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Phylogeny of the túngara frog genus *Engystomops* (=*Physalaemus pustulosus* species group; Anura: Leptodactylidae)

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Abstract

We present a phylogeny of the Neotropical genus *Engystomops* (=*Physalaemus pustulosus* species group) based on sequences of ~2.4 kb of mtDNA, (12S rRNA, valine-tRNA, and 16S rRNA) and propose a phylogenetic nomenclature. The phylogeny includes all described taxa and two unnamed species. All analyses indicate that *Engystomops* is monophyletic and contains two basal allopatric clades. Clade I (Edentulus) includes *E. pustulosus* and the Amazonian *E. petersi* + *E.* cf. *freibergi*. Clade II (Duovox) includes all species distributed in W Ecuador and NW Peru. Brevivox, a clade of small-sized species is strongly supported within Duovox. Populations of *Engystomops pustulosus* fall into two well-supported clades, each of which occupies two disjunct portions of the species range. Overall, our phylogeny is congruent with most previous hypotheses. This study is among the few published species-level phylogenies of Neotropical amphibians derived from molecular datasets. A review of the proportion of new species detected by similar studies suggests that the increasing use of molecular techniques will lead to the discovery of a vast number of species of Neotropical amphibians.
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1. Introduction

Physalaemus and *Engystomops* are closely related genera of frogs of the subfamily Leptodactylinae; until recently these were allocated into a single genus (*Physalaemus*) with 49 species (updated from Frost, 2004) and four species groups (Cannatella and Duellman, 1984; Lynch, 1970): *P. biligonigerus*, *P. cuvieri*, *P. pustulosus*, and *P. signifer* group. In a taxonomic review, Nascimento et al. (2005) resurrected the genus *Engystomops* for the species of the *P. pustulosus* group and defined seven species groups within *Physalaemus*. *Engystomops* is distributed from central Veracruz (Mexico) to the Amazon Basin and the lowlands of western Ecuador and NW Peru.

Engystomops has been a model system in studies of sexual selection and animal communication since the 1980s (e.g., Bosch et al., 2000; Cannatella et al., 1998; Ryan, 1983; Ryan and Drewes, 1990; Ryan and Rand, 1995; Tarano and Ryan, 2002; Wilczynski et al., 2001). The systematics of *Engystomops* was reviewed by Cannatella and Duellman (1984), who recognized four species and provided morphological evidence for the group's monophyly. Sister species status was established for (*E. petersi* + *E. pustulosus*) and (*E. coloradorum* + *E. pustulatus*). Ryan and Rand (1993) presented a phylogeny based on unpublished morphological characters, allozyme variation and 12S mtDNA sequences (pers. com. from D.C. Cannatella et al.; Fig. 1B). Their phylogeny differed from that implied by Cannatella and Duellman (1984) in placing *E. pustulosus* as sister taxon to the remaining species instead of to *E. petersi*. Cannatella et al. (1998) included two additional species and analyzed morphology, behavior, allozyme variation and 12S rRNA

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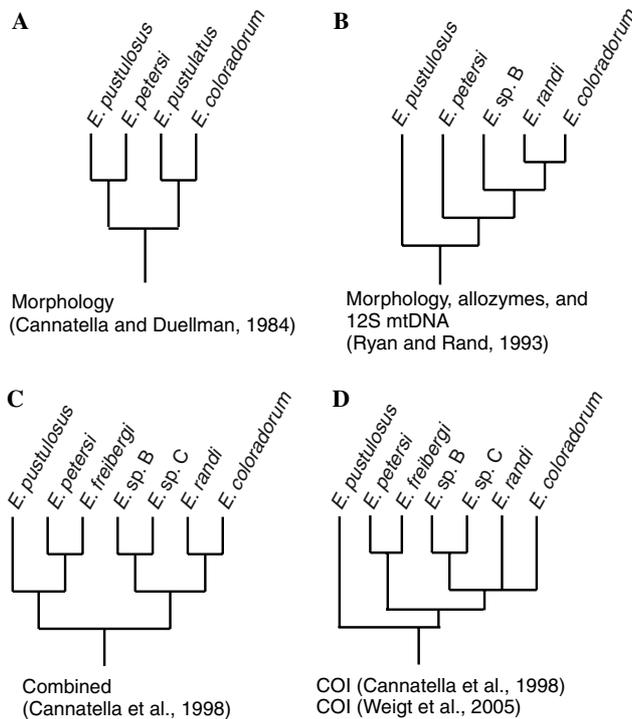*E-mail address:* sron@mail.utexas.edu (S.R. Ron).

Fig. 1. Previous phylogenetic hypotheses for *Engystomops*.

and COI mtDNA sequences. The combined analysis of all characters placed *E. pustulosus* as sister taxon to the clade (*E. petersi* + *E.* cf. *freibergi*). However, their COI mtDNA data partition supported *E. pustulosus* as sister taxon to all species of the group (Figs. 1C and D). A recent phylogeny based on COI mtDNA shows the same basal position for *E. pustulosus* (Weigt et al., 2005). To demonstrate the taxonomic status of the cryptic *E. guayaco*, Ron et al. (2005) included a brief phylogeny based on a subset of the mtDNA data presented here (five species). Because that analysis is congruent with our results, we will not discuss it further.

Taxon sampling influences tree topology (Zwickl and Hillis, 2002) and the interpretation of character evolution (Ackerly, 2000). Comprehensive taxon sampling for phylogenetic inference is particularly important in model systems, like *Engystomops*, where large datasets need to be analyzed in an evolutionary framework. The earliest studies on communication and sexual selection in *Engystomops* had the virtue of being among the first comparative analyses of behavioral characters that used explicit phylogenetic methods (e.g., Ryan and Rand, 1995). Unfortunately, the phylogenies used in those studies have incomplete taxon sampling and/or conflicting topologies (Fig. 1). For example, the influential concept of the Sensory Exploitation Hypothesis, which posits that a male secondary sexual trait can evolve to take advantage of pre-existing female sensory biases (Ryan, 1990) was based on character reconstructions on a phylogeny that only included the four species of *Engystomops* known at the time (Fig. 1A). Since then, the number of species of *Engystomops* has more than doubled. The addition of these new taxa could plausibly compromise

support for the Sensory Exploitation Hypothesis, depending on the resulting new topology and character state distributions of the male secondary sexual trait and female mate choice in the added species.

