

**Molecular Phylogenetics of *Pristimantis* (Anura: Strabomantidae) and the origin
and diversification of Central American species**

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ABSTRACT

Pristimantis (Anura: Strabomantidae) represents an exceptionally diverse group among Neotropical anurans, but the evolutionary relationships among subgeneric groups are poorly known. Using both original and published multilocus DNA sequence data, we developed a novel phylogenetic hypothesis for this genus. Ingroup sampling included 30.9% of the described species (265 individuals from 132 species, of which 156 individuals from 34 species are new data). Genetic data included three mitochondrial (COI, 12S, 16S) and two nuclear markers (Rag-1 and Tyr) for a total of ~4279 base pairs. Phylogenies were inferred using parsimony, maximum likelihood and Bayesian analyses of individual genes and combined data sets. The new phylogenetic hypothesis conflicts with most recognized taxonomic groupings. Within *Pristimantis*, the *peruvianus* group was the only group recovered, whereas the "*conscipillatus*", "*curtipes*", "*devillei*", "*frater*", "*lacrimosus*", "*myersi*", "*orestes*", "*surdus*", and "*unistrigatus*" were not supported as natural groups. The molecular phylogeny suggests that the colonization of Central America by South American *Pristimantis* involved perhaps 13 independent events. Our results suggest alternative interpretations of *Pristimantis* taxonomy, character evolution, and biogeography, topics that now demand more extensive evaluation in future studies.

Keywords: Anura, Strabomantidae, *Pristimantis*, Terrarana, Colombia, Central America, Great American Biotic Interchange, Molecular phylogenetics.

INTRODUCTION

Pristimantis Hedges et al. (2008) is a Strabomantidae genus which has been assigned to Terrarana (*sensu* Hedges et al. 2008), with 427 species is the genus of amphibian with the greatest number of species in the Americas (Lynch 1986, Lynch & Duellman 1997, Campbell 1999, Duellman 1999, Hedges 1999, Frost 2008, AmphibiaWeb 2008, Hedges et al. 2008). However, from an evolutionary and taxonomic viewpoint, this clade of frogs is one of the least known of the major groups of vertebrates. Although new species are recognized and described annually, taxonomists have been incapable of agreeing for the most part on how to organize those species to better reflect their evolutionary history (e.g., Lynch 1976, Savage 1987, Lynch & Duellman 1997, Frost et al. 2006; Hedges et al. 2008). The taxonomic confusion in part shows the insufficiency of characters available for studying the group and the plasticity of the few “useful” characters (Hedges et al. 2008).

During the past five years Terrarana systematics has undergone something of a revolution as the molecular work has been joined by newly developed methods of analysis. Until recently, nine studies using DNA sequences have been undertaken with these frogs, Darst & Cannatella (2004), Crawford & Smith (2005), Frost et al. (2006), Padial et al. (2007), Padial et al. (2008), Heinicke et al. (2007), Crawford et al. (2007), Hedges et al. (2008), and Wang et al. (2008). Excluding the Heinicke et al. (2007) and Hedges et al. (2008) studies, the majority of these investigations have had limited taxon sampling, including less than 5% of all “eleutherodactylines.” Lynch & Duellman (1997) based exclusively on inspection of morphological characters, such as relative length of digits assigned the species of “*Eleutherodactylus*” to 12 groups: *myersi*, *sulcatus*, *cerasinus*, *conspicillatus*, *curtipes*, *dolops*, *devillei*, *loustes*, *orestes*, *surdus*, *diastema* and *unistrigatus*. Although they considered each species group as monophyletic, they did not present a formal phylogenetic analysis. Subsequently, based on molecular data and phylogenetic analyses Crawford & Smith (2005) elevated to *Craugastor* to the generic rank, leaving the remaining eleutherodactylines species as a monophyletic group. Afterward Heinicke et al. (2007) restricted “*Eleutherodactylus*” to the South American clade of 87 species

(representing 397 species) as *Pristimantis*. Hedges et al. (2008) proposed the South American clade division in three subgenera (*Hypodyction*, *Pristimantis* and *Yunganastes*) and 427 species.

Pristimantis sensu Hedges et al. (2008) is distributed in Central American (eastern Honduras, Panamá, Honduras and Costa Rica), South America (Colombia, Ecuador, Peru, Bolivia, Amazonian Brazil and the Guianas), Trinidad and Tobago, Grenada, and the Lesser Antilles (AmphibiaWeb 2008). This genus is most diverse in northwestern South America, where its distribution includes the lowlands to elevations of about 4000 m in the Andes in Colombia, Ecuador, and Peru (Frost 2008).

Studying the fauna shared between Colombia and Central American is motivated by the role of the Panamá Isthmus as a land bridge and a driver of expansion, extinction, and the Great American Biotic Interchange (Marshall et al. 1982, Marshall 1988, Simpson 1940, Webb 1978, Webb & Rancy 1996) roughly three million years ago (Coates et al. 2004). Molecular data have been used to investigate the impact the origin of the Isthmus of Panama on marine organisms (Lessios 1979, Bermingham & Lessios 1993, Knowlton et al. 1993), aquatic organisms (Bermingham & Martin 1998) and terrestrial organisms (Zamudio & Greene 1997, Zeh et al. 2003, Weigt et al. 2005, Wang et al. 2008). An interesting model to study the role of Isthmus of Panama is the genus *Pristimantis*, because it is a terrestrial organism distributed in Central American and South America, intolerant of salt water, unable to fly, and restricted to forested habitats.

The geographic distribution of *Pristimantis* has showed that it was originated in South America (Vanzolini & Heyer 1985, Duellman 2001, Savage 2002, Heinicke et al. 2007, Hedges et al. 2008, Wang et al. 2008). Therefore, we could distinguish one biogeographical hypothesis by focusing on one important questions concerning to Central American samples. (1) Do all endemic Central American samples form a monophyletic clade relative to South American samples?. To answer this question, we inferred the spatial and genealogical history based on mitochondrial and nuclear genes of *Pristimantis* and compared our results to previous work as well as the geological history of Central America isthmo.

METHODS

Taxon sampling

Throughout this work, we used the taxonomic classification proposed by Hedges et al. (2008). In total, we sampled 156 individuals representing 34 species (Appendix 1). Additional sequences representing 98 species and 109 individuals were downloaded from GenBank (NCBI; Appendix 2). We include 10 species as outgroups, *Craugastor daryi*, *C. longirostris* (Craugastoridae); *Agalychnis callidryas*, *Litoria caerulea* (Hylidae); and *Lynchius flavomaculatus*, *L. nebulanastes*, *Oreobates cruralis*, *O. saxatilis*, *Phrynopus auriculatus*, *P. bracki* (Strabomantidae). Recent studies based on molecular data (Hedges et al. 2008) support the hypothesis that *Lynchius*, *Oreobates* and *Phrynopus* genus are the sister species of *Pristimantis*. Other group proposed to be closely related to Strabomantidae is Craugastoridae. We used some species of Hylidae as more divergent outgroups to root the phylogeny.

Specimens were collected in 7 countries (Appendix 1). Museum material was made available for morphological and molecular examination by the Circulo Herpetológico, Panama (CH); Colección herpetológica, Universidad Industrial de Santander, Colombia (UIS-H), Museum Bolivia Herpetology, Bolivia (MBH); Museo de Herpetología de la Universidad de Antioquia, Colombia (MHUA), and the Museo de Zoología de la Pontificia Universidad Católica del Ecuador, Ecuador (QCAZ). Tissue samples were obtained from specimens listed in Appendix 1. Tissues were collected in the field and preserved in 99% ethanol or a 20% solution of dimethylsulfoxide (DMSO) saturated with NaCl (Amos & Hoelzel 1991, cited in Amos et al. 1992) with the addition of 0.125 M EDTA. Specimens were deposited at public research institutions. Voucher numbers, locality information, and GenBank accession numbers for each specimen are listed in Appendix 1.

Laboratory techniques

We sequenced the following three mitochondrial genes: 16S rRNA (16S), 12SrRNA (12S), and COI. For a subset of samples we also obtained DNA sequence data from two nuclear exon gene regions: the recombination activating gene 1 (Rag-1) and the tyrosinase gene (Tyr) (Table1). The mitochondrial genes were chosen because they display a slower rate of evolution in an attempt to avoid saturation problems (Heinicke et al. 2007). The fragment of the nuclear gene Tyr was chosen because it has been useful in other studies (Bossuyt & Milinkovitch 2001, Frost et al. 2006, Hedges et al. 2008, Heinicke et al. 2007). The fragment of the nuclear gene Rag-1 is from the relatively faster-evolving first half of the gene (Hedges et al. 2008, Heinicke et al. 2007).

The molecular procedure was performed at the Instituto de Genética, Universidad de los Andes (Colombia) and the Instituto Smithsonian de Investigaciones Tropicales (Panamá). Genomic DNA was extracted from liver and/or thigh muscle tissue using Qiagen QIAamp tissue kit. PCR amplification of gene fragments was performed in 12.5-ml reactions using 0.125 Qiagen Taq, 1.25 µl Buffer 10X with 1.5mM of MgCl₂, 1.25µl dNTPs at 2mM, 0.625 µl forward and reverse primers at 10 mM, and 1 ml of extracted DNA (more for low-quality tissue). Standard reaction conditions were an initial hold for 5 min at 94°C; followed by 29 cycles of 94°C/30 s, 55°C (for 16S and 12S) 52°C (for COI) and 60°C (Rag-1 and Tyr) for 30 s and 72°C/60 s. After 29 cycles, a final hold of 72°C/7 min was performed before terminating the reaction at 4°C. For low- or nonyielding samples, annealing temperature was dropped from 60°C to 46°C. Primers used in PCR reactions were obtained from the literature or designed in the lab (see Table 1). Polymerase chain reaction (PCR) products were cleaned by gel slicing and agarose digest, or Exo I/SAP digest. For each individual, both H (heavy) and L (light) strands were sequenced directly. Cycle sequencing reactions were completed using the corresponding PCR primers and BigDye Terminator (Applied Biosciences), with a standard cycle sequencing profile (96°C/60 s; 30 cycles of 96°C/10 s, 50°C/15 s and 60°C/4 min; and 72°C/7min). DNA sequencing was performed with an ABI Prism 3100 sequencer (PE Applied Biosystems).

The DNA sequences were analyzed with Sequencher 4.2 (Gene Codes Corporation) and Geneious 3.7.0 (Biomatters Ltda). The alignments were conducted initially using MAFFT version 6 (Katoh 2002) under default parameters. Manual adjustments using MacClade ver. 4.07 (Maddison & Maddison 2000) were particularly important in protein coding genes (COI, Rag-1 and Tyr) to maintain reading frames. The 16S and 12S genes are a highly conserved mitochondrial marker but mutations are common in some variable regions, corresponding to loops in the ribosomal RNA structure. Therefore these alignments were inspected for errors and compared against secondary structure models available from the European ribosomal RNA database. Regions of uncertain homology were excluded from preliminary analysis using G-block 0.91b (Castresana 2000), and manual pruning guided by the results obtained therein. GenBank accession numbers will be provided for all DNA sequences obtained for this study upon acceptance of this manuscript, and all alignments will be made available at TreeBase (<http://www.treebase.org>) (accession numbers: xxxxxxxxx)

Phylogenetic analyses

With our data and Gen-Bank information, we constructed four matrices, the first one with 243 individuals and 2503 ribosomal base pairs, the second one with 243 individuals and 3115 mitochondrial base pairs, the third one with 80 individuals and 4279 mitochondrial and nuclear characters), the last one 132 species (265 individuals and 4279 mitochondrial base pairs).

Phylogenetic analyses were conducted using maximum parsimony (MP), maximum likelihood (ML), and Bayesian methods for individual genes, as well as for the concatenated dataset (see data partitions). For MP analysis we performed a heuristic search with 10000 replicates of random taxon-addition and TBR branch swapping, with gaps treated as missing characters, using PAUP* version 4.0b10 (Swofford 2002) as implemented in CIPRES portal. Non-parametric bootstrap values (Felsenstein 1985) were

obtained with 5000 replicates, each having ten replicates of random taxon-addition.

