TTU47517/TK13558	Mexico, Yucatan	JQ731826	JQ765471	JQ765481
Eumops nanus	TTU47516/TK13557	Mexico, Yucatan	JQ731829	JQ765475	JQ765480

APPENDIX.—Continued.

Species	Catalog no./Tissue no.[a]	Locality	GenBank accession nos. Cytb	ND1	βFib
Eumops patagonicus	TTU80582/TK64367	Paraguay, Neembucu	JQ731830	HQ671535	JQ765491
Eumops patagonicus	TTU62499/TK34937	Paraguay, Pte. Hayes	JQ731833	JQ765472	JQ765490
Eumops patagonicus	TTU80491/TK64850	Paraguay, Pte. Hayes	JQ731831	JQ765474	JQ765489
Eumops patagonicus	TTU80620/TK64364	Paraguay, Neembucu	JQ731828	JQ765473	HQ671612
Eumops perotis	ASNHC13295/ASK6271	USA, Texas	JQ731825	JQ765454	JQ765479
Eumops perotis	ASNHC12238/ASK5379	USA, Texas	JQ731805	JQ765455	HQ671613
Eumops underwoodi	TTU29311/TK12366	Nicaragua, Boaco	JQ731807	JQ765457	HQ671614
Eumops underwoodi	TTU29322/TK12144	Mexico, Chiapas	JQ731808	JQ765458	JQ765478
Eumops wilsoni	TTU103278/TK134816	Ecuador, Guayas	JQ731804	JQ765465	JQ765486
Eumops wilsoni	TTU103466/TK134989	Ecuador, Guayas	JQ731827	HQ671536	JQ765485
Nyctinomops macrotis	ASNHC11533/ASK5445	USA, Texas	JQ731803	HQ671549	HQ671619
Nyctinomops femorosaccus	ASNHC11528/ASK5446	USA, Texas	JQ731802	HQ671548	HQ671620
Tadarida brasiliensis	ASNHC9561/ASK3904	USA, Texas	JQ731812	JQ765466	JQ765494
Tadarida brasiliensis	OMNH23758/OCGR1848	Argentina, Tucuman	–	JQ765467	–
Molossus ater	ASNHC7008/FN32848	Mexico, Yucatan	–	JQ765453	JQ765495
Promops centralis	ROM106020/F40274	Ecuador, Napo	–	JQ765452	HQ671615

[a]Institutional acronyms: Angelo State University Natural History Collection, San Angelo, Texas (ASNHC); Royal Ontario Museum, Toronto, Ontario, Canada (ROM); University of Kansas Natural History Museum, Lawrence, Kansas (KU); Natural Science Research Laboratory, Museum of Texas Tech University, Lubbock, Texas (TTU); Sam Noble Oklahoma Museum of Natural History, University of Oklahoma, Norman, Oklahoma (OMNH); Carnegie Museum, Pittsburgh, Pennsylvania (CM).