The existence of undescribed species of *Engystomops* has been previously reported (e.g., Cannatella et al., 1998; Ryan and Rand, 2001) and recent fieldwork has confirmed and expanded the list of new species (Ron et al., 2004, 2005). The present study is an effort to provide a complete phylogeny for all extant species of *Engystomops* (described as well as new, but as yet undescribed, species; 10 or 11 in total). The phylogeny is based on analyses of ∼2.4 kb from three mitochondrial genes (ribosomal RNA genes, and the valine tRNA gene). In combination with the wealth of available data on call evolution and female mate preference, this new phylogeny presents new opportunities to expand, complement, and reevaluate previous analyses of sexual selection and the evolution of communication in this model clade.

As exemplified by the recent discovery of morphologically cryptic species in *Engystomops* (Ron et al., 2004, 2005), the use of genetic markers in systematics has an enormous potential to facilitate the global inventory of biodiversity. The revolution that systematics is experiencing will be crucial for management and conservation of biotic resources considering that probably <10% of species on the planet have been discovered and as few as <1% are known beyond a succinct anatomical description (Wilson, 2005). Although the taxonomic deficit seems to be less severe among terrestrial vertebrates, sampling of amphibians and reptiles inhabiting highly diverse regions in the Neotropics is far from complete (Duellman, 1999; Rodrigues, 2005). Although it is clear that the inventory of species of tropical amphibians and reptiles is still inadequate, the extent of this inadequacy is unknown. In this paper, we also combine our results with those from other species-level phylogenies of Neotropical amphibians to estimate the potential impact of molecular systematics on the discovery of new species in the tropics.

2. Materials and methods

2.1. Taxa sampled

We sampled 36 populations of *Engystomops* from throughout the Neotropical Region belonging to eight described and at least two unnamed species (Table 1; Fig. 2). Tissue samples (liver and muscle) were stored in 95% ethanol, tissue buffer, or DMSO buffer. Sample sources and sequence accession numbers are listed in Table 1. All available information suggests that *Engystomops* is monophyletic and sister to *Physalaemus* (Cannatella and Duellman, 1984; Nascimento et al., 2005; Ron et al., 2005; Tarano and Ryan, 2002). The monophyly of *Engystomops* has been corroborated by phylogenetic analyses of ∼2400 bp of mtDNA that included 25 species of *Physalaemus* (DCC, unpublished data). For the outgroup, we used six species representing all three remaining species groups

Table 1
Localities, voucher information, and GenBank accession numbers for the specimens and sequences used in the phylogenetic analyses

Species	Locality No.[a]	Locality	Catalog or sample No.[b]	Accession No.
P. nattereri	—	Brazil: São Paulo: Luis Antônio	ACJ 95.267	DQ337208
P. signifer	—	Brazil: Rio de Janeiro: Seropédica, Hôrto Florestal Santa Cruz	TNHC 60073	DQ337209
P. albonotatus	—	Argentina: Chaco: San Fernando, 4.5 km SE Resistencia, S 27°25′55.6″; W58°54′46.7″	DCC-NB 019	DQ337210
P. enesefae	—	Venezuela: Guárico: Calabozo	MR 005	DQ337211
P. biligonigerus	—	Argentina: Salta: Finca San Ramón, 1 km N La Merced, S 24°57′8.6″, W65°29′32.5″	DCC-NB 012	DQ337212
P. barrioi	—	Brazil: São Paulo: Parque Nacional Serra do Bocaina, 1 km before entrance	TNHC 60105	DQ337213
E. pustulatus	16	Ecuador: Los Ríos: Patricia Pilar	QCAZ 19606	DQ337214
E. pustulatus	17	Ecuador: Guayas: Cerro Blanco	QCAZ 23420	DQ337215
E. sp. B	18	Peru: Lambayeque: Olmos, 8.5 km N Motupe	MR 726	DQ337216
E. sp. D	19	Ecuador: El Oro: Puyango	QCAZ 26981	DQ337217
E. sp. D	20	Ecuador: Loja: Zapotillo	QCAZ 26959	DQ337218
E. guayaco	21	Ecuador: Guayas: 11km N Cerro Masvale	QCAZ 23533	DQ337219
E. guayaco	22	Ecuador: Guayas: Naranjal	QCAZ 23652	DQ337220
E. coloradorum	23	Ecuador: Pichincha: road between Santo Domingo and Alluriquín	QCAZ 19294	DQ337221
E. coloradorum	24	Ecuador: Pichincha: 4 km NW La Florida	QCAZ 19418	DQ337222
E. montubio	25	Ecuador: Guayas: Balsas	QCAZ 23323	DQ337223
E. montubio	26	Ecuador: Manabí: Pedernales	QCAZ 23199	DQ337224
E. randi	19	Ecuador: El Oro: Puyango	QCAZ 23768	DQ337225
E. randi	17	Ecuador: Guayas: Cerro Blanco	QCAZ 23425	DQ337228
E. randi	30	Ecuador: Guayas: Cerro Masvale	QCAZ 19559	DQ337227
E. randi	29	Ecuador: El Oro: Pasaje	QCAZ 19602	DQ337226
E. cf. *freibergi*	31	Brazil: Acre: Reserva Extrativista do Alto Juruá, Tejo River	ZUEC 9511	DQ337229
E. petersi	32	Ecuador: Pastaza: El Puyo	QCAZ 26210	DQ337230
E. petersi	33	Ecuador: Napo: Cando	QCAZ 11965	DQ337231
E. petersi	34	Ecuador: Napo: Napo-Galeras, Ishquiñambi	QCAZ 14723	DQ337232
E. petersi	35	Ecuador: Orellana: Estación Científica Yasuní, Universidad Católica del Ecuador	QCAZ 12128	DQ337233
E. petersi	36	Ecuador: Sucumbíos: La Selva	QCAZ 23976	DQ337234
E. pustulosus	1	Venezuela: Guárico: Calabozo	LLV4	DQ337235
E. pustulosus	2	Venezuela: Sucre: Carupano	CARU	DQ337236
E. pustulosus	3	Colombia: Tolima: Mariquita	COL1	DQ337237
E. pustulosus	4	Venezuela: Zulia: Maracaibo Lake, 2.5 km from Santa Elena	SEV1	DQ337238
E. pustulosus	5	Panama: Panama: Kent's Marsh Gamboa	KM91	DQ337239
E. pustulosus	6	Panama: Veraguas: Santiago	SANT1-PAN	DQ337240
E. pustulosus	7	Panama: Chiriquí: Puerto Armuelles	PA2-PAN	DQ337241
E. pustulosus	8	Panama: San Blas: Nusagandi	NUS1-PAN	DQ337242
E. pustulosus	9	Panama: Panama: Perlas Islands	REY1-PAN	DQ337243
E. pustulosus	10	Costa Rica: Guanacaste: Liberia	LW96	DQ337244
E. pustulosus	11	Nicaragua: Managua: Managua	LW1083	DQ337245
E. pustulosus	12	El Salvador: San Miguel	LW1067	DQ337246
E. pustulosus	13	Mexico: Chiapas: Puerto Madera	LW1033	DQ337247
E. pustulosus	14	Mexico: Chiapas: Tehuantepec	LW1022	DQ337248
E. pustulosus	15	Mexico: Veracruz: Laguna Verde	LW101A	DQ337249

