

Species status of *Syrrhophus guttilatus* and *S. marnocki*

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Abstract

Mitochondrial sequence data were used to examine relationships of *S. guttilatus* and *S. marnocki*. In all types of phylogenetic analysis, populations in the Chisos Basin of Big Bend National Park form a distinct monophyletic unit separate from a clade containing *S. marnocki* and *S. guttilatus* in Mexico. These results suggest that it is appropriate to resurrect the name *S. gaigae* for the populations in Big Bend National Park as they form a distinct species separate from *S. guttilatus* in Mexico. Mexican *S. guttilatus* group with *S. marnocki* suggesting that these taxa are conspecific but more data are needed to resolve this issue. Populations of *S. marnocki* in the Edwards Plateau show a surprising high degree of divergence (4% uncorrected p) and perhaps additional cryptic taxa in the Edwards Plateau await discovery.

Introduction

The genus *Syrrhophus* contains approximately 17 species of leptodactylid frogs distributed from Texas south along the Atlantic coast of Mexico and with scattered populations of multiple species along the Mexican Pacific versant (Lynch, 1970). Species of *Syrrhophus* are small and nondescript leptodactylid frogs that have received little attention with regard to their behavior and ecology but attention has been focused on trying to disentangle their cryptic relationships (Wright and Wright, 1949; Lynch, 1968; Lynch, 1970).

Within Texas, *Syrrhophus* is currently represented by 3 species; *S. cystignathoides*, *S. marnocki*, and *S. guttilatus*. *S. cystignathoides* is found primarily along the Atlantic

versant of Mexico but also occurs naturally in extreme southern Texas (Dixon, 2000). Recently, this species has been accidentally introduced into various Texas cities probably as a result of the potting plant industry (Dixon, 2000). *S. marnocki* is endemic to the Edwards Plateau of Texas but *S. guttilatus* occurs mostly in Mexico extending north into Texas in Brewster, Pecos, and Presidio Counties.

Distinct diagnostic characters readily distinguish *S. cystignathoides* from the other two *Syrrhophus* however, the characters used to differentiate *S. marnocki* and *S. guttilatus* are questionable. *S. cystignathoides* has a first finger longer than the second whereas in the other two Texas species, the first finger is shorter than or equal to the second finger (Lynch, 1970). Distinguishing *S. guttilatus* from *S. marnocki* is based on three characters: 1) vermiculate dorsal pattern, 2) an interorbital bar, and 3) a supratympanic stripe (Lynch, 1970). Fieldwork in various parts of the Edwards Plateau and at Big Bend National Park has revealed that these characters do not distinguish the two taxa (pers. obs.). Examining specimens of *S. guttilatus* from Tamaulipas, Mexico (N=12) revealed that only approximately 17% had an interorbital bar, the supratympanic stripe was not evident, and there was no consistent vermiculate dorsal pattern. The interorbital bar is regarded as the most important character diagnosing *S. guttilatus* from *S. marnocki* (Lynch, 1970). This character is not obvious in the field for northern populations or a sample of Mexican *S. guttilatus* museum specimens. Characters defined by Lynch (1970) to distinguish the two taxa therefore appear unreliable and suggest a more complex taxonomic problem.

Assuming that *S. guttilatus* and *S. marnocki* are closely related and with the distribution data, I propose three hypotheses to test the validity of these taxa. First,

because of the lack of observable, diagnostic characters and because the distribution of the two species are adjacent with each other (Fig. 1), *S. guttilatus* is conspecific with *S. marnocki*. Second, both *S. guttilatus* and *S. marnocki* are “good” species but lack divergent, observable morphological characters and finally third, Texas *S. guttilatus* are a novel species from Mexican *guttilatus* but no observed morphological characters are apparent that distinguish Texas *S. guttilatus* from *S. marnocki*. A novel species hypothesis is interesting to consider because a large gap exists between Texas and Mexican *S. guttilatus* (Fig. 2) implying that little to no genetic exchange is possible between the two populations.

Molecular approaches provide powerful tools for delimiting species and revealing cryptic taxa (Hillis et al., 1996). Despite the vast quantity of molecular data available, no molecular data exist for *S. guttilatus* and *S. marnocki*. In this paper, I use a portion of *cytb* mtDNA to aid in revealing the taxonomic status of *S. guttilatus* and *S. marnocki* and provide support for the novel species hypothesis.