For ML and Bayesian analysis, we used Modeltest version 3.7 (Posada & Crandall 1998) to evaluate different models of evolution. We selected the model obtained by means of the criterion of Akaike or AIC (Akaike 1973) as this allowed us to simultaneously compare non-nested models, assess model selection uncertainty, and allowed for the estimation of model parameters using all available models (Posada & Buckley 2004). Maximum likelihood analyses were run in RAxML 7.0.0 (Randomized Accelerated Maximum Likelihood, available at <http://www.phylo.org>) (Stamatakis et al. 2008), which uses GTRCAT as an approximation for GTR+Gamma. Node support was assessed via 1000 bootstrap replicates.

We conducted Bayesian phylogenetic analyses using MrBayes, version 3.1 (Ronquist & Huelsenbeck 2003) as implemented in CIPRES portal, under the same model as in the ML analysis. We conducted two parallel runs of the MCMC algorithm for 10 million generations each, sampled one tree per 1000 generations. All runs employed four chains with Metropolis coupled MCMC heating. In all searches stationarity of the Markov Chain was determined as the point when sampled log likelihood values plotted against generation time reached a stable mean equilibrium value; "burn-in" data sampled from generations preceding this point were discarded (Huelsenbeck & Ronquist 2001). The graphics were visualized using Tracer 1.3 (Rambaut & Drummond 2003).

Initially each gene fragment was analyzed individually. Topologies resulting from each gene were compared to detect areas of incongruence that were strongly supported by bootstrap values and/or posterior probabilities (Wiens 1998). We did not employ the Incongruence Length Difference (ILD) test because its power to detect incongruence is extremely low when the incongruence is caused by different topologies, when the number of informative sites is small, and the heterogeneity of among-site substitution rate is

large. Besides the incongruence caused by unequal branch lengths does not appear to be detected easily by the ILD test (Barker & Lutzoni 2002, Darlu & Lecomte 2002).

Our goal was to analyze the data jointly. Because our combined data set is comprised of one protein-coding mitochondrial gene (COI), two ribosomal genes with secondary structure (12S and 16S), and two nuclear genes (Rag-1 and Tyr), we suspected that application of a single nucleotide substitution model was unlikely to provide a particularly good fit to the data (e.g., Nylander et al. 2004, Brandley et al. 2005). Therefore, we partitioned the data set by gene (12S, 16S, COI, Rag-1 and Tyr), but analyze the data jointly.

Divergence times

Times of divergence were estimated for the 80-species data set by using the program BEAST v1.4.6 (Drummond & Rambaut 2007). The assumed topology was from the five-gene ML analysis. The dataset was analyzed under the GTR+Gamma model with a relaxed clock, allowing branch lengths to vary according to an uncorrelated Lognormal distribution (Drummond et al. 2006). This program co-estimates phylogeny and divergence times under a new class of relaxed clock models. For species-level phylogenies, the Yule tree prior that assumes a constant speciation rate per lineage was used, as suggested by Drummond et al. (2006).

The “treeModel.RootHeight” prior (i.e., the age at the root of the tree) was set to 57 million years (with a standard deviation of 14 million years), in accordance with results from Roelants et al. (2007). We used the Central American clade (*Craugastor*) as calibration point (42 million years with a standard deviation of 11 million years) according to Heinicke et al. (2007). All other priors were left to the defaults in BEAST. Parameters were estimated using 2 independent runs of 1 million generations each (with a pre-run burn-in of 10000 generations), with parameters sampled every 1000 generations. Convergence was checked in the Tracer v1.4.6 program and summary trees were

generated using TreeAnnotator v1.4.6, both part of the BEAST package.

Hypothesis testing

Phylogenetic Predictions:

We tested predictions from the possibility that the taxonomic groups "*conscipillatus*", "*curtipes*" "*devillei*", "*frater*", "*lacrimosus*", "*myersi*" "*orestes*", and "*unistrigatus*" among *Pristimantis* genus were monophyletic. Seven *a priori* tree topologies were constructed with MacClade ver. 4.07 (Maddison & Maddison 2000). Every one topology presented each individual group as monophyletic, without any relationship among the groups. With the topologies with constrain is running a new ML search. The significance of the difference in the sum of site-wise log-likelihoods for all trees is evaluated by bootstrap sampling of site scores with 1000 replicates and then calculating how far the observed differences are from the mean of the bootstrap replicates (Shimodaira & Hasegawa 1999).

Biogeographic Predictions:

We generated a biogeographical hypothesis from distributional data for *Pristimantis* and from geological data on the formation of the Isthmus (Coates & Obando 1996, Coates et al. 2004). *Pristimantis* is thought to have origin in South America (Vanzolini & Heyer 1985, Duellman 2001, Savage 2002, Heinicke et al. 2007, Hedges et al. 2008, Wang et al. 2008). Today *Pristimantis* is distributed on Central American and South America. However, the incomplete isthmus upheaval before 3.1 Mya (Coates & Obando 1996) could represent a significant dispersal barrier between South America and Central America. Therefore, to achieve its present distribution we assume that the ancestor of Centroamerica *Pristimantis* likely had one dispersal history. It may have dispersed along Panamá Isthmus and invaded the Central America land. Therefore our *a priori* hypothesis is that all Central America species form a monophyletic clade relative to South American samples.

This hypothetical topology was compared to the topology obtained with ML using the paired-sites test (SH) of Shimodaira & Hasegawa (1999) as implemented in PAUP version 4.0b10 (Swofford 2002). The SH tests whether a ML phylogenetic tree estimated under a topological constraint is significantly worse than the optimal (unconstrained) tree.

RESULTS AND DISCUSSION

Phylogenetic analysis

The final data set for all five gene regions comprised 4279 base pairs (612 from COI, 1543 from 16S, 960 from 12S, 633 from Rag-1 and 531 from Tyr), from 265 individuals and 132 species (Figure 4, 5).

We show Maximum parsimony (MP) and likelihood (ML) trees for 80-data set (Figs. 1 and 2) because it is the data set with the complete data (4279 characters), except for COI gene and include bootstrap confidence values and Bayesian confidence values (posterior probabilities) on nodes. Bayesian inference analyses yielded trees very similar in topology to those obtained from Maximum likelihood. The data set was combined because strongly supported conflicts were absent when comparing the individual gene trees.

For ML and Bayesian analyses, ModelTest selected in the majority of cases the GTR+I+G model as optimal for each gene (Table 2). Base composition, gamma parameters, and a proportion of invariant sites for each of the data sets are presented in Table 2. The A–G and C–T substitution (see Table 2, bold letter) rate parameters have higher relative importance values than the transversion parameters. This indicates that for these data it is important to allow the two transition types to have different rates, more so than the transversion types. As expected, mitochondrial genes presented more variability among taxa than nuclear genes (Table 3).

In general in all topologies we observe that *Pristimantis* was a monophyletic genus with significant support (100%) in the MP, ML and Bayesian analysis. In all trees, it appears as a close relative of the clade containing *Lynchius*, *Oreobates*, and *Phrynopus*, with support (100%) in the MP, ML and Bayesian analysis (Figure 1-2). Hedges et al. (2008) proposed that there are three subgenera named as *Hypodictyon*, *Pristimantis* and *Yunganastes* within *Pristimantis*. Based on our preliminary topologies (figures 1-4) these clades are not recovered.

Within *Pristimantis*, the *peruvianus* group was the only group recovered and well supported (100%) (Figure 1-2) while "*conscipillatus*", "*curtipes*", "*devillei*", "*frater*", "*lacrimosus*", "*myersi*", "*orestes*", "*surdus*", and "*unistrigatus*" appear as non-natural groups. For the *chalceus*, *galdi*, and *orcesi* groups, we only have one species representing each one of these groups; therefore we cannot infer anything about monophyly of these groups. Comparing the Figure 1 hypothesis with the *a priori* topology with each one of the groups as monophyletic is possible concluded that each topology with each one of the groups as monophyletic was rejected with $P < 0.05$.

Based on our molecular phylogenies (Figs. 1–2 and supplementary material Figs. 3-8) many of the species groups recovered here are demonstrably not monophyletic. Our hypotheses disagree with the previously defined morphological species groups (Lynch & Duellman 1997).

Our results show that the *Pristimantis* groups proposed by Lynch & Duellman (1997) based on morphological characters are phenetic groups. Therefore, is necessary to search for morphological homologous characters that allow the definition of the groups proposed by the molecular data. These discrepancies and the limited taxon sampling (132 species) make it difficult to define subdivisions within *Pristimantis*. We refrain from defining other groups until DNA sequence data become available for a larger proportion of the *Pristimantis* genus.

Divergence Times and Biogeographical history

Dates of divergence were obtained by using the 80 data set (Figure 2). Our results indicate that the *Pristimantis* genus diverged from other eleutherodactylines in the Eocene 44 Mya (with credibility interval CI of 32-60 Mya) and began an explosive diversification 34 Mya (CI=26-44 Mya). The geologic epoch is concordant with the times presented by Heinicke et al. (2007). Most of the basal branches of eleutherodactylines with some dating to the early Cenozoic (49.79 Mya, CI=37.18-68.67 Mya) occur in South America (Heinicke et al. 2007). This shows that South America was the place of origin for the genus *Pristimantis*, according to Vanzolini & Heyer (1985), Duellman (2001), Savage (2002), Heinicke et al. (2007), Hedges et al. (2008), and Wang et al. (2008). The rapid diversification within this genus began 34 Mya and has continued to the present (Figure 2). The enormous diversity of species within this clade (427 species) according to Hedges et al. (2008) is linked with the beginning Andean uplift (Lynch 1986), which occurred in the last 10-20 million years (Gregory-Wodzicki 2002, MacFadden 2006). During this period, mountain building and associated climatic changes resulted in repeating patterns of habitat isolation and speciation in these amphibians (Lynch & Duellman 1997).

An amazing pattern in our results is the apparent successful colonization of some species of the *Pristimantis* genus into Central American. The molecular data suggest a complex history at least of 13 separate invasions from South America to Central American (Figure 2). Comparing this hypothesis with the hypothetical topology with all Central American groups monophyletic using SH tests it is possible to conclude that the topology which assumes only one dispersal event from South America to Central American can be rejected with $P < 0.05$.

These invasions occurred mainly 5-25 Mya in the Miocene epoch, which show that the Centroeamerican *Pristimantis* species already occupied the isthmian landscape prior to the completion of the isthmian land bridge 2.8-3.1 Mya (Coates & Obando 1996). Although

these divergence times of Centroamerican species seem very great, it corresponds with divergence times calculated for other groups as túngara frogs (Weigt et al. 2005), *P. ockendeni* (Elmer et al. 2007), *P. ridens* (Wang et al. 2008), freshwater fishes (Bermingham & Martin 1998), and vipers (Zamudio & Greene 1997).

Actually, there is geological information that supports the dispersion towards Centroamerica from Southamerican *Pristimantis*. The fossilized mammals of raccons and giant ground sloths suggest that southern Central America had a dry-land connection (continuous peninsula) to North America during the middle Miocene (Whitmore & Stewart 1965, Kirby & MacFadden 2005). This peninsula may have received in the late Miocene anuran colonist from South America, such as *P. ridens* (Wang et al. 2008), and the túngara frog (Weigt et al. 2005). *Pristimantis* could have arrived in Central America before the completion of the land bridge by rafting (Vences et al. 2004), or through a period of low sea levels in the end of the Miocene when the sea level was approximately 60 m below today's level (Bermingham & Martin 1998). However, the actual distribution of some species such as *P. taeniatus* (L clade) is also compatible with dispersal over land after the emergence of the Isthmus of Panamá (aprox. 3 Mya).

Other scenario although unlikely is that these dispersal events probably occurred over water along islands in the West Indies (Heinicke et al. 2007; Hedges et al. 2008).