[a] Numbers correspond to those in Figs. 2 and 3.

[b] *Abbreviations:* ACJ, A. Cardoso field No.; DCC, D.C. Cannatella field No.; MJ, M.J. Ryan field No.; QCAZ, Museo de Zoología Pontificia Universidad Católica del Ecuador; DCC-NB, N. Basso field No.; TNHC, Texas Memorial Museum; ZUEC, Museu de História Natural, Universidade Estadual de Campinas. Specimens for *E. pustulosus* samples were not preserved and its catalog numbers are L.W. Weigt vial codes.

recognized in *Physalaemus* (groups as defined by Lynch, 1970): (1) *P. albonotatus*, *P. barrioi*, and *P. enesefae* (= *P. fischeri*) from the *P. cuvieri* group; (2) *P. biligonigerus* and *P. nattereri* from the *P. biligonigerus* group; and (3) *P. signifer* from the *P. signifer* group. For ease of comparison, our nominal species *E.* sp. B is the same as "*P.* sp. B" in Cannatella et al. (1998). In Cannatella et al. (1998) two species were misnamed: "*Physalaemus pustulatus*" is in fact *E. randi* whereas "*P.* sp. C" is *E. pustulatus*.

2.2. DNA extraction, amplification, purification, and sequencing

Total DNA was extracted from muscle and liver tissue preserved in ethanol and tissue storage buffer using DNeasy (Qiagen) or Viogene DNA extraction kits. Polymerase chain reaction (PCR) was used to amplify a 2.4-kb region that included 12S rRNA, valine-tRNA, and 16S rRNA genes. We amplified the segment using 4–6 overlap-
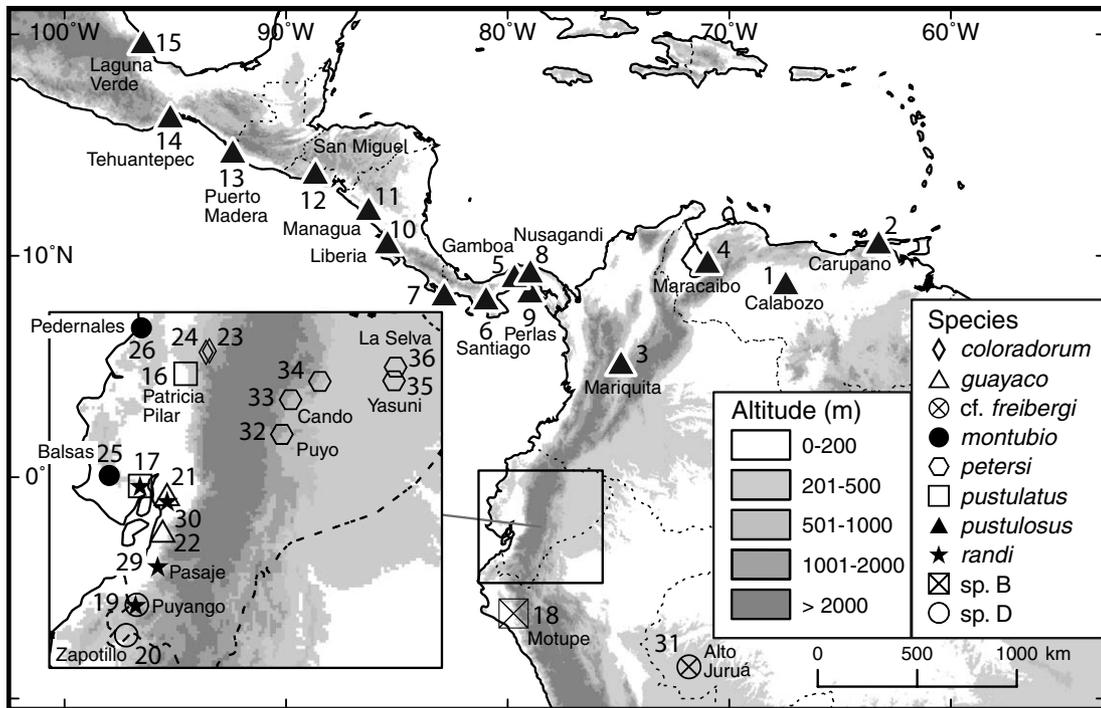
Fig. 2. *Engystomops* sampled in the study. Locality numbers correspond to those in Fig. 3 and Table 1.