Materials and Methods

Tissues consisting of liver or muscle were collected from specimens from three localities in central Texas and from Big Bend National Park. Tissues were collected and placed in either Longmire’s solution or 96% EtOH but one specimen consisted of a buccal swab (Goldberg et al., 2003). Muscle tissue from Mexican *S. guttilatus* were collected from specimens preserved in isopropyl alcohol and housed in the Universidad Autonoma de Neuvo Leon collection of amphibians and reptiles. Localities for specimens are displayed in Figure 3 and exact locality data can be found in Appendix 1.

An 820+ bp region of the cytochrome b gene was targeted to resolve the taxonomic status of *Syrrhophus guttilatus* and *S. marnocki*. DNA was extracted from tissues using a QIAGEN kit and following manufacturer protocols (QIAGEN Corp, Inc.). The cytochrome b fragment was amplified using hot start *Taq* polymerase under touchdown PCR cycling and with Gludge and MVZ16 primers (Table 1). The first cycle began with a flexible annealing environment (93°C, 3'; 55°C, 45"; 68°C, 1') followed by successive cycles of increasingly stringent annealing temperatures (1°C decrease in annealing temperature/cycle to end at 45°C, 30" over 20 cycles). PCR products were gel extracted using a QIAGEN gel extraction kit and stored in -80°C. PCR products were then ligated to a kanamycin resistant vector and heat shocked into *E. coli* following standard protocols (Karcher, 1995). Colonies were grown in SOC growth medium and spread onto LB + kanamycin antibiotic plates then grown overnight. Two clones per sample were picked and plasmids extracted from the cells using a QIAGEN plasmid prep kit. Cycle sequencing was conducted using a Thermo Sequence Cycle Sequencing Kit (USB Corporation) and reactions propagated under the following cycling conditions: 95°C, 2'; 55°C, 30"; 72°C, 1.5' followed by 49 cycles of 95°C, 30"; 55°C, 30"; 72°C, 1.5'. Products were then sequenced on a Lycor 4200 sequencer.

Sequences were compared to sequences available in Genbank using the BLAST search algorithm (Altschul et al., 1990) to confirm or reject whether the sequence was the fragment of interest and not a potential contaminant. Sequences were aligned using Sequencher 4.1 (Genes Corp., Inc.) and electrochromatograms checked by eye for miscalled base pairs. Two *Leptodactylus* sequences comprising a smaller portion of the cytochrome b region were downloaded from Genbank and used to align sequences of

Syrrhophus. The Sequencher consensus alignment was exported to PAUP* version 4.0b10 (Swofford, 2003) for use in phylogenetic analysis.

I performed distance, parsimony, maximum likelihood, and Bayesian analyses to generate phylogenetic hypotheses from the sequence data. Both *Leptodactylus* and *S. cystignathoides* were used as outgroups in distance and parsimony analyses but only *S. cystignathoides* was used as the outgroup in maximum likelihood and Bayesian analyses. *Syrrhophus cystignathoides* was recovered as sister to the ingroup and had the same amount of contiguous sequence data as the other *Syrrhophus*. Minimum evolution analyses were performed under two different DNA/RNA substitution criteria. In the first minimum evolution analysis, I used the distance optimality criteria in PAUP* and used the uncorrected p DNA/RNA substitution setting. In the second minimum evolution analysis, I used the distance optimality criteria in PAUP* with the LogDet/Paralinear function. Trees generated under the distance optimality criterion used the TBR branch-swapping algorithm and neighbor-joining constraint. Maximum parsimony analyses were conducted using exhaustive search criteria.

I used the program Modeltest v. 3.06 (Posada and Crandall, 1998) to calculate the best model from 56 models of sequence evolution. From the model that produced the best log-likelihood score, I then set the R-matrix, proportion of invariable sites, and the gamma shape distribution parameter into the maximum likelihood settings of PAUP*. Maximum likelihood searches employed the GTR model, used empirical nucleotide frequencies, and did not enforce a molecular clock (Hasegawa et al., 1985).

For the parsimony and maximum likelihood analyses, I assessed clade and node support through nonparametric bootstrapping (Felsenstein, 1985). Bootstrap searches

were conducted heuristically with 10,000 pseudoreplicates for maximum parsimony and 1,000 pseudoreplicates for maximum likelihood with 10 replicate random taxa additions.