Traditionally the colonization of a continent by an insular lineage has been considered rare, and subsequent diversification is thought to be even rarer. Several explanations based on faunal saturation on the mainland and competitive disadvantage of island species have been advanced to explain this pattern (Nicholson et al. 2005). The model of one-way colonization from continent to islands, and the ecological hypotheses designed to explain it, must be revised as more studies reveal more than one invasion (Raxworthy et al. 2002, Filardi & Moyle 2005, Dávalos 2007). However according to our results the colonization through the western islands is less possible, because this hypothesis requires the migration of the species through the islands and then admit several events of extinction.

Thus, in order to clarify the groups and the relationship among the species groups, additional studies are needed, including more taxa and morphological and molecular approaches (microsatellites). Although our results are preliminary, the phylogenetic and

biogeographic hypotheses are based in the most parsimonious cladogram, which is the less refuted with the current evidence, and “this cladogram is only the focus of the next round of testing, and so it goes” (Kluge 1997:93).

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TABLES

Table 1.

Genes and primers employed in this study.

Primer	Primer sequence (5' ----3')	Source
Mitochondrial COI		
dgLCO-1490	GGTCAACAAATCATAAAGAYATYGG	
dgHCO-2198	TAAACTTCAGGGTGACCAAARAAYCA	
LCO-1490	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)
HCO-2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)
Mitochondrial 16S		
16Sar-L	CGCCTGTTTATCAAAAACAT	Palumbi et al. (1991)
16Sbr-H	CCGGTCTGAACTCAGATCACGT	Palumbi et al. (1991)
Mitochondrial 12S		
12H10	CACYTTCCRGTRCRYTTACCRGTACGACTT	Heinicke et al. (2007)
12.1L4E	TACACATGCAAGTYTCCGC	Heinicke et al. (2007)
Nuclear RAG1		
R182	GCCATAACTGCTGGAGCATYAT	Heinicke et al. (2007)
R270	AGYAGATGTTGCCTGGGTCTTC	Heinicke et al. (2007)
Nuclear Tyr		
Tyr1C	GGCAGAGGAWCRTGCCAAGATGT	Bossuyt & Milinkovitch (2000)
Tyr1G	TGCTGGGCRTCTCTCCARTCCCA	Bossuyt & Milinkovitch (2000)

Table 2.

Estimated parameters for Bayesian analyses. Parameters were calculated using Mr. ModelTest 3.7 (Posada & Crandall, 1998). AIC= Akaike information criterion; I=Proportion of invariable sites; Gamma= Gamma distributed rate variation among sites.

Gen	Best-fit Model	AIC score	-ln likelihood	I	Gamma	Rate Matrix						Base frequency			
						AC	AG	AT	CG	CT	GT	A	C	G	T
COI	GTR+I+G	27593.7	13786.9	0.4311	0.5459	0.5680	11.2552	0.4570	0.4277	6.3348	1.0000	0.3069	0.3028	0.1010	0.2893
12S	GTR+I+G	63217.2	31598.6	0.2627	0.7028	2.6346	9.1316	2.6776	0.4414	20.9307	1.0000	0.3888	0.2278	0.1621	0.2213
16S	GTR+I+G	84298.7	42139.3	0.2706	0.6188	3.3087	8.6510	3.3805	0.7856	23.7068	1.0000	0.4110	0.2126	0.1459	0.2305
Rag_1	GTR+I+G	8824.5	4402.3	0.3280	1.6718	1.2761	4.1305	0.6188	1.4669	5.6222	1.0000	0.3329	0.2123	0.1756	0.2793
Tyr	HKY+I+G	8914.6	4451.3	0.3356	1.1107			Ti/tv ratio 2.5277				0.2560	0.2399	0.2111	0.2931

Table 3.

Proportion of invariable, variable un-informative and parsimony informative (PI) characters.

Gen	Alignment positions	No. of invariable sites	Proportion of invariable sites	No. of variable sites are un-PI	Proportion of variable sites are un-PI	No. of PI	Proportion of PI
COI	612	286	0.47	19	0.03	307	0.50
12S	960	287	0.30	105	0.11	568	0.59
16S	1543	496	0.32	142	0.09	905	0.59
Rag_1	633	299	0.47	79	0.13	255	0.40
Tyr	531	253	0.48	67	0.12	211	0.40

APPENDICES

Appendix 1. For each specimen, extraction number or tissue collection number, museum voucher, source, locality and GenBank accession number are reported. Acronyms for museums are: CH = Circulo Herpetologico, Panama; MBH= Museum Bolivia Herpetology, Bolivia; MHUA= Museo de Herpetología de la Universidad de Antioquia, Colombia, QCAZ= Museo de Zoología de la Pontificia Universidad Católica del Ecuador, Ecuador; UIS-H= Colección herpetológica, Universidad Industrial de Santander, Colombia. The abbreviations for the individuals field series are as follows: AJC=Andrew Jackson Crawford; CJD= Claudia Juliana Dulcey; DL= David Laurencio; EMM= Eliana Maria Muñoz; ENS= Eric N. Smith; KRL= Karen R. Lips; NRPS= Nelsy Rocio Pinto Sanchez; RC= Rances Caicedo; SMG= Sandra M. Gallo.

Species	Extraction number	Museum voucher	Origin	Country	Department/Province	Municipality	Mitochondrial genes			Nuclear genes	
							COI (614 bp)	16S (621 bp)	12S (806 bp)	Rag1 (620 bp)	Tyr (500 bp)
<i>Craugastor bufoniformis</i>		AJC 1144	This study	Panamá	Panamá	Altos del Maña, ~7.5 km NE de El Valle de Anton, corregimiento de Chamé	X	X	X	0	0
<i>Craugastor cf. longirostris</i>	E-76	AJC 1336	This study	Colombia	Antioquia	Maceo	X	X	X	0	0
<i>Craugastor cf. longirostris</i>	E-78	AJC 1193	This study	Colombia	Chocó	Nuqui	X	X	X	0	0
<i>Diasporus diastema</i>		AJC 0540	This study	Costa Rica	Puntarenas	Finca Sergio Jimenez, 6.5 km por air SSE de Aguitas, Drake, Osa	X	X	0	0	X
<i>Diasporus gularis</i>	E-77	AJC 1187	This study	Colombia	Chocó	Nuqui	X	X	0	0	X

<i>Diasporus hylaeformis</i>		AJC 0468	This study	Costa Rica	Alajuela	Estación Biológica Alberto Ml. Brenes, Reserva Biológica San Ramón	0	X	X	0	0
<i>Diasporus hylaeformis</i>		AJC 0474	This study	Costa Rica	Cartago	Tapantí, Cantón Paraíso	X	0		0	0
<i>Diasporus vocator</i>		AJC 0127	This study	Costa Rica	Puntarenas	Las Cruces	X	X	X	X	X
<i>Diasporus vocator</i>		CH 4784	This study				X	X	X	X	X
<i>Pristimantis achatinus</i>		AJC 0573	This study	Panamá	Darién	Caná main camp, Sendero Boca de Cupe	X	X	X	X	X
<i>Pristimantis affinis</i>	E-50	NRPS0031	This study	Colombia	Cundinamarca	Parque Nacional Natural Chingaza	X	X	X	X	0
<i>Pristimantis altae</i>		AJC 0398	This study	Costa Rica	Alajuela	Monumento Natural Histórico LaPaz (MINAE), ~16km por rd. NW de San Ramón, Canton LaPaz	X	X	X	X	0
<i>Pristimantis bogotensis</i>	E-51	NRPS0033	This study	Colombia	Cundinamarca	Parque Nacional Natural Chingaza	X	X	X	X	X
<i>Pristimantis caryophyllaceus</i>		AJC 0187	This study	Panamá	Chiriquí	Fortuna	X	X	X	X	X
<i>Pristimantis caryophyllaceus</i>		AJC 0486	This study	Costa Rica	San José	Rio Gacho, Los Juncos, Cascajal, Cantón Vázquez de Coronado	X	X	X	X	0
<i>Pristimantis caryophyllaceus</i>		CH 6367	This study	Panamá	Darién	Caná, Laguna	X	X	X	X	X
<i>Pristimantis cerasinus</i>		AJC 0071	This study	Costa Rica	Heredia	La Selva	X	X	X	X	X
<i>Pristimantis cerasinus</i>		AJC 0527	This study	Costa Rica	Limón	"Vuelta de Queque" Rio Siquirres trail, Guayacan, Siquirres	X	X	X	X	0
<i>Pristimantis cerasinus</i>		AJC 1132	This study	Panamá	Panamá	Altos del Maña, ~7.5 km NE de El Valle de Anton, corregimiento de Chamé	0	X	X	X	0

<i>Pristimantis cerasinus</i> (white on thighs)	AJC 1142	This study	Panamá	Panamá	Altos del María, ~7.5 km NE de El Valle de Anton, corregimiento de Chamé	0	X	X	0	X
<i>Pristimantis cruentus</i>	AJC 0234	This study	Panamá	Chiriquí	Fortuna	X	X	X	X	X
<i>Pristimantis cruentus</i>	AJC 0394	This study	Costa Rica	Alajuela	Monumento Natural Histórico La Paz (MINAE), ~16km por rd. NW de San Ramón, Canton La Paz	0	0	0	0	0
<i>Pristimantis cruentus</i>	AJC 0460	This study	Costa Rica	Alajuela	Estación Biológica Alberto Ml. Brenes, Reserva Biológica San Ramón	X	X	X	X	0
<i>Pristimantis cruentus</i>	AJC 0475	This study	Costa Rica	Cartago	Tapantí, Cantón Paraíso	X	X	X	X	X
<i>Pristimantis cruentus</i>	AJC 0480	This study	Costa Rica	Heredia	La Paz Waterfall Gardens	X	X	X	X	0
<i>Pristimantis cruentus</i>	AJC 0524	This study	Costa Rica	Limón	Base de Volcan Turrialba, along rd. Guácimo, Canton Guácimo	X	X	X	X	0
<i>Pristimantis cruentus</i>	AJC 1204	This study	Panamá	Panamá	Altos del María, Río María, Distrito de Chamé	0	X	X	0	0
<i>Pristimantis cruentus</i>	KRL 0685	This study	Panamá	Coclé	Parque Nacional Omar Torrijos H., El Copé				X	X
<i>Pristimantis cruentus</i> (big & plain)	AJC 0603	This study	Panamá	Darién	Caná, Pirre high camp	0	X	X	0	0
<i>Pristimantis cruentus</i> (cf. rojo tomate)	CH 6721	This study	Panamá	Panamá	Refugio ANAM, Cerro Brewster "hacia Cerro Guajará". Límite P.N. Chagres,	X	X	X	X	X
<i>Pristimantis cruentus</i> (golden venter)	AJC 0581	This study	Panamá	Darién	Caná, Pirre high camp, kitchen.	0	X	0	X	X