ping DNA fragments using primers listed in Darst and Cannatella (2004). We use the following combination of eight primers to amplify four overlapping PCR products of ≈600 bp (notation and number follows Goebel et al., 1999 and publication above): MVZ59 (#29)-tRNAval (#73); L1091 (#46)-16SH; 12SM-16Sar (#88); and 16SC-16Sbr (#96). All PCR products were amplified under standard conditions and with the following PCR profile: (1) initial heating for 2 min at 94 °C; (2) 37 cycles of: 94 °C for 30 s, 45–48 °C for 30 s, and 72 °C for 60 s; and (3) final extension for 8 min at 72 °C. PCR products were visualized on an agarose/TBE gel and single fragments were excised and purified using QIAquick (Qiagen) and Gel-M (Viogene) gel extraction kits following the manufacturer's specifications. Purified PCR products were sequenced in both directions using ABI Prism BigDye Terminator chemistry (versions 2.0 and 3.0; Applied Biosystems). Sequenced products were cleaned using CentriSep columns (Princeton Separations) with Sephadex G-50 (Sigma–Aldrich) and then run on a capillary automated sequencer (ABI 3100; Applied Biosystems). Sequences were edited for ambiguities and sequence errors using Sequencher (versions 4.1 and 4.2; Gene Codes). A continuous sequence was generated from the 8 to 12 overlapping fragments obtained.

2.3. Sequence alignment and analyses

We analyzed 2422 bp of the mitochondrial genes12S rRNA, valine–tRNA, and 16S rRNA. Preliminary alignment was done with CLUSTAL X 1.8 (Thompson et al., 1997). The sequence matrix was imported to MacClade (version 4.06; Maddisson and Maddison, 2000) and the

ambiguously aligned regions were adjusted manually to produce a parsimonious alignment (i.e., informative sites minimized). Phylogenetic analyses were carried out with both the entire matrix (2422 bp) and with a subset that excluded ambiguously aligned regions (65 bp deleted). The MP, ML, and Bayesian analyses of the subset matrix resulted in the same topologies and similar support to those from the entire matrix; bootstrap values for supraspecific clades in the MP analysis were the same except for three clades (1% lower in two, 4% higher in one). All results reported hereafter are those derived from analyses of the entire matrix.

Maximum parsimony (MP) and maximum likelihood (ML) analyses were performed with PAUP* 4.0b10 (Swofford, 2002). For the MP analyses, trees were found with the heuristic search algorithm using tree bisection–reconnection algorithm branch swapping. One thousand replicate searches, starting from a random tree, were carried out. Characters were unordered, equally weighted and optimized with accelerated character transformation (ACCTRAN). Clade support was evaluated with nonparametric bootstrapping (Felsenstein, 1985) with heuristic searches (1000 pseudoreplicates, with 10 random addition-sequence replicates each). Patristic distances (i.e., total length of branches between each pair of taxa in the tree) were obtained from the MP tree in PAUP*.

For the ML analyses, Modeltest v. 3.5 (Posada and Crandall, 1998) was used to find the best model of character evolution of the data. To find the ML tree, we performed iterated searches. On each iteration, model parameters were set to estimates from the previous iteration, the starting tree was found via stepwise addition and latter rearranged by

branch-swapping with the tree bisection–reconnection algorithm. Parameters for the first iteration were estimated from the most parsimonious tree with the best likelihood score. Iterations were continued until additional iterations yielded identical trees. This searching procedure (often termed "successive-approximations") has been demonstrated to be as reliable as full-optimization ML searches to find optimal trees (Sullivan et al., 2005).

Bayesian analyses were conducted using pMrBayes 3.0b4 (Altekar et al., 2004; Ronquist and Huelsenbeck, 2003) on a Macintosh G5 using two parallel processors, enabled by Pooch 1.5.5 (daugerresearch.com, Dauger Research). Six Markov chains were utilized in three analyses, the temperature parameter was set at 0.08 for Analysis 1 and 0.15 for Analyses 2 and 3, the prior for the rate matrix was a uniform dirichlet and all topologies were equally probable a priori. Analysis 1 ran for 3×10^6 generations and Analyses 2 and 3 for 1.5×10^6 generations. For each analysis, the chain was sampled every 10^3 generations. The exchange rate among adjacent chains varied from 0.68 to 0.74 for Analysis 1 and 0.44–0.61 for Analyses 2 and 3, indicating well-mixed chains. Convergence was determined by examining plots of parameters (likelihood score, six rate variables, four nucleotide frequencies, shape parameter of the gamma distribution, and proportion of invariant sites) against generation number. The first one third of sampled trees were discarded as the burn-in and the remaining trees were used for estimating Bayesian posterior probabilities by a majority-rule consensus procedure in PAUP*.

Although useful in Bayesian phylogenetic analysis, a mixed model was not used for the 12S and 16S genes because the same model (GTR+Γ+I) was estimated for each gene and separate analyses of 12S and 16S yielded the same tree.

2.4. Testing previous hypotheses

We conducted parametric bootstrap tests (Huelsenbeck and Hillis, 1996) to evaluate whether our dataset can statistically reject a given topological hypothesis. Specifically, we tested the topology that combines all species of *Engystomops* except *E. pustulosus* in a clade (e.g., Figs. 1A and C). To apply the test we: (1) introduced a topological constraint to find the shortest tree compatible with the constrained topology (null hypothesis); (2) estimated ML parameters of a model of evolution from the observed dataset and the best constrained tree; (3) simulated 1000 replicate datasets (using Seq-Gen v.1.3.2; Rambaut and Grassly, 1997) based on the model parameters from step 3 and the best constrained tree; (4) found the best MP tree and the best MP constrained tree for each replicate dataset and calculated the difference in steps between both trees; (5) compared the observed difference in steps (best tree vs. constrained tree from observed data) with the (null) distribution of the differences obtained in step 4; we rejected the null hypothesis if the observed value was >95% of the values from the null distribution.

3. Results

3.1. Phylogenetic relationships

The MP strict consensus, ML tree, and Bayesian majority-rule consensus resulted in fully compatible topologies except for the placement of populations of *E. pustulosus* (Fig. 3). The MP analysis of 2422 characters (900 variable, 755 parsimony-informative) yielded four most parsimonious trees of length 2645 (CI = 0.499, RI = 0.818; Fig. 3). The log-likelihood scores of the parsimony trees ranged from −15540.99 to −15538.83 (only 2.5–4.7 log-likelihood units from the score of the ML tree). The four most parsimonious trees differed in their placement of *E. coloradorum* (either as sister taxon to *E. guayaco* or to the clade (*E. montubio + E. randi*)) and the intraspecific placement of two *E. pustulosus* populations from eastern Central America.

According to the Akaike information criterion (Akaike, 1974), the model with the best fit is GTR+Γ+I. Maximum likelihood analysis under that model resulted in a tree with ln $L = -15536.32$ (Fig. 3; shape parameter with four discrete rate categories = 0.65348; proportion of invariable sites = 0.429389; estimated nucleotide frequencies: A = 0.36420, C = 0.17661, G = 0.16541, T = 0.29378). The ML tree and the Bayesian consensus place *E. coloradorum* as the sister species of *E. guayaco*. However, the clade (*E. coloradorum + E. guayaco*) lacks strong support (Bayesian posterior probability = 0.92). All other supra-specific clades are well-supported (Fig. 3). The Bayesian tree topologies for all three analyses were identical. Of the 40 internal nodes, only 7 had posterior probabilities less than 1.0 and the posterior probabilities differed by 0.02 at one node and 0.01 in the other six, indicating adequate convergence of the Markov chains.

Two allopatric basal clades are defined within *Engystomops*: one contains all species distributed in the lowlands (up to 1000 m of altitude) west of the Andes in Ecuador and northern Peru; the other contains *E. pustulosus* (Central America and northern South America) and the Amazonian *E. petersi* and *E.* cf. *freibergi* (Figs. 3–5). Within the W Ecuador-Peru clade, there is a clade of small sized species (mean male snout-vent length <23 mm) that includes *E. coloradorum* and the recently described *E. guayaco*, *E. montubio*, and *E. randi*.

All analyses show two well-supported basal clades among the 15 samples of *E. pustulosus*: (1) includes all the samples from the western range of the species (i.e., Costa Rica, Nicaragua, El Salvador and Mexico; 10–15 in Fig. 2); (2) includes samples from the eastern range (Panama, Colombia, and Venezuela; 1–9). Patristic distances between both clades ranged from 32 to 44 (uncorrected $p = 0.031$–0.043); patristic distances within clades ranged from 3 to 36 (uncorrected $p = 0.005$–0.031). A genetic divide among populations is evident in central Costa Rica. For example, Liberia (W Costa Rica) and Laguna Verde (SE Mexico) belong to the same clade, have a patristic distance of 9 (uncorrected $p = 0.015$), and are
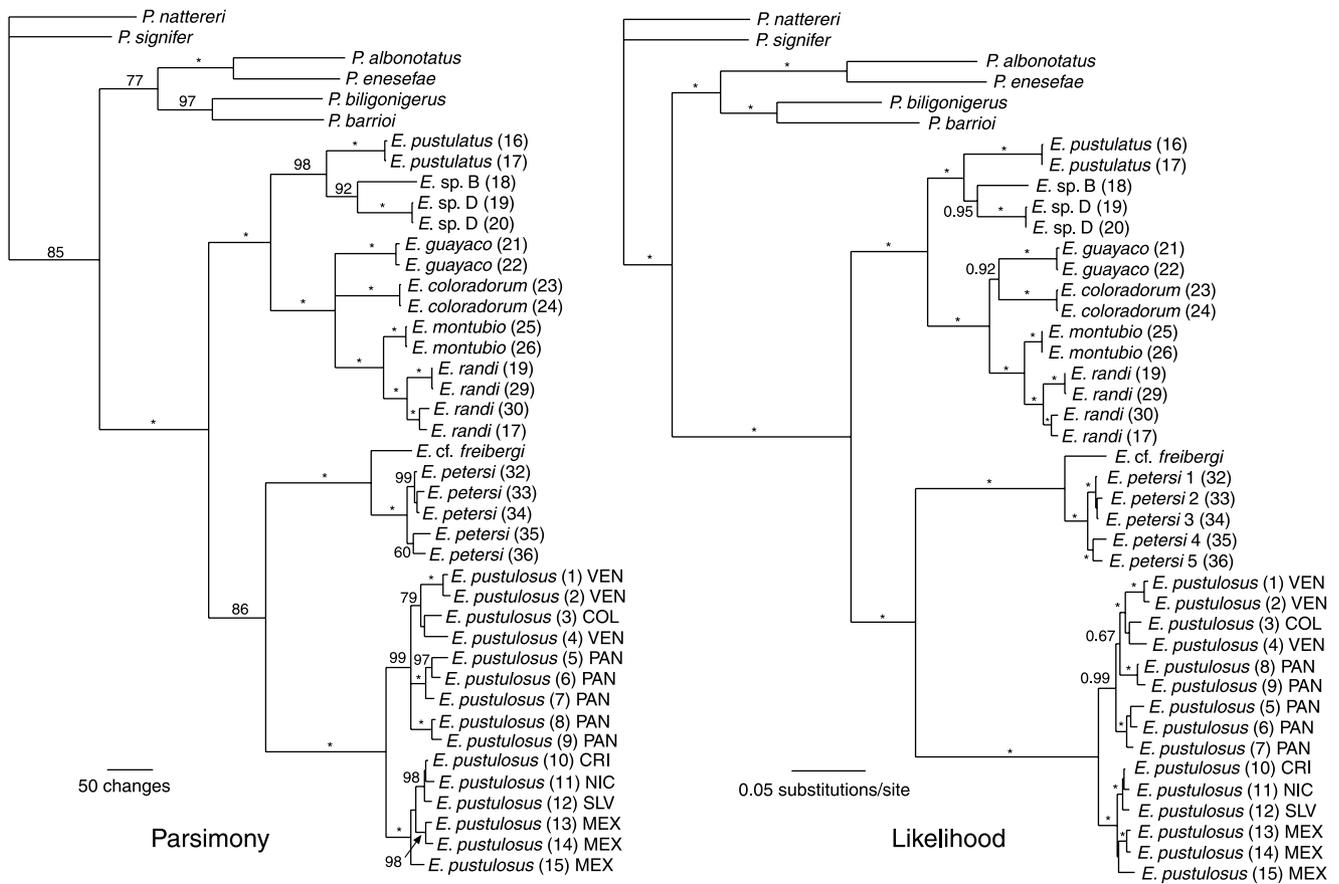
Fig. 3. Phylogenetic relationships for members of *Engystomops*. Left: strict consensus from four most parsimonious trees (TL = 2645 steps; 755 parsimony-informative characters; CI = 0.499, RI = 0.818). Numbers at branches are nonparametric bootstrap values (from 1000 pseudoreplicates); asterisks represent 100 values. Right, maximum likelihood tree (ln L = −15536.32) obtained under the GTR+Γ+I model of character evolution. Numbers on branches are Bayesian posterior probabilities; asterisks represent 1.0 values. *Abbreviations:* COL, Colombia; CRI, Costa Rica; MEX, Mexico; NIC, Nicaragua; PAN, Panama; SLV, El Salvador; and VEN, Venezuela. Numbers in parentheses correspond to those of localities in Fig. 2 and Table 1.