Bayesian analyses were conducted using MrBayes v.3.0b4 (Huelsenbeck and Ronquist 2001) with 1,000,000 generations and sample frequency of 100. A burnin of 1,000 was set by performing preliminary Bayesian runs and examining the point at which likelihood scores stabilized. A Bayesian tree was generated using the last 9,001 trees.

Results

The *cyt b* dataset consisted of 826 characters, 537 that were constant, 117 variable, and 172 that were parsimony informative characters. For distance analyses, both uncorrected p and LogDet/Paralinear criteria produced the same tree (Fig. 4). *Srryhopus guttilatus* from Texas form a distinct monophyletic unit separate from *S. guttilatus* in Mexico and *S. marnocki* and *S. cystignathoides* is sister to this clade.

Parsimony analysis found a single most parsimonious tree with a length of 383 steps, CI=0.89, R=0.870, RC=0.775, and HI=0.110 (Fig. 5). Bootstrap evaluation found strong support for all nodes of the topology and this topology is identical to the topology recovered under the distance optimality criterion.

Modeltest selected the TVM + Γ model of sequence evolution and the best log likelihood score was -2276.0186. The maximum likelihood tree recovered a strong phylogenetic break between the Big Bend and other populations however support for the topology comprising the Terrell County sample and the (Bandera Co., TX, Tamaulipas, MX) clade collapses under bootstrap support (Fig. 6).

Bayesian analysis revealed a similar topology as the other analyses but a low posterior probability value resulted for the same (Terrell Co, TX, (Bandera Co., TX, Tamaulipas,

MX)) node as found using likelihood analysis (Fig. 7). The phylogenetic break between the Big Bend clade and the (TX, MX) clade is strongly supported.

Examining uncorrected p pairwise distances shows that the Big Bend populations have between approximately 11 and 13% sequence divergence compared to other ingroup *Syrrhophus* (Table 2). Populations among Texas have about 4% sequence divergence whereas the Bandera County and Tamaulipas Mexico sample are only 0.3% divergent.

Discussion

The novel species hypothesis is supported, suggesting that populations of *Syrrhophus* from Big Bend National Park represent a distinct species. All phylogenetic analyses render *S. guttilatus* polyphyletic. Big Bend populations formed a well supported distinct monophyletic unit in all analyses separate from *S. guttilatus* populations in Mexico. This clade is also substantially divergent from other *S. guttilatus* and *S. marnocki* (Table 2). *S. marnocki* and populations of Mexican *S. guttilatus* form a monophyletic unit suggesting that these populations are either one or multiple taxa.

Populations of *Syrrhophus* from Big Bend were formerly recognized as a distinct species (*S. gaigae*) based on smaller body size and larger head size compared to *S. marnocki* (Schmidt and Smith, 1944). However, Milstead et al. (1950) synonymized *S. gaigae* with *S. marnocki* because they could not verify the characters used to describe the species. Specifically, they compared specimens of *S. marnocki* and *S. gaigae* and showed that head width was a function of body size although sample size of Big Bend was four compared to the 66 *S. marnocki*. Additionally, no multivariate analyses have been performed on the morphological characters of *Syrrhophus*. Multivariate analyses of

morphological data should prove useful in exploring further trends resulting from the deep phylogenetic break that occurs in the Chisos Basin of Big Bend National Park.

The similarity between the Bandera Co., TX and Tamaulipas, MX samples is surprising given the several hundred kilometer gap between the populations. These two populations are only 0.3% sequence divergence suggesting two possible scenarios. First, an yet unidentified biogeographic mechanism has forced these populations to be recently derived, or second the Mexico sample is a containment. Additional samples from Mexico are necessary to eliminate the latter scenario.

Divergences within the three sampled Texas populations are surprising high (4%) and this warrants further study. Possibly, several cryptic taxa remain to be discovered among *Syrrhophus* inhabiting the Edwards Plateau of Texas.

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Appendix 1

Syrrhophus cystignathoides-JHM 1390-USA; TX; Tarrant County; Bedford.
S. marnocki-MF2493-USA; TX; Bandera County; Lost Maples State Natural Area, 5.0 rd
mi N of Vanderpool on FM 187; MF 7401-USA; TX; Terrell County, Independence
Creek Nature Preserve; MF 6216-USA; TX; Travis County; Austin; MF 5022, MF3737,
Syrrhophus guttilatus-USA; TX; Brewster County; Big Bend National Park; Chisos
Basin; UNAL 5419; Mexico; Tamaulipas; Miquihuana; El Aserradero.