<i>Pristimantis cruentus</i> (museosus?)		AJC 1128	This study	Panamá	Panamá	Altos del María, ~7.5 km NE de El Valle de Anton, corregimiento de Chamé	X	X	X	0	X
<i>Pristimantis cruentus</i> (pop-eyed)		AJC 0985	This study	Panamá	Panamá	Burbayar Lodge, Km 15 on El Llano-Cartí Road, Distrito de Chepo, Corregimiento El Llano	0	0	0	0	X
<i>Pristimantis cruentus</i> (pop-eyed)		AJC 1133	This study	Panamá	Panamá	Altos del María, ~7.5 km NE de El Valle de Anton, corregimiento de Chamé	0	X	0	0	X
<i>Pristimantis cruralis</i>		MBH 5699	This study	Bolivia			0	0	0	0	X
<i>Pristimantis elegans</i>	E-53	NRPS0052	This study	Colombia	Cundinamarca	Parque Nacional Natural Chingaza	0	0	0	0	X
<i>Pristimantis fenestratus</i>		MBH 5712	This study	Bolivia			0	0	0	0	X
<i>Pristimantis gaigeae</i>	E-32	AJC 1360	This study	Colombia	Tolima	Ruinas Falan	X	0	0	0	X
<i>Pristimantis gaigeae</i>	E-79	AJC 1339	This study	Colombia	Antioquia	Maceo	X	X	0	0	X
<i>Pristimantis gaigeae</i>		CH 6471	This study				X	X	X	0	X
<i>Pristimantis gaigeae</i>		KRL 8880	This study	Panamá	Coclé	Parque Nacional Omar Torrijos H., El Copé	0	0	X	0	X
<i>Pristimantis grupo diastema</i>	E-75	AJC 1335	This study	Colombia	Antioquia	Maceo	0	X	X	X	X
<i>Pristimantis latidiscus</i> (morfol)	E-54	NRPS0054	This study	Colombia	Valle del Cauca	El Cairo	X	X	X	X	X
<i>Pristimantis latidiscus</i> (morfol)	E-56	NRPS0056	This study	Colombia	Valle del Cauca	El Cairo	X	X	X	X	X
<i>Pristimantis librarius</i>	E-6	QCAZ 25852	This study	Ecuador	Napo		X	X	X	0	0
<i>Pristimantis cruentus</i>	E-80	AJC 1344	This study	Colombia	Antioquia	Maceo	X	X	X	0	0
<i>Pristimantis martiae</i>	E-5	QCAZ 17998	This study	Ecuador	Napo		X	X	X	X	X
<i>Pristimantis martiae</i>	E-9	QCAZ 18018	This study	Ecuador	Napo		X	X	0	X	X
<i>Pristimantis medemi</i>	E-44	NRPS0087	This study	Colombia	Villavicencio	Restrepo	X	X	0	X	X
<i>Pristimantis mercedesae</i>		MBH 4695	This study	Bolivia	Chuquisaca	Vecindad de El Palmar	0	0	0	X	X
<i>Pristimantis miyatai</i>	E-11	RC 610	This study	Colombia	Santander	Floridablanca	X	X	X	X	X

<i>Pristimantis miyatai</i>	E-12	RC 613	This study	Colombia	Santander	Floridablanca	X	X	X	X	X
<i>Pristimantis sp. (morfo1)</i>	E-42	NRPS0083	This study	Colombia	Villavicencio		X	X	X	X	X
<i>Pristimantis sp. (morfo2)</i>	E-43	NRPS0085	This study	Colombia	Villavicencio	Restrepo	X	X	X	X	X
<i>Pristimantis sp. (morfo3)</i>	E-45	NRPS0090	This study	Colombia	Villavicencio	Restrepo	X	X	X	X	X
<i>Pristimantis moro</i>		AJC 1753	This study	Panamá	Panamá	Urbanizacion de los Altos de Cerro Azul, Distrito de Chilibre	X	X	X	0	0
<i>Pristimantis moro</i>		AJC 1860	This study	Panamá	Darién	Serrania de Pirre	X	X	X	0	X
<i>Pristimantis museosus</i>		AJC 1143	This study	Panamá	Panamá	Altos del María, ~7.5 km NE de El Valle de Anton, corregimiento de Chamé	0	0	0	X	X
<i>Pristimantis museosus</i>		AJC 1210	This study	Panamá	Panamá	Altos del María, Río María, Distrito de Chamé	X	X	0	0	X
<i>Pristimantis nervicus</i>	E-52	NRPS0048	This study	Colombia	Cundinamarca	Parque Nacional Natural Chingaza	X	X	0	0	X
<i>Pristimantis ockendeni</i>	E-4	QCAZ 25766	This study	Ecuador	Napo		X	X	0	X	X
<i>Pristimantis ockendeni</i>	E-10	QCAZ 25428	This study	Ecuador	Orellana		X	X	0	0	X
<i>Pristimantis paisa</i>	E-60	NRPS0060	This study	Colombia	Antioquia	Caldas	X	X	0	0	X
<i>Pristimantis paisa</i>	E-72	NRPS0062	This study	Colombia	Antioquia	Caldas	0	0	0	0	0
<i>Pristimantis pardalis</i>		AJC 0188	This study	Panamá	Chiriquí	Fortuna	X	X	0	X	X
<i>Pristimantis pardalis</i>		AJC 1208	This study	Panamá	Panamá	Altos del María, Río María, Distrito de Chamé	X	X	0	X	X
<i>Pristimantis pardalis</i>		CH 6284	This study	Panamá	Darién	Tent camp, Reserva Natural Privada Chucanti, Corregimiento Río Congo Arriba, Distrito de Chepigana	X	X	0	X	X
<i>Pristimantis pardalis</i>		KRL 0690	This study	Panamá	Coclé	Parque Nacional Omar Torrijos H., El Copé	X	X	0	X	X
<i>Pristimantis pirrensis</i>		AJC 0594	This study	Panamá	Darién	Caná, Pirre high camp	X	X	X	X	X

<i>Pristimantis pirrensis</i>		AJC 1863	This study	Panamá	Darién	Serrania de Pirre	X	X	X	X	X
<i>Pristimantis platydactylus</i>		MBH 5746	This study	Bolivia			X	X	X	X	X
<i>Pristimantis ptochus</i>	E-58	NRPS0058	This study	Colombia	Valle del Cauca	El Cairo	X	X	X	0	X
<i>Pristimantis quaquaversus</i>	E-7	QCAZ 16150	This study	Ecuador	Sucumbios		X	X	X	X	X
<i>Pristimantis quaquaversus</i>	E-8	QCAZ 25676	This study	Ecuador	Pastaza		X	X	X	X	X
<i>Pristimantis rhabdolaemus</i>		MBH 5589	This study	Bolivia			0	0	X	0	0
<i>Pristimantis ridens</i>	E-31	AJC 1359	This study	Colombia	Tolima	Ilanito, cerca a Falan	0	0	X	0	0
<i>Pristimantis ridens</i>	E-71	AJC 1373	This study	Colombia	Tolima		0	0	X	0	0
<i>Pristimantis ridens</i>		AJC 0126	This study	Costa Rica	Puntarenas	Rio Claro, lado Pacifico	X	X	X	X	X
<i>Pristimantis ridens</i>		AJC 0216	This study	Panamá	Panamá	Altos de Campana	X	X	X	X	X
<i>Pristimantis ridens</i>		AJC 0336	This study	Panamá	Panamá	Altos de Campana National Park (Sendero de Intepretación)	X	X	0	X	X
<i>Pristimantis ridens</i>		AJC 0356	This study	Costa Rica	Limón	"Utopia" En el borde Occidental del Rio Blanco, 3.2 km (~ 6 km W de Guápiles)	X	X	X	X	X
<i>Pristimantis ridens</i>		AJC 0522	This study	Costa Rica	Heredia	S. Holdridge, EB La Selva, Puerto Viejo, Sarapiquí	X	X	0	X	X
<i>Pristimantis ridens</i>		DL 401	This study	Costa Rica	San José	Parque Nacional Carara, Bajo Carara.	X	X	X	X	X
<i>Pristimantis ridens</i>		ENS 10647	This study	Honduras	Olancho	Sierra de Bataderos	0	0	X	0	0
<i>Pristimantis ridens</i>		ENS 10722	This study	Honduras	Olancho	Sierra de Agalta	X	X	0	X	X
<i>Pristimantis ridens (cf. molinoi)</i>		AJC 0211	This study	Panamá	Kuna Yala	Nusagandi	X	X	X	X	X
<i>Pristimantis signifer</i>	E-55	NRPS0055	This study	Colombia	Valle del Cauca	El Cairo	X	X	0	0	X
<i>Pristimantis sp</i>	E-18	EMM-232	This study	Antioquia	San Rafael	Sector Papayos, Central Hidroeléctrica Jaguas	X	X	X	X	X
<i>Pristimantis sp</i>	E-21	EMM-233	This study	Colombia	Antioquia	San Rafael	X	X	X	X	X

<i>Pristimantis sp</i>	E-22	EMM-247	This study	Colombia	Antioquia	San Rafael	X	X	X	X	X
<i>Pristimantis sp</i>	E-26	EMM/	This study	Colombia	Antioquia	San Rafael	0	X	X	X	X
<i>Pristimantis sp</i>	E-46	NRPS0016	This study	Colombia	Cundinamarca	Yacopi	X	X	X	X	X
<i>Pristimantis sp</i>	E-47	NRPS0017	This study	Colombia	Cundinamarca	Yacopi	X	X	X	X	X
<i>Pristimantis sp</i>	E-48	NRPS0019	This study	Colombia	Cundinamarca	Yacopi	X	X	X	0	X
<i>Pristimantis sp</i>	E-49	NRPS0022	This study	Colombia	Cundinamarca	Yacopi	X	0	X	X	X
<i>Pristimantis sp</i>	E-57	NRPS0057	This study	Colombia	Valle del Cauca	El Cairo	X	X	X	X	X
<i>Pristimantis sp</i>	E-59	NRPS0059	This study	Colombia	Valle del Cauca	El Cairo	X	X	X	X	X
<i>Pristimantis sp</i>	E-61	NRPS0067	This study	Colombia	Antioquia	Caldas	X	X	0	X	X
<i>Pristimantis sp</i>	E-62	NRPS0001	This study	Colombia	Antioquia	Anori	X	0	X	X	X
<i>Pristimantis sp</i>	E-63	NRPS0002	This study	Colombia	Antioquia	Anori	X	X	X	X	X
<i>Pristimantis sp</i>	E-64	NRPS0007	This study	Colombia	Antioquia	Anori	X	X	X	X	X
<i>Pristimantis sp</i>	E-65	NRPS0010	This study	Colombia	Antioquia	Amalfi	X	X	0	X	X
<i>Pristimantis sp</i>	E-66	NRPS0011	This study	Colombia	Antioquia	Amalfi	X	X	X	0	0
<i>Pristimantis sp</i>	E-67	NRPS0013	This study	Colombia	Antioquia	Amalfi	0	X	X	0	0
<i>Pristimantis sp</i>	E-68	NRPS0016	This study	Colombia	Antioquia	Amalfi	X	0	X	0	0
<i>Pristimantis sp</i>	E-69	NRPS0017	This study	Colombia	Antioquia	Amalfi	0	X	X	X	X
<i>Pristimantis sp</i>	E-70	NRPS0019	This study	Colombia	Antioquia	Amalfi	0	X	X	0	X
<i>Pristimantis sp</i>	E-73	NRPS0072	This study	Colombia	Antioquia	Urrao	X	X	X	0	X
<i>Pristimantis sp.</i>		AJC 1670	This study	Panamá	Darién	Tent camp, Reserva Natural Privada Chucanti, Corregimiento Rio Congo Arriba, Distrito de Chepigana	0	0	X	X	X
<i>Pristimantis sp. (cf. cruentus)</i>		AJC 0217	This study	Panamá	Chiriquí		X	X	0	X	X
<i>Pristimantis sp. (cf. ridens)</i>	E-74	AJC 1191	This study	Colombia	Chocó	Nuqui	X	X	X	X	X
<i>Pristimantis suetus</i>	E-3	MHUA 0440	This study	Colombia	Antioquia	Guatapé	X	X	X	0	0
<i>Pristimantis taeniatus</i>	E-1	MHUA 0426	This study	Colombia	Antioquia	Maceo	0	X	X	X	0
<i>Pristimantis taeniatus</i>	E-2	MHUA 0455	This study	Colombia	Antioquia	Amalfi	X	X	X	0	0