1600 km apart; Liberia and Armuelles (W Panama) belong to different clades, have a patristic distance of 34 (uncorrected $p = 0.023$) even though they are separated by only 391 km (Fig. 2).

An additional species showing well-supported allopatric clades is *E. randi*. These clades are separated by a narrow stretch of lowland east of Golfo de Guayaquil in Ecuador (Fig. 5). Distances between the northern and southern clade of *E. randi* range from 10 to 14 (uncorrected $p = 0.020$–0.027).

Patristic distances (MP) ranged from 1 (between both populations of *E. coloradorum*; uncorrected p distance <0.001) to 292 (between *E. enesefae* and *E. pustulosus* from Carupano, Venezuela; uncorrected $p = 0.178$). The minimum patristic distance between two unambiguously recognized species is 20 (*E. montubio* and *E. randi*; uncorrected $p = 0.029$); those between *E.* cf. *freibergi* (Alto Juruá, Brazil) and *E. petersi* from eastern Ecuador range from 29 to 33 (uncorrected $p = 0.039$–0.041).

We present a phylogenetic classification that provides unranked names for well-supported clades within *Engystomops* (Appendix A; Fig. 6). The hierarchical position of each name is denoted by its indentation in the Appendix A.

3.2. Testing previous hypotheses

The only major incongruence with previous phylogenies is seen in those derived exclusively or mainly from sequences of 12S rRNA and COI genes. They show *E. petersi* as sister taxon to the clade Duovox rather than to *E. pustulosus* (Cannatella et al., 1998; Ryan and Rand, 1993; Weigt et al., 2005). The parametric bootstrap test for monophyly of ((*E. petersi* + *E. freibergi*) + Duovox) shows that this null hypothesis can be rejected with high confidence ($p = 0.004$; Fig. 7).

4. Discussion

4.1. Systematics

Our phylogeny, including all known species of *Engystomops*, is consistent with most previous systematic reviews (e.g., Cannatella and Duellman, 1984; Cannatella et al., 1998). Clades recovered previously on the basis of morphological (Cannatella and Duellman, 1984; Cannatella et al., 1998) and molecular datasets have high support in our phylogeny (Cannatella et al., 1998; Ron et al., 2005). The only exception is that our data reject the hypothesis
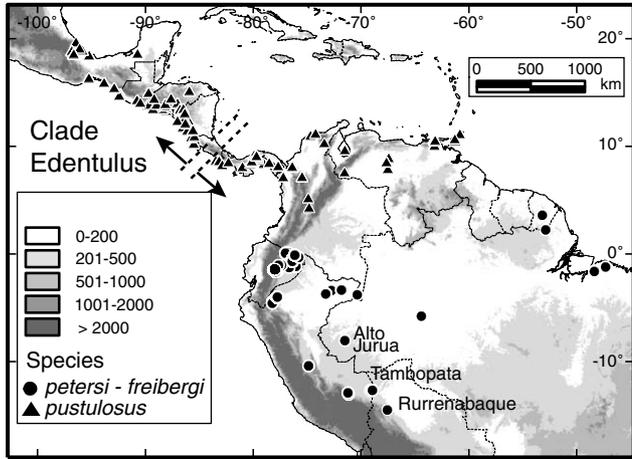
Fig. 4. Distribution of the clade Edentulus based on museum and litera-ture records (Cannatella and Duellman, 1984; California Academy of Sci-ences; Museo de Zoología Pontificia Universidad Católica del Ecuador; Museum of Comparative Zoology Harvard University, Museum Michi-gan State University, Natural History Museum University of Kansas, Museum of Vertebrate Zoology University of California Berkeley, and Texas Cooperative Wildlife Collection Texas A&M University). The dashed lines in Central America demarcate the gap in the distribution of *Engystomops pustulosus*. Populations on each side of this gap fall into each of two genetically divergent clades.