Table 1. Primer sequences used in this study.

Primer Name	Sequence 5'-3'	F/R
Gludge	TGACTTGAARAACCA YCGTTG	F
MVZ16	AAATAGGAARTATCAYTCTGGTTTRAT	R

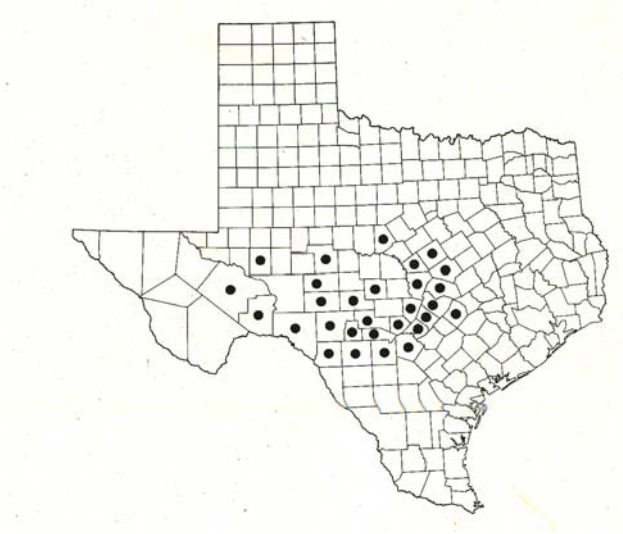
Table 2. Uncorrected p distance values for pairwise comparisons. Bold represents Big Bend populations.

	<i>S. cystig.</i>	Austin, TX	Terrell Co., TX	Tama, MX	Bandera Co., TX	Big Bend #1	Big Bend #2	Big Bend #3
<i>S. cystig.</i>	---							
Austin, TX	0.19095	---						
Terrell Co., TX	0.20135	0.04071	---					
Tama, MX	0.19733	0.04123	0.04433	---				
Bandera Co., TX	0.20136	0.04194	0.04383	0.00371	---			
Big Bend#1	0.20957	0.11792	0.12446	0.11088	0.11707	---		
Big Bend #2	0.21240	0.11949	0.12545	0.11332	0.11809	0.00615	---	
Big Bend #3	0.20919	0.11597	0.12200	0.10983	0.11464	0.00246	0.00366	---

Fig. 1. (A) Distribution of *Syrrhophus marnocki* and (B) *Syrrhophus guttilatus* in Texas.

Modified from Dixon (2000)

A



B

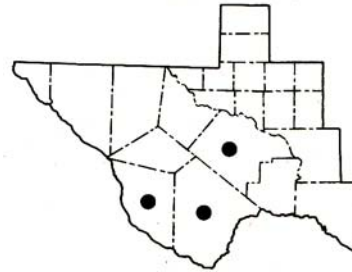


Fig. 2. Distribution of *S. guttilatus* in Texas and Mexico (Lynch, 1970).

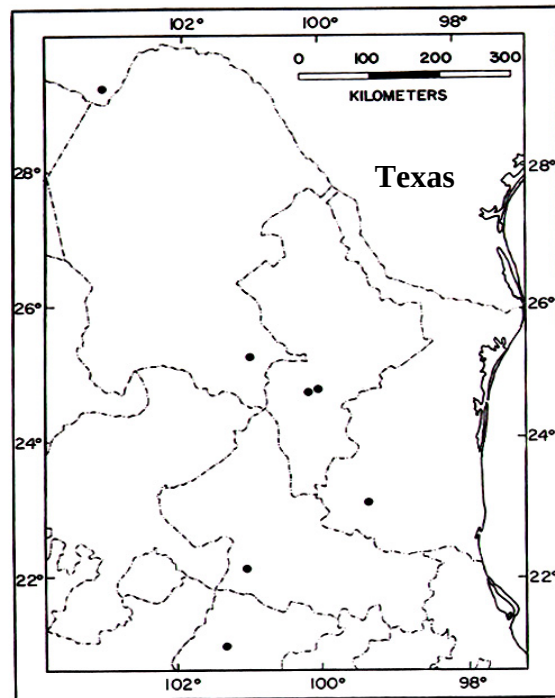


Fig. 3. Map showing localities of *Syrrhophus guttilatus* (squares) and *S. marnocki* (circles) used in this study.



Figure 4. Minimum evolution tree constructed with the LogDet/Paralinear algorithm. The uncorrected p neighbor-joining tree had an identical topology.

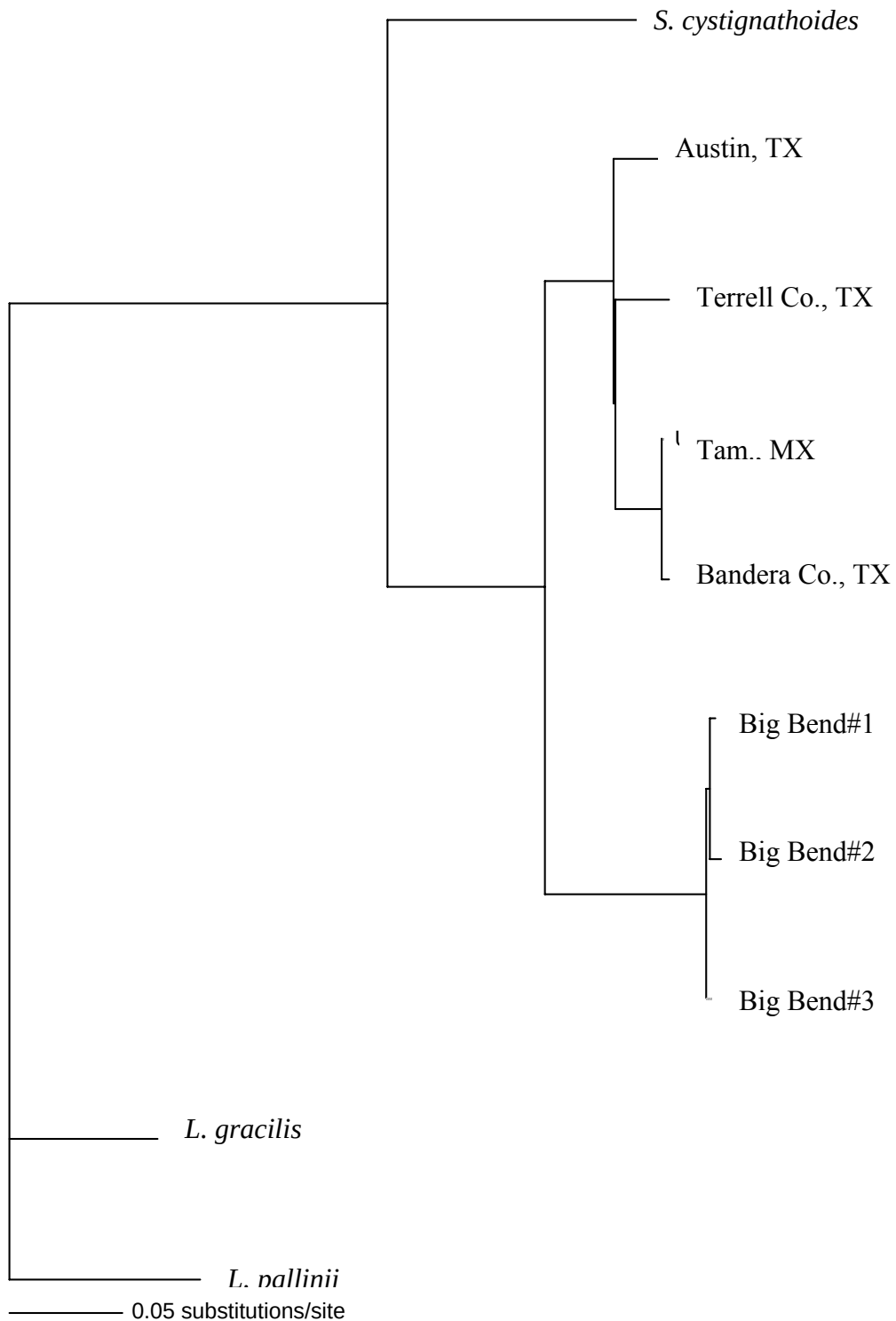


Figure 5. Maximum parsimony phylogram generated using the exhaustive search criterion. Numbers over nodes represent results of nonparametric bootstrap analysis using 10,000 pseudoreplicates with 10 random taxon additions. Length=383 steps; CI=0.890; RI=0.870; RC=0.775; and HI=0.110).

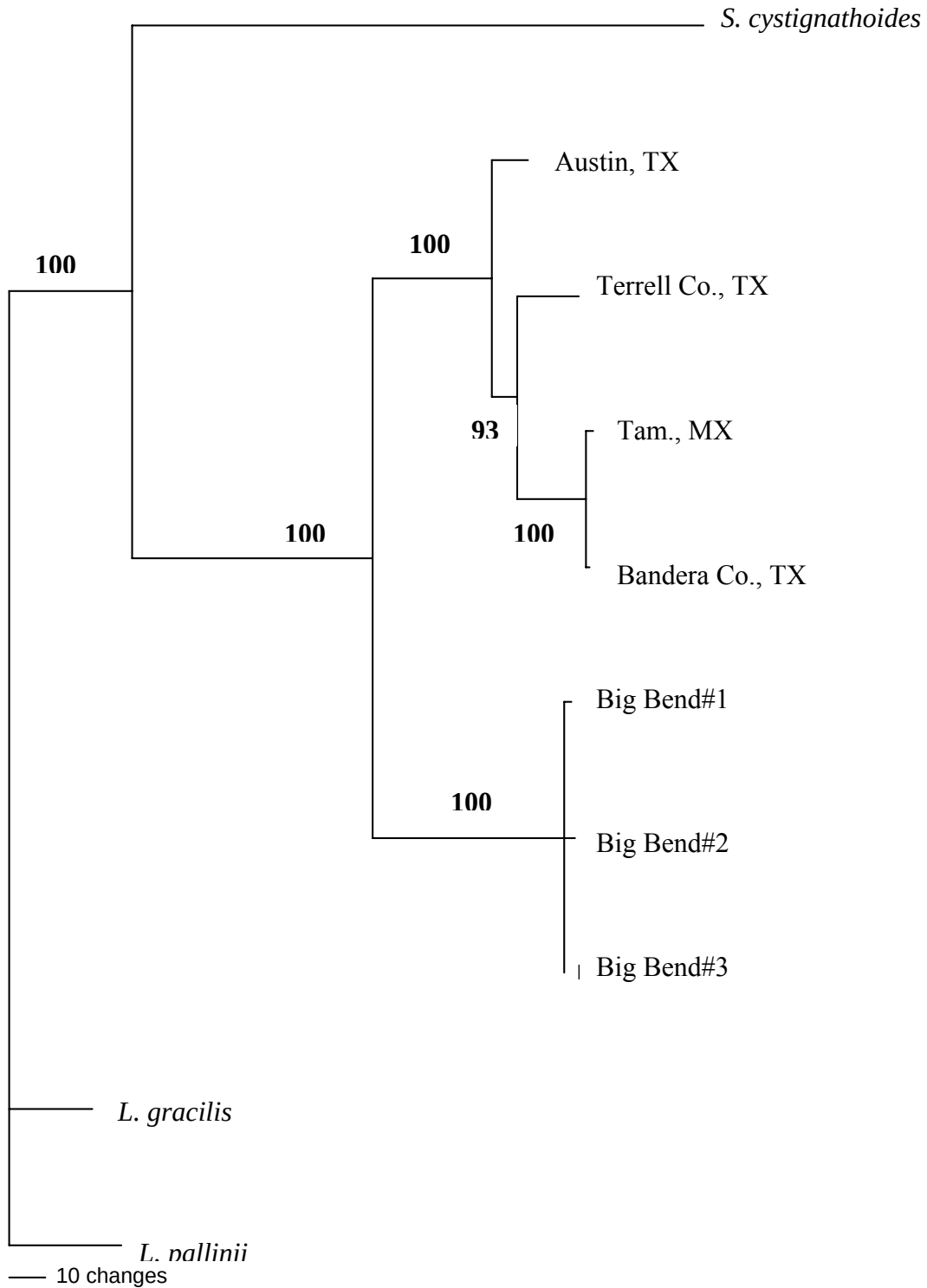


Figure 6. Maximum likelihood cladogram generated with 1,000 pseudoreplicates and 10 random taxa additions. Numbers over nodes are bootstrap values. Cladogram generated with the TVM + Γ log likelihood score -2276.0186 as found using Modeltest 3.06 and Aike's Information Criterion.

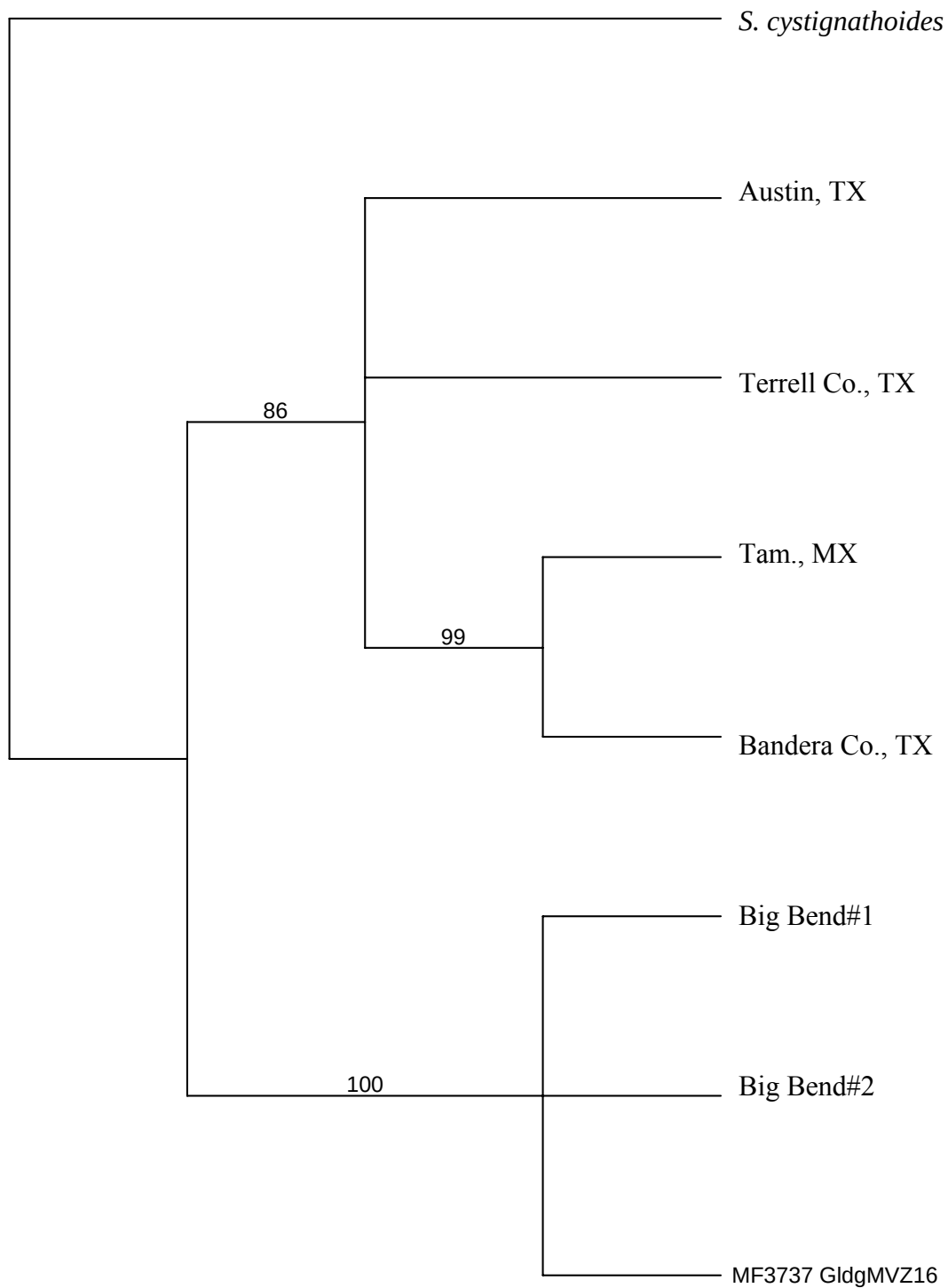


Figure 7. Bayesian tree from 1,000,000 generations and generated Big Bend#3 trees. Values above nodes are posterior probability values.