<i>Pristimantis taeniatus</i>	E-13	CJD 069	This study	Colombia	Santander		X	X	X	0	X
<i>Pristimantis taeniatus</i>	E-14	SMG 325	This study	Colombia	Antioquia	San Rafael	X	X	0	X	0
<i>Pristimantis taeniatus</i>	E-16	CJD-070	This study	Colombia	Santander	Floridablanca	X	X	X	X	X
<i>Pristimantis taeniatus</i>	E-19	CJD-068	This study	Colombia	Santander		X	X	X	X	X
<i>Pristimantis taeniatus</i>	E-23	CJD-067	This study	Colombia	Santander		X	X	0	X	X
<i>Pristimantis taeniatus</i>	E-24	CJD-071	This study	Colombia	Santander		X	X	X	0	X
<i>Pristimantis taeniatus</i>	E-29	AJC 1353	This study	Colombia	Tolima	Falan, no ruins	X	0	X	X	0
<i>Pristimantis taeniatus</i>	E-33	AJC 1363	This study	Colombia	Tolima	Falan, no ruins	X	X	X	0	X
<i>Pristimantis taeniatus</i>	E-34	AJC 1368	This study	Colombia	Tolima	Falan, no ruins	X	X	X	X	X
<i>Pristimantis taeniatus</i>	E-35	AJC 1372	This study	Colombia	Tolima	Ruinas Falan	X	X	X	X	X
<i>Pristimantis taeniatus</i>	E-36	RC 611	This study	Colombia	Santander	Floridablanca	X	0	X	X	0
<i>Pristimantis taeniatus</i>	E-37	RC 612	This study	Colombia	Santander	Floridablanca	X	0	X	X	X
<i>Pristimantis taeniatus</i>		AJC 1126	This study	Panamá	Colón	Occidente de Gatun	X	X	X	X	X
<i>Pristimantis taeniatus</i>		AJC 1683	This study	Panamá	Darién	Cana, Campo Principal	X	X	X	X	X
<i>Pristimantis taeniatus</i>		AJC 1782	This study	Panamá	Panamá	Urbanización de los Altos de Cerro Azul, Distrito de Chilibre	X	X	0	X	X
<i>Pristimantis taeniatus</i>		AJC 1839	This study	Panamá	Darién	Cana, Sendero Mina	X	X	X	X	X
<i>Pristimantis taeniatus</i>		AJC 1961	This study	Panamá	Panamá	Refugio ANAM, Cerro Brewster "hacia Cerro Guajará". Límite P.N. Chagres,	X	X	X	0	0
<i>Pristimantis taeniatus</i>		AJC 1977	This study	Panamá	Panamá	Estación Río Chico de la ACP, Río Chagres norte, arriba del lago Alajuela. Corregimiento de Chilibre, Distrito de Panamá.	X	X	0	0	0

<i>Pristimantis taeniatus</i>		AJC 1993	This study	Panamá	Panamá	Estación Río Chico de la ACP, Río Chagres sur, arriba del lago Alajuela. Corregimiento de Chilibre, Distrito de Panamá.	X	X	X	X	X
<i>Pristimantis taeniatus</i>		AJC 1994	This study	Panamá	Panamá	Estación Río Chico de la ACP, Río Chagres sur, arriba del lago Alajuela. Corregimiento de Chilibre, Distrito de Panamá.	X	X	0	0	0
<i>Pristimantis taeniatus</i>		AJC 1995	This study	Panamá	Panamá	Estación Río Chico de la ACP, Río Chagres sur, arriba del lago Alajuela. Corregimiento de Chilibre, Distrito de Panamá.	X	X	X	X	X
<i>Pristimantis taeniatus</i>		CH 4945	This study	Panamá	Panamá		X	X	X	X	X
<i>Pristimantis taeniatus</i>		CH 4999	This study	Panamá	Panamá		X	X	X	X	X
<i>Pristimantis taeniatus</i>		CH 5067	This study	Panamá	Panamá		X	X	X	X	X
<i>Pristimantis taeniatus</i>		CH 5585	This study	Panamá	Panamá		X	X	X	X	X
<i>Pristimantis taeniatus</i>		CH 6796	This study	Panamá	Panamá	Urbanización de los Altos de Cerro Azul, Distrito de Chilibre	X	X	X	X	X
<i>Pristimantis taeniatus</i> (cf. <i>P. quidditus</i>)		AJC 1835	This study	Panamá	Darién	Caná, Sendero Mina	X	X	X	X	X
<i>Pristimantis taeniatus</i> (cf. <i>P. diastema</i>)		CH 6393	This study	Panamá	Darién	Serranía de Pirre	0	X	X	X	X
<i>Pristimantis viejas</i>	E-15	EMM-250	This study	Colombia	Antioquia	San Rafael	X	X	X	X	X
<i>Pristimantis viejas</i>	E-17	EMM-248	This study	Colombia	Antioquia	San Rafael	X	0	X	0	0
<i>Pristimantis viejas</i>	E-20	EMM-249	This study	Colombia	Antioquia	San Rafael	X	0	X	0	0
<i>Pristimantis viejas</i>	E-25	SMG 326	This study	Colombia	Antioquia	San Rafael	X	0	X	0	0

<i>Pristimantis viejas</i>	E-28	AJC 1352	This study	Colombia	Tolima	Falan, no ruins	X	0	X	0	0
<i>Pristimantis viejas</i>	E-30	AJC 1355	This study	Colombia	Tolima	Falan, no ruins	X	0	X	0	0
<i>Pristimantis zophus</i>	E-39	NRPS0071	This study	Colombia	Antioquia	Urrao	X	X	X	X	X
<i>Pristimantis zophus</i>	E-38	NRPS0070	This study	Colombia	Antioquia	Urrao	X	X	X	X	X
<i>Pristimantis zophus</i>	E-40	NRPS0074	This study	Colombia	Antioquia	Urrao	X	X	0	0	0
<i>Pristimantis zophus</i>	E-41	NRPS0076	This study	Colombia	Antioquia	Urrao	X	X	0	0	0

Appendix 2.

GenBank accession numbers for downloaded sequences. All sequences were obtained from GenBank.

Species	Extraction voucher	Museum voucher	Mitochondrial genes			Nuclear genes		Origin
			COIbp	12S	bp16S	Rag_1	Tyr	
<i>Pristimantis pluvicanorus</i>		AMNH-A 165195	n/a	AY843586		n/a	AY844035	Faivovich et al., 2005
<i>Pristimantis pluvicanorus</i>	n/a	n/a	n/a	AY843586		n/a	n/a	Faivovich et al., 2005
<i>Pristimantis malkini</i>	267642	QCAZ28296	n/a	EU186663		n/a	n/a	Hedges et al., 2008
<i>Pristimantis terraebolivaris</i>	102301	n/a	n/a	EU186650		n/a	n/a	Hedges et al., 2008
<i>Pristimantis zeuctotylus</i>	268013	ROM43978	n/a	EU186678		n/a	n/a	Hedges et al., 2008
<i>Pristimantis galdi</i>	267975	QCAZ32368	n/a	EU186670		EU186746	EU186767	Hedges et al., 2008
<i>Pristimantis cf_mendax</i>	267140	MTD45080	n/a	EU186659		n/a	n/a	Hedges et al., 2008
<i>Pristimantis simonsii</i>	267961	KU212350	n/a	EU186665		n/a	n/a	Hedges et al., 2008
<i>Pristimantis albertus</i>	171100	KU291675	n/a	EU186695		n/a	n/a	Hedges et al., 2008
<i>Pristimantis cf_rhabdolaemus</i>	267143	MTD45073	n/a	EU186660		n/a	n/a	Hedges et al., 2008
<i>Pristimantis altamazonicus</i>	267204	KU215460	n/a	EF493670		EF493441	EU186778	Hedges et al., 2008
<i>Pristimantis ardalonychus</i>	267959	KU212301	n/a	EU186664		n/a	n/a	Hedges et al., 2008
<i>Pristimantis caryophyllaceus</i>	268029	MVZ203810	n/a	EU186686		n/a	n/a	Hedges et al., 2008

<i>Pristimantis crucifer</i>	268105	KU177733	n/a	EU186736	EU186718	n/a	n/a	Hedges et al., 2008
<i>Pristimantis cruciocularis</i>	171097	KU291673	n/a	EU186656		n/a	n/a	Hedges et al., 2008
<i>Pristimantis diadematus</i>	267967	KU221999	n/a	EU186668		n/a	n/a	Hedges et al., 2008
<i>Pristimantis eriphus</i>	267976	QCAZ32705	n/a	EU186671		n/a	n/a	Hedges et al., 2008
<i>Pristimantis inguinalis</i>	268010	ROM40164	n/a	EU186676		n/a	n/a	Hedges et al., 2008
<i>Pristimantis jester</i>	268091	ROM43302	n/a	EU186734	EU186716	n/a	n/a	Hedges et al., 2008
<i>Pristimantis marmoratus</i>	268090	ROM43913	n/a	EU186692		n/a	n/a	Hedges et al., 2008
<i>Pristimantis minutulus</i>	171117	KU291677	n/a	EU186657		n/a	n/a	Hedges et al., 2008
<i>Pristimantis prolatus</i>	268107	KU177433	n/a	EU186701		n/a	n/a	Hedges et al., 2008
<i>Pristimantis rhabdocnemus</i>	171063	KU291651	n/a	EU186724	EU186706	n/a	n/a	Hedges et al., 2008
<i>Pristimantis saltissimus</i>	268092	ROM43310	n/a	EU186693		n/a	n/a	Hedges et al., 2008
<i>Pristimantis dendrobatoides</i>	268093	ROM43318	n/a	EU186735	EU186717	n/a	n/a	Hedges et al., 2008
<i>Pristimantis pulvinatus</i>	268114	KU181015	n/a	EU186741	EU186723	n/a	n/a	Hedges et al., 2008
<i>Pristimantis chalconus</i>	267865	KU177638	n/a	EF493675		n/a	n/a	Heinicke et al., 2007
<i>Pristimantis bipunctatus</i>	171021	KU291638	n/a	EF493702			EF493492	Heinicke et al., 2007
<i>Pristimantis caprifer</i>	267880	KU177680	n/a	EF493391		n/a	n/a	Heinicke et al., 2007
<i>Pristimantis citriogaster</i>	267201	KU212278	n/a	EF493700		n/a	n/a	Heinicke et al., 2007
<i>Pristimantis condor</i>	267212	KU217857	n/a	EF493701		EF493443	EF493504	Heinicke et al., 2007
<i>Pristimantis conspicillatus</i>	267636	QCAZ28448	n/a	EF493529		EF493437	EF493499	Heinicke et al., 2007

<i>Pristimantis fenestratus</i>	266046	MHNSM9298	n/a	EF493703	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis lymani</i>	267220	KU218019	n/a	EF493392	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis skydmainos</i>	266052	MHNSM10071	n/a	EF493393	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis buckleyi</i>	267210	KU217836	n/a	EF493350	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis cryophilus</i>	267214	KU217863	n/a	EF493672	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis curtipes</i>	267215	KU217871	n/a	EF493513	EF493435	EF493497	Heinicke et al., 2007	
<i>Pristimantis gentryi</i>	267230	KU218109	n/a	EF493511	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis appendiculatus</i>	267866	KU177637	n/a	EF493524	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis devillei</i>	267216	KU217991	n/a	EF493688	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis quinquesimus</i>	267872	KU179374	n/a	EF493690	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis truebae</i>	267229	KU218013	n/a	EF493512	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis vertebralis</i>	267870	KU177972	n/a	EF493689	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis ockendeni</i>	267253	KU222023	n/a	EF493519	EF493434	EF493496	267254	Heinicke et al., 2007
<i>Pristimantis bromeliaceus</i>	171051	KU291702	n/a	EF493351	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis schultei</i>	267199	KU212220	n/a	EF493681	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis festae</i>	267247	KU218234	n/a	EF493515	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis leoni</i>	267437	KU218227	n/a	EF493684	EF493433	EF493495	Heinicke et al., 2007	
<i>Pristimantis ocreatus</i>	267439	KU208508	n/a	EF493682	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis pyrhomerus</i>	267441	KU218030	n/a	EF493683	n/a	n/a	Heinicke et al., 2007	

<i>Pristimantis orcesi</i>	267221	KU218021	n/a	EF493679	n/a	n/a	Heinicke et al., 2007
<i>Pristimantis thymelensis</i>	267644	QCAZ16428	n/a	EF493516	EF493442	EF493503	Heinicke et al., 2007
<i>Pristimantis melanogaster</i>	267438	MHNSM-WED56846	n/a	EF493826	EF493664	n/a	Heinicke et al., 2007
<i>Pristimantis orestes</i>	267249	KU218257	n/a	EF493388	n/a	n/a	Heinicke et al., 2007
<i>Pristimantis simonbolivari</i>	267248	KU218254	n/a	EF493671	n/a	n/a	Heinicke et al., 2007
<i>Pristimantis aniptopalmodus</i>	171070	KU291627	n/a	EF493390	n/a	n/a	Heinicke et al., 2007
<i>Pristimantis peruvianus</i>	266050	MHNSM9267	n/a	EF493707	EF493436	EF493498	Heinicke et al., 2007
<i>Pristimantis rhabdolaemus</i>	267875	KU173492	n/a	EF493706	n/a	n/a	Heinicke et al., 2007
<i>Pristimantis sagittulus</i>	171098	KU291635	n/a	EF493705	EF493439	EF493501	Heinicke et al., 2007
<i>Pristimantis stictogaster</i>	171080	KU291659	n/a	EF493704	EF493445	EF493506	Heinicke et al., 2007
<i>Pristimantis toftae</i>	267206	KU215493	n/a	EF493353	n/a	n/a	Heinicke et al., 2007
<i>Pristimantis colomai</i>	267635	QCAZ17101	n/a	EF493354	EF493440	EF493502	Heinicke et al., 2007
<i>Pristimantis cremnobates</i>	267878	KU177252	n/a	EF493528	EF493424	EF493486	Heinicke et al., 2007
<i>Pristimantis cruentus</i>	267876	AMNH12444-448	n/a	EF493697	n/a	n/a	Heinicke et al., 2007
<i>Pristimantis latidiscus</i>	267219	KU218016	n/a	EF493698	n/a	n/a	Heinicke et al., 2007
<i>Pristimantis ridens</i>	267877	AMNH-A124551	n/a	EF493355	n/a	n/a	Heinicke et al., 2007
<i>Pristimantis achatinus</i>	267208	KU217809	n/a	EF493827	EF493660	n/a	Heinicke et al., 2007
<i>Pristimantis actites</i>	267209	KU217830	n/a	EF493696	EF493432	EF493494	Heinicke et al., 2007
<i>Pristimantis crenunguis</i>	267879	KU177730	n/a	EF493693	EF493666	n/a	Heinicke et al., 2007

<i>Pristimantis labiosus</i>	267640	QCAZ19771	n/a	EF493694	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis lanthanites</i>	267252	KU222001	n/a	EF493695	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis w-nigrum</i>	n/a	n/a	n/a	AY326004	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis duellmani</i>	267444	KU217998	n/a	n/a	n/a	EF493438	EF493500	Heinicke et al., 2007
<i>Pristimantis duellmani</i>	n/a	n/a	n/a	AY326003	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis surdus</i>	267871	KU177847	n/a	EF493687	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis acerus</i>	267207	KU217786	n/a	EF493678	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis cajamarcensis</i>	267211	KU217845	n/a	EF493823	EF493663	n/a	n/a	Heinicke et al., 2007
<i>Pristimantis calcarulatus</i>	267868	KU177658	n/a	EF493523	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis celator</i>	267874	KU177684	n/a	EF493685	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis ceuthospilus</i>	267198	KU212216	n/a	EF493520	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis chloronotus</i>	n/a	n/a	n/a	AY326007	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis croceinguinis</i>	267213	KU217862	n/a	EF493669	EF493665	n/a	n/a	Heinicke et al., 2007
<i>Pristimantis dissimulatus</i>	267867	KU179090	n/a	EF493522	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis euphronides</i>	266624	BWMC6918	n/a	EF493527	EF493427	EF493489	Heinicke et al., 2007	
<i>Pristimantis glandulosus</i>	267217	KU218002	n/a	EF493676	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis imitatrix</i>	267205	KU215476	n/a	EF493824	EF493667	n/a	n/a	Heinicke et al., 2007
<i>Pristimantis inusitatus</i>	267218	KU218015	n/a	EF493677	n/a	n/a	Heinicke et al., 2007	
<i>Pristimantis livellus</i>	267200	KU212226	n/a	EF493521	n/a	n/a	Heinicke et al., 2007	

<i>Pristimantis luteolateralis</i>	267863	KU177807	n/a	EF493517	n/a	n/a	Heinicke et al., 2007
<i>Pristimantis nyctophylax</i>	267869	KU177812	n/a	EF493526	EF493425	EF493487	Heinicke et al., 2007
<i>Pristimantis parvillus</i>	267864	KU177821	n/a	EF493351	n/a	n/a	Heinicke et al., 2007
<i>Pristimantis petrobardus</i>	267202	KU212293	n/a	EF493825	EF493367	n/a	Heinicke et al., 2007
<i>Pristimantis phoxocephalus</i>	267222	KU218025	n/a	EF493349	n/a	n/a	Heinicke et al., 2007
<i>Pristimantis pycnodermis</i>	267223	KU218028	n/a	EF493680	n/a	n/a	Heinicke et al., 2007
<i>Pristimantis rhodoplichus</i>	267250	KU219788	n/a	EF493674	n/a	n/a	Heinicke et al., 2007
<i>Pristimantis riveti</i>	267224	KU218035	n/a	EF493348	n/a	n/a	Heinicke et al., 2007
<i>Pristimantis rozei</i>	102308	Novoucher	n/a	EF493691	EF493429	EF493491	Heinicke et al., 2007
<i>Pristimantis shrevei</i>	266036	Novoucher	n/a	EF493692	n/a	n/a	Heinicke et al., 2007
<i>Pristimantis spinosus</i>	267225	KU218052	n/a	EF493673	n/a	n/a	Heinicke et al., 2007
<i>Pristimantis subsigillatus</i>	267246	KU218147	n/a	EF493525	n/a	n/a	Heinicke et al., 2007
<i>Pristimantis supernatis</i>	n/a	n/a	n/a	AY326005	n/a	n/a	Heinicke et al., 2007
<i>Pristimantis thymalopsoides</i>	267873	KU177861	n/a	EF493514	n/a	n/a	Heinicke et al., 2007
<i>Pristimantis unistrigatus</i>	267227	KU218057	n/a	EF493387	EF493444	EF493505	Heinicke et al., 2007
<i>Pristimantis urichi</i>	101646	USNM336098	n/a	EF493699	EF493426	EF493488	Heinicke et al., 2007
<i>Pristimantis verecundus</i>	267646	QCAZI2410	n/a	EF493686	n/a	n/a	Heinicke et al., 2007
<i>Pristimantis versicolor</i>	267228	KU218096	n/a	EF493389	EF493431	EF493493	Heinicke et al., 2007
<i>Pristimantis walkeri</i>	267231	KU218116	n/a	EF493518	EF493428	EF493490	Heinicke et al., 2007

<i>Pristimantis wiensi</i>	267251	KU219796	n/a	EF493377	EF493668	n/a	n/a	Heinicke et al., 2007
<i>Pristimantis festae</i>		KU218234	n/a	EF493515		n/a	n/a	Heinicke et al., 2007
<i>Agalychnis callidryas</i>	n/a	n/a	n/a	DQ283423		n/a	DQ283018	Heinicke et al., 2007
<i>Agalychnis callidryas</i>	n/a	n/a	n/a	n/a	n/a	EF493362	n/a	Heinicke et al., 2007
<i>Craugastor daryi</i>	267858	UTA57940	n/a	EF493531		EF493452	EF493480	Heinicke et al., 2007
<i>Craugastor longirostris</i>	267853	KU177803	n/a	EF493395		EF493454	EF493482	Heinicke et al., 2007
<i>Litoria caerulea</i>	n/a	n/a	AY883980	AY843692		n/a	AY844131	Heinicke et al., 2007
<i>Litoria caerulea</i>	267887	novoucher	n/a	n/a	n/a	EF493446	n/a	Heinicke et al., 2007
<i>Phrynopus auriculatus</i>	171082	KU291634	n/a	EF493708		n/a	n/a	Heinicke et al., 2007
<i>Phrynopus bracki</i>	171045	USNM286919	n/a	EF493709		EF493421	EF493507	Heinicke et al., 2007
<i>Rana catesbeiana</i>	n/a	n/a	n/a	DQ283257		n/a	DQ282959	Heinicke et al., 2007
<i>Rana catesbeiana</i>	266591	N0_voucher	n/a	n/a	n/a	EF493448	n/a	Heinicke et al., 2007
<i>Lynchius flavomaculatus</i>	267966	KU218210	n/a	EU186667		EU186745	EU186766	Hedges et al., 2008
<i>Lynchius nebulanastes</i>	268115	KU181408	n/a	EU186704		n/a	n/a	Hedges et al., 2008
<i>Oreobates cruralis</i>	267962	KU215462	n/a	EU186666		EU186743	EU186764	Hedges et al., 2008
<i>Oreobates saxatilis</i>	267960	KU212327	n/a	EU186726	EU186708	EU186742	EU186763	Hedges et al., 2008

FIGURES

Figure 1. Major clades of *Pristimantis* frogs. MP of 80 individuals of frogs including eight out-group species. Bootstrap supports are presented. The groups are named according to Hedges et al., (2008).

Figure 2. A time tree of *Pristimantis* frogs. The tree topology is derived from a Bayesian analysis of 80 taxa. Support values are indicated at nodes (ML/Bayesian posterior probability). Calibration nodes are indicated by an asterisk. Branches with blue color show the Central American species.

Figure 3. Maximum likelihood phylogeny of 265 individuals of frogs is presented. The data set consists of 4279 base pairs of aligned DNA sequences, including mitochondrial DNA (12S rRNA, 16S rRNA, and COI) and nuclear DNA (Rag-1 and Tyr genes). Posterior probabilities are shown. First segment (top) of tree. (B) Second segment of tree. (C) Third segment (bottom) of tree.

Figure 4. Maximum likelihood phylogeny of 265 individuals of frogs is presented. The data set consists of 4279 base pairs of aligned DNA sequences, including mitochondrial DNA (12S rRNA, 16S rRNA, and COI) and nuclear DNA (Rag-1 and Tyr genes). Posterior probabilities are shown. The terminal taxa names are collapsed. First segment (top) of tree. (B) Second segment of tree. (C) Third segment (bottom) of tree.

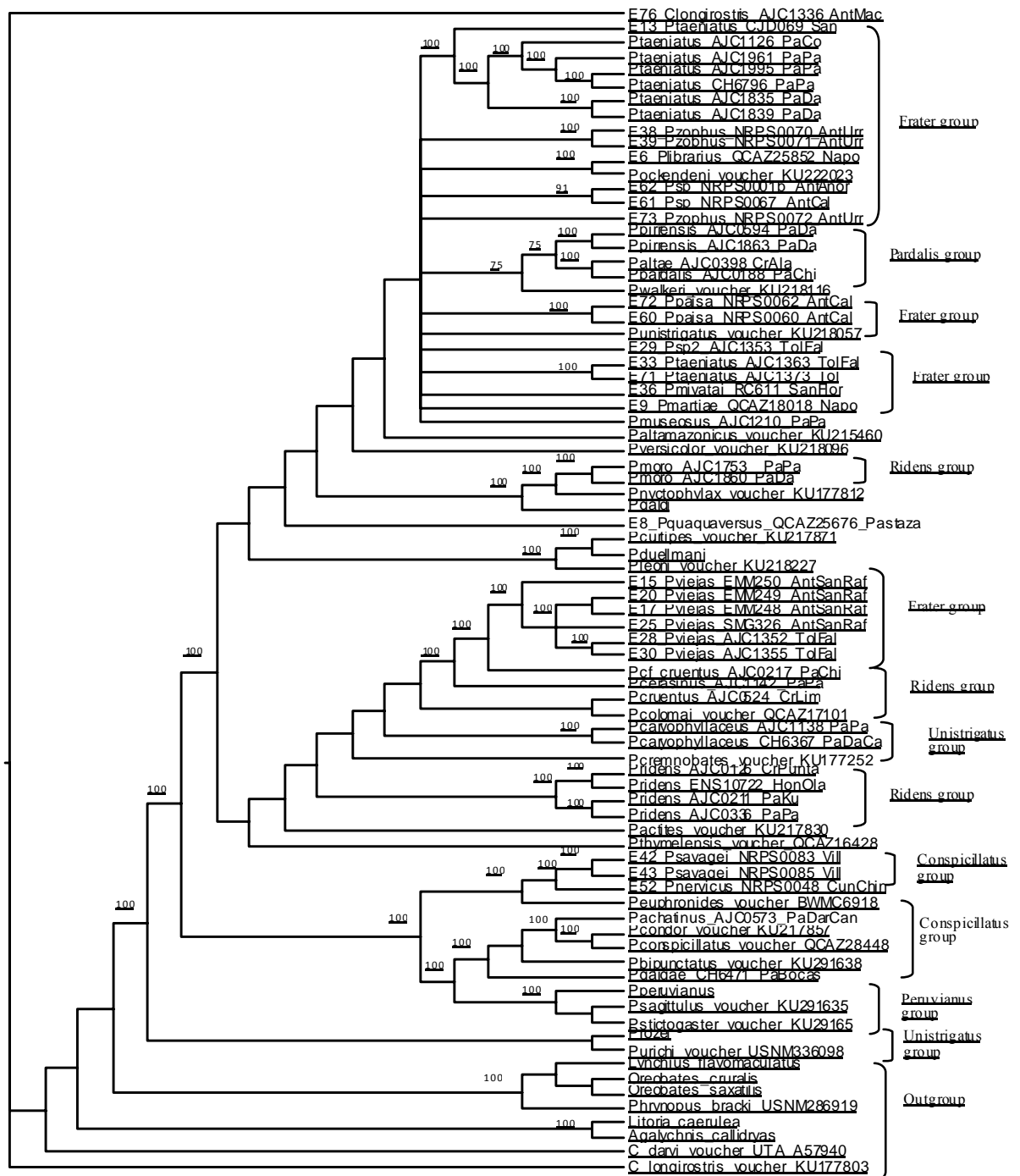


Figure 1.

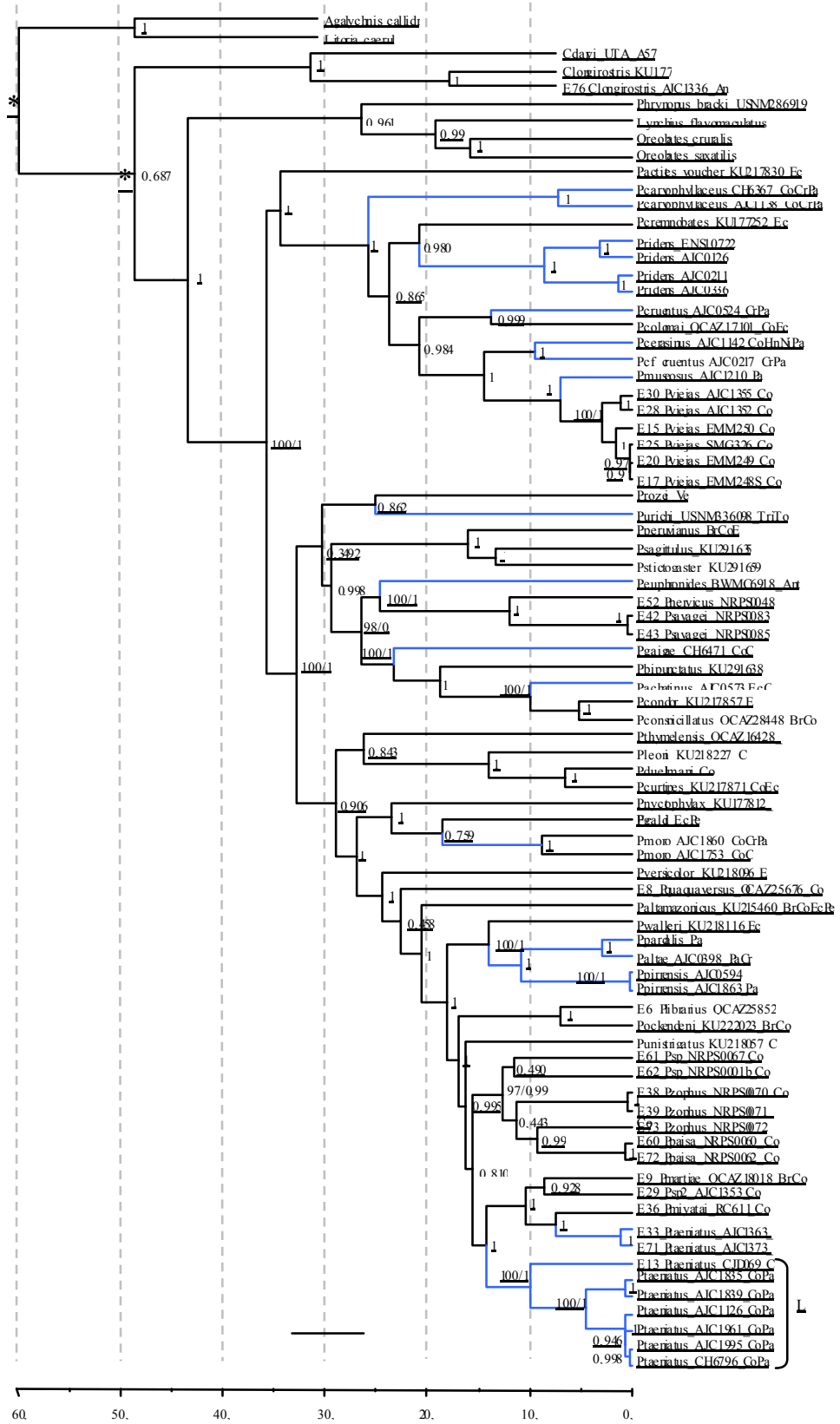


Figure2.

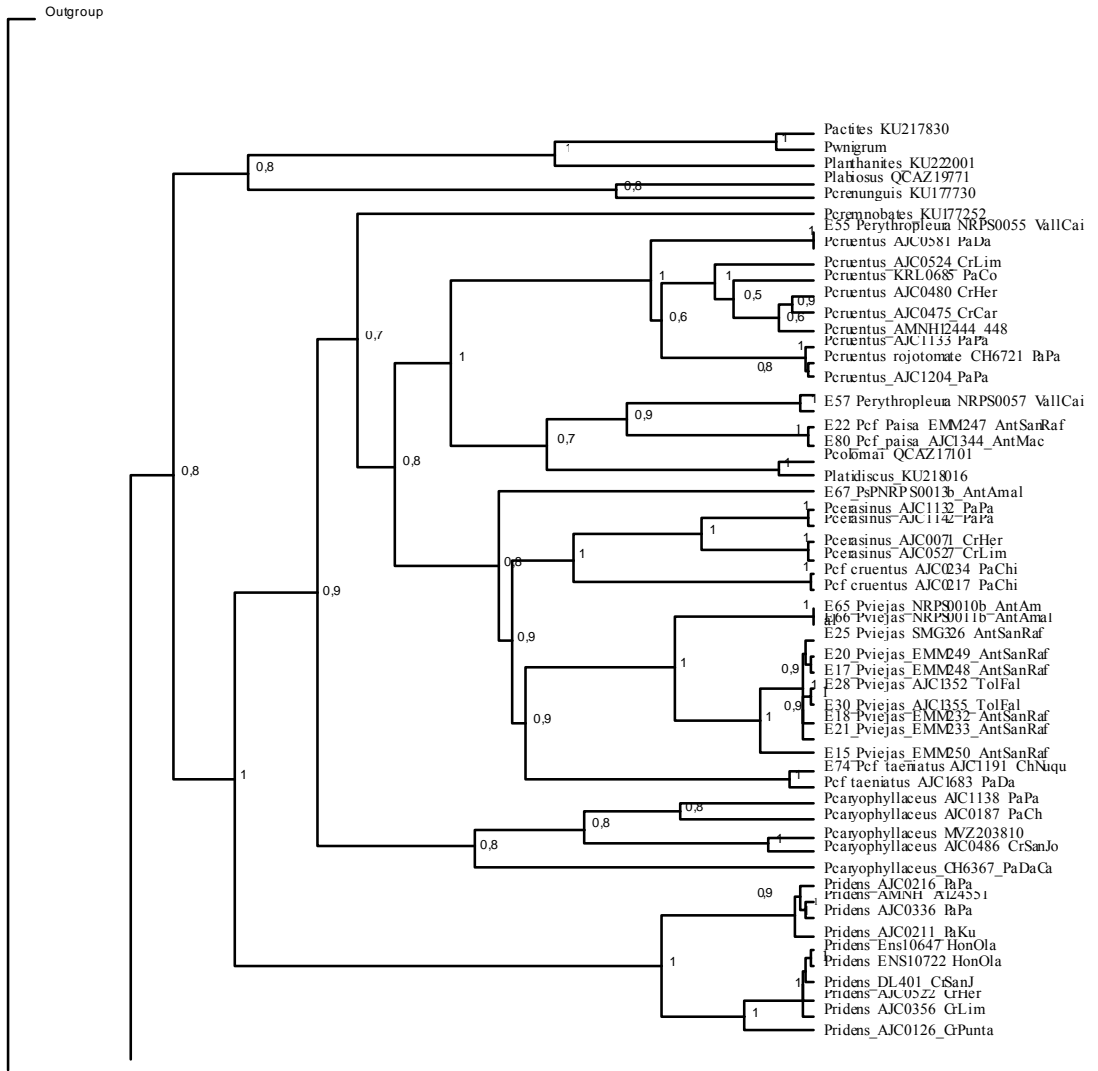


Figure3a.



Figure3b.

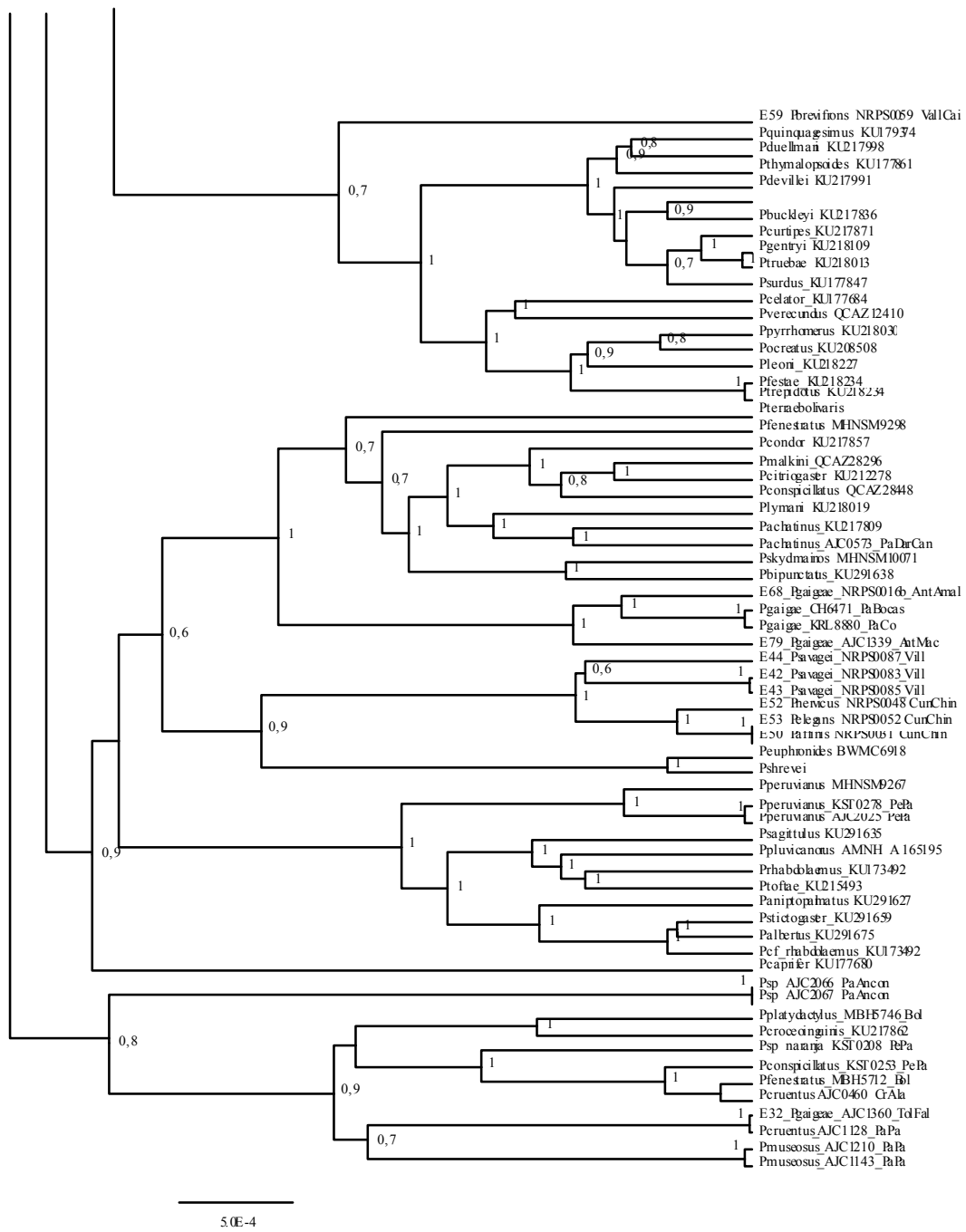


Figure3c.

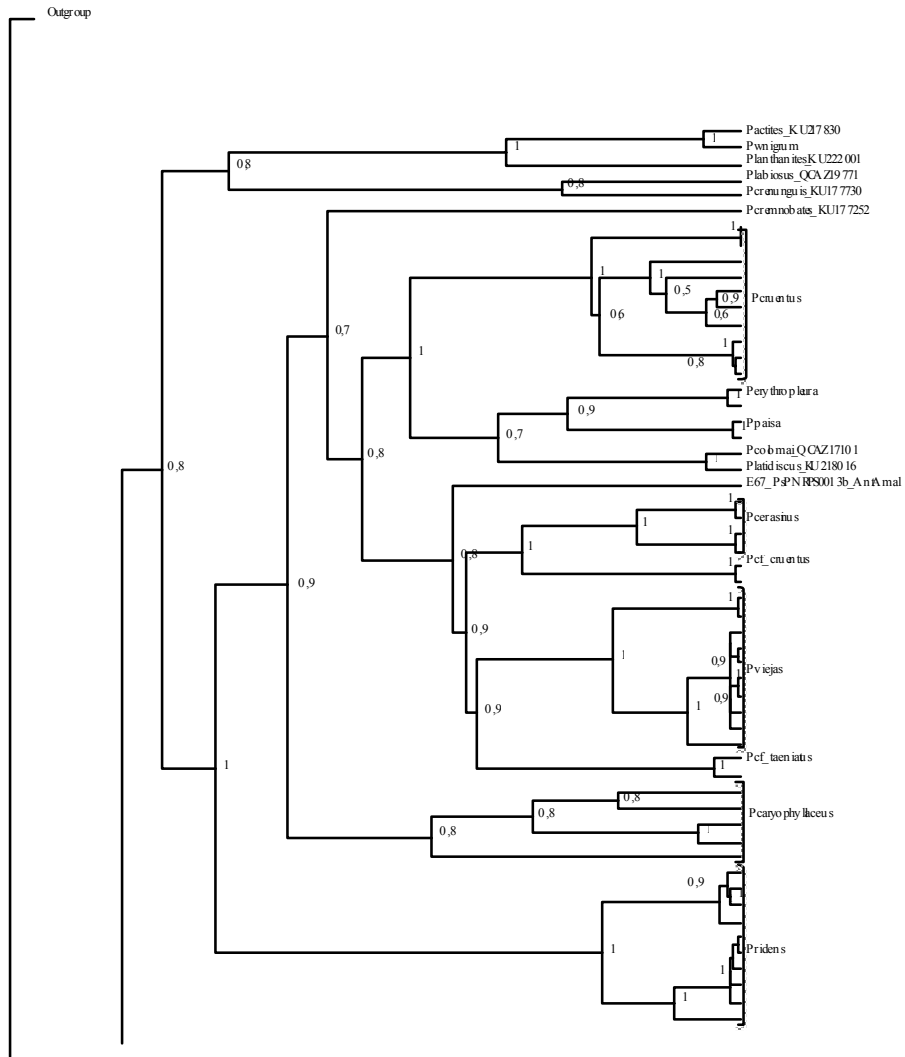


Figure4a.

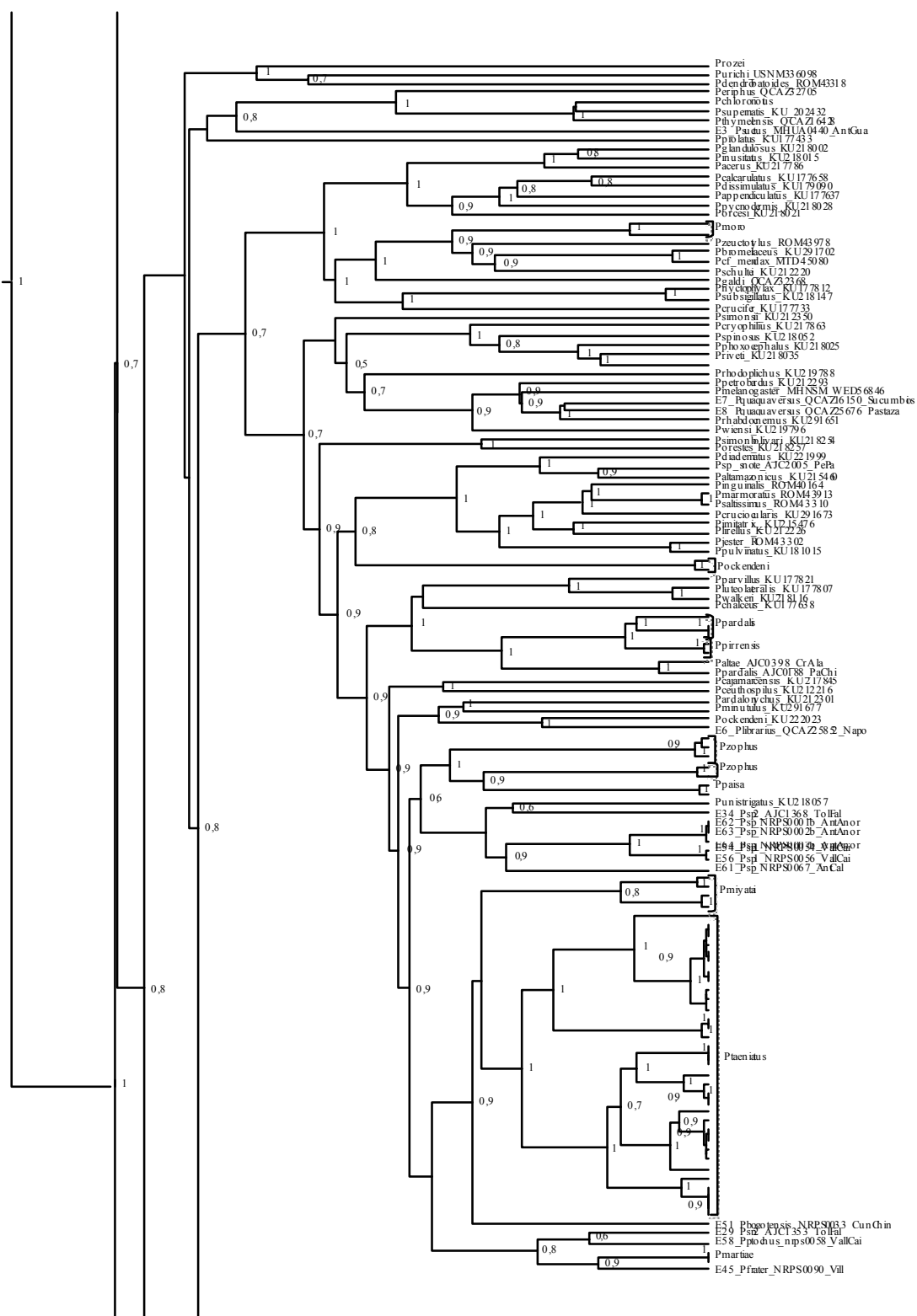


Figure4b.

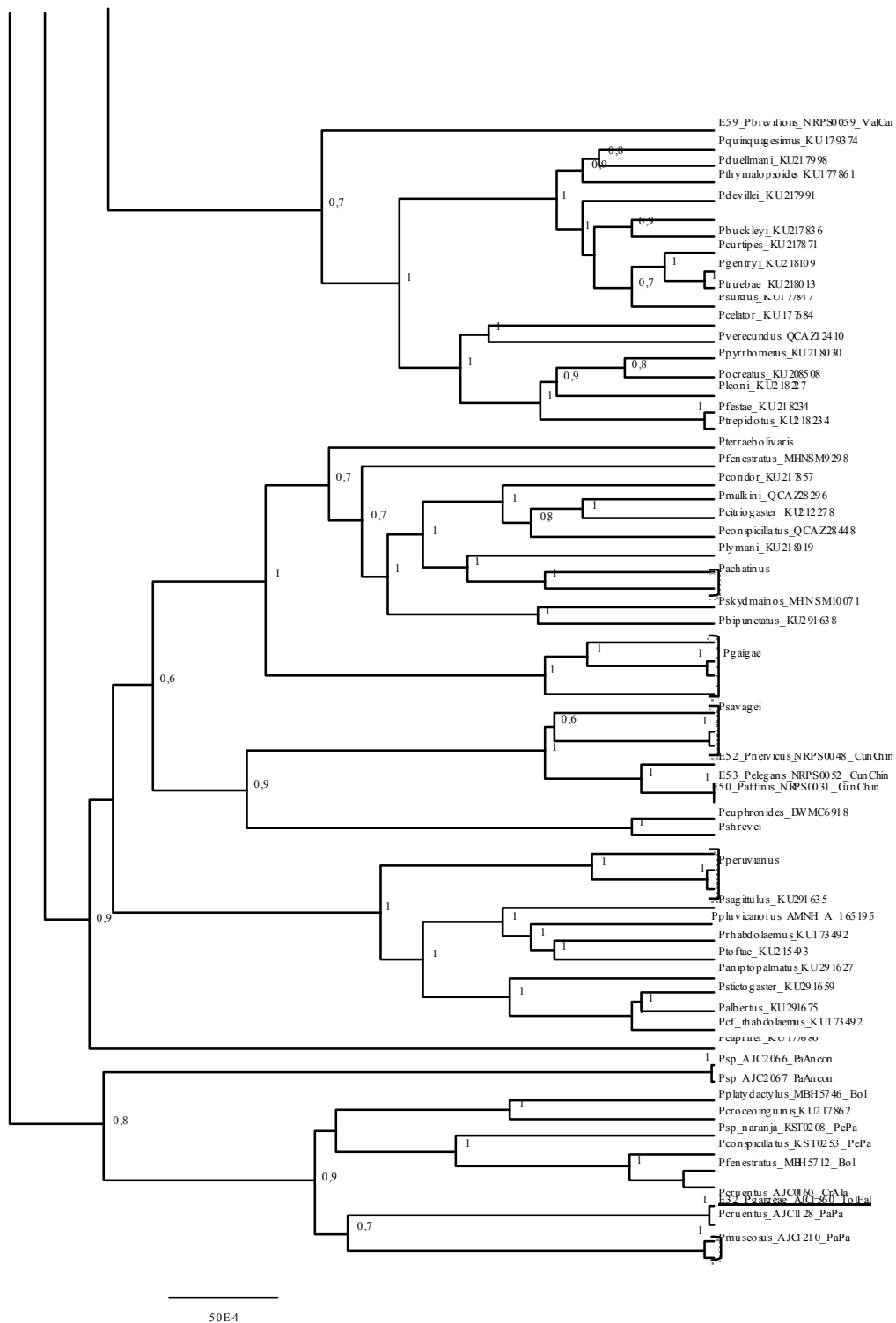


Figure4c.