Fig. 5. Distribution polygons of Duovox based on museum records and the literature (Ron et al., 2004, 2005; Museo de Historia Natural Universi-dad Nacional Mayor de San Marcos, Museum of Vertebrate Zoology University of California Berkeley).
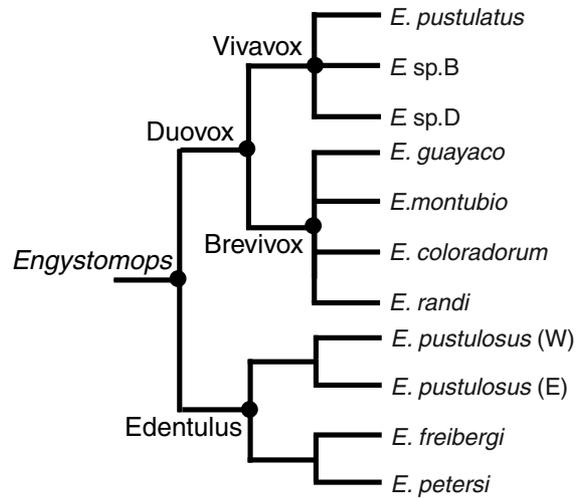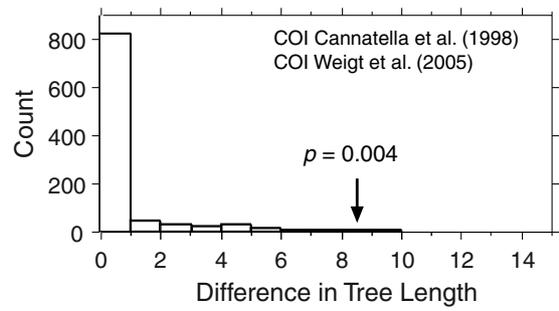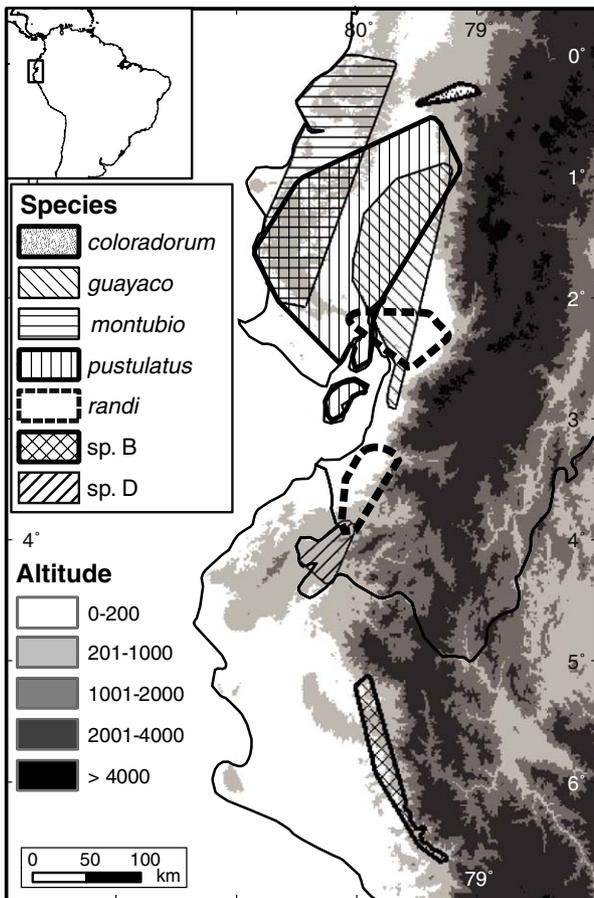

Fig. 6. Phylogenetic classification for *Engystomops*.

Fig. 7. Parametric bootstrap test for monophyly of ((*E. petersi* + *E. freibergi*) + Duovox) (H_0). The graph shows the difference in steps (parsimony) between the null and test hypothesis for 1000 replicates. The observed difference (arrow) was >99.5% of the values from the null distri-bution (H_0 rejected).

that *E. pustulosus* is the sister taxon to a clade comprising the remaining species of *Engystomops* (Figs. 1 and 7).

Monophyly of *Engystomops* is well-supported by our analysis and the inclusion of mtDNA sequences from addi-tional species of *Physalaemus* does not alter that outcome (DCC, unpublished data; Tarano and Ryan, 2002). As far as we know, *Engystomops* monophyly has not been ques-tioned since it was first proposed by Lynch (1970). There-fore, the resurrection of the genus *Engystomops* as proposed by Nascimento et al. (2005) is logically consistent with the phylogeny. Although the taxonomic change was not a strict requirement of the phylogeny, the increase in the informativeness of the classification is desirable consid-ering the large species content of the former "*Physalaemus*" (almost 50 species).

4.1.1. Clade Edentulus

Edentulus is distributed in Central America, northern South America, and the Amazon Basin (Fig. 4) and is allo-patric to its sister clade, Duovox. The clade is supported by at least three morphological synapomorphies, including the absence of teeth (Cannatella et al., 1998).

Our analysis shows two well-supported allopatric clades within *E. pustulosus*. Each occupies one of two disjunct

portions of the distribution range of *E. pustulosus*. The disjunction is 175 km in length, in central Costa Rica, between Barranca and Puerto Cortés (both in Puntarenas Province; Savage, 2002; Fig. 4). The western clade is distributed from southern Mexico to western Costa Rica; the eastern clade ranges from eastern Costa Rica to northern Colombia and Venezuela (Fig. 4; Weigt et al., 2005). Our results are consistent with evidence of genetic distinctiveness between both ranges in allozymes and CO I sequences (Ryan et al., 1996; Weigt et al., 2005).

Several lines of evidence suggest that *E. pustulosus* is composed of at least two cryptic species. The high support for both clades (bootstrap 99 and 100) and their concordance with geography (i.e., allopatric, with an intervening barrier to gene flow) indicate that each basal clade represents a separate species, according to the criteria of Wiens and Penkrot (2002). The use of mtDNA for species delimitation is controversial because its uniparental inheritance does not encapsulate the complete organismal history (but see Wiens and Penkrot, 2002). However, nuclear markers have uncovered the same two genetic clusters (i.e., eastern and western separated by a distributional gap in central Costa Rica; Weigt et al., 2005) suggesting that the divergence between both clades is not an artifact of differential gene flow and dispersal in females or a mismatch between the mtDNA tree and the population histories.

Species status for each clade also is suggested by a putative long period of divergence between both clades of 6 and 10 Ma (Weigt et al., 2005) and by patristic distances higher than those reported between uncontroversial sister species in Duovox (*E. montubio* and *E. randi*). Although previously overlooked, there is at least some level of morphological differentiation as well. A reanalysis of datasets (Freeman, 1967 and Cannatella and Duellman, 1984) of average body size from 34 populations and 669 individuals shows significant differences between both ranges (western populations are smaller; ANOVA $p < 0.001$, df = 33).

Ryan et al. (1996) and Weigt et al. (2005) have asserted that the allozyme differentiation between both ranges is within the limits of inter-population variation. That interpretation has been questioned (Wynn and Heyer, 2001) and is not readily supported by the observed pattern of genetic differentiation. If each clade is granted species status, the binomial *E. pustulosus* should be applied to the eastern lineage (type locality for *E. pustulosus* is "New Grenada, on the River Truando" in Colombia; Cope, 1864 in Cannatella and Duellman, 1984). No binomial is available for the western lineage and therefore the species awaits description.

Subsequent to Lynch's (1970) review, most Amazonian *Engystomops* have been assigned to *E. petersi*. The use of *E. freibergi* has been restricted to the type locality "Río Runerrabaque [= Rurrenabaque], Río Beni, Bolivia" and a few localities in SE Peru (Fig. 4). Cannatella and Duellman (1984) placed *E. freibergi* as a junior synonym of *E. petersi* because its diagnostic characters were dubious. However, Cannatella et al. (1998) recognized *E. freibergi* as a valid name for Southern Peruvian populations based on genetic distances, differences in male advertisement calls, and geographic proximity to the type-locality of *E. freibergi*; specimens and sequences from the type locality were not available. Similarly, our tentative assignment of the Alto Juruá population to *E. freibergi* has been based exclusively on geographic proximity to *E. freibergi*'s type locality (Fig. 4) and high levels of genetic differentiation relative to populations near the type locality of *E. petersi*, in Amazonian Ecuador. Differentiation in male advertisement calls can be indicative of prezygotic reproductive isolation in anurans (Gerhardt and Huber, 2002) and is extensive among some populations of Amazonian *Engystomops*. Calls from the single southern population sampled by Cannatella et al. (1998) [from Tambopata, Peru] were known to have an additional high frequency suffix (absent in the single northern population sampled, in Ecuador). Additional data has shown that the high frequency suffix is also present in *E. petersi* populations from Ecuador (see below) and therefore is not suitable to diagnose *E. freibergi*.

Regardless of nomenclature, available information suggests that Amazonian *Engystomops* are a species complex. Our samples include five populations from NW Amazonia and one population in Alto Juruá, Acre, Brazil (Fig. 2). The NW Amazonian populations form one clade with high bootstrap support (100), sister to the Alto Juruá population. The large genetic differentiation between the NW Amazonian clade and Alto Juruá (patristic distances 29–33) suggests that each represents a separate species. Cytological studies have shown highly divergent chromosomal morphology and C-banding patterns among specimens from a single locality in Acre, Brazil, indicating the co-occurrence of two species (Lourenço et al., 1999). A conspicuous difference among populations is the addition of a high-frequency suffix to the call. The suffix is present in Yasuní (Ecuador; Fig. 2) and Tambopata (Peru; Fig. 4) but absent in Cando, Ishquiñ-ambi, La Selva, and Puyo (Ecuador; Fig. 2; Boul and Ryan, 2004; SRR, unpublished). Significant inter-population differences also are evident in fundamental frequency (e.g., La Selva vs. Yasuní) and duration of the call (e.g., Yasuní vs. Tambopata; Boul, 2003). *Engystomops pustulosus* also can add a high frequency suffix to their calls but this capacity seems to be present in all populations (Ryan et al., 1996).

The distribution of Amazonian *Engystomops* extends over more than 3 million km^2 and has been sparsely sampled (Fig. 4). Although the populations included in our phylogeny represent a small portion of the distribution, they show considerable genetic divergence and call differentiation. On that basis, we predict that analyses encompassing a larger geographic area will reveal the existence of even more distinct lineages with complex patterns of call variation and distribution. A comprehensive analysis of the phylogeny and phylogeography of Amazonian *Engystomops* is currently underway (W. C. Funk, pers. comm.).

4.1.2. Clade Duovox

Despite its considerable species diversity, Duovox has a relatively restricted distribution (lowlands of western Ecuador and NW Peru; Fig. 5). Habitat types range from tropical deciduous dry forest to tropical evergreen moist forest. All species in this clade are locally abundant in human-disturbed regions and it seems unlikely that the widespread conversion of natural vegetation into agricultural lands has had a negative impact on their populations (Ron et al., 2005).

Duovox monophyly is strongly supported by our molecular data and morphological characters (Cannatella et al., 1998; Ron et al., 2005). Morphological synapomorphies for the group are the absence of tarsal tubercle, and a narrow stalk of the alary process of the hyoid (Cannatella et al., 1998; Ron et al., 2005).

Until 2004, only two species of Duovox had been described, *E. pustulatus* and *E. coloradorum*. Traditionally, all Duovox lacking autapomorphies of *E. coloradorum* have been assigned to *E. pustulatus*. Incorrect assignment to *E. pustulatus* might be a consequence the brief species description based on a single juvenile specimen (Ron et al., 2004).

Engystomops pustulatus is a large species compared to its congeners (male SVL 25.17–29.88). It has a distribution restricted to western Ecuador (Fig. 5; Ron et al., 2004, 2005). Although with a conspicuously different advertisement call and external morphology, *Engystomops randi* has been incorrectly referred to *E.* "*pustulatus*" in most publications (e.g., Cannatella and Duellman, 1984; Ryan and Rand, 1993, 1995; Cannatella et al., 1998). Comparison of the type material of *E. pustulatus* with the newly collected series enabled us to detect this misidentification.

Specimens morphologically similar to *E. pustulatus* from NW Peru have been considered a distinct species (e.g., Cannatella et al., 1998; Ryan and Rand, 2001). Our phylogenetic and subsequent morphological analyses (SRR, unpublished) confirm that those populations belong to an undescribed species with a distribution restricted to NW Peru (*E.* sp. B in Figs. 3 and 5). Based on its high patristic distances (47–48), we suggest that its sister taxon (*E.* sp. D in Figs. 3 and 5) is also an undescribed species, known from few localities in SW Ecuador. *Engystomops* sp. B has been mistakenly referred as *E.* "*pustulatus*" (e.g., Cannatella and Duellman, 1984; Frost, 2004; Nascimento et al., 2005; Savage, 2002).

Species of the clade Brevivox have a smaller size than other *Engystomops* (Ron et al., 2004, 2005). Except for the distinctive *E. coloradorum*, this group is morphologically conservative, to the extent that species identification based on external morphology is challenging (Ron et al., 2005). Moreover, reproduction in *E. guayaco*, *E. montubio*, and *E. randi* takes place by night, during the same season, and in similar microhabitat. In regions of sympatry, this results in males from closely related species calling next to each other in reproductive aggregations. Reproductive syntopy should exert strong selective pressures favoring interspecific divergence in advertisement call and female call preference in sympatric species. The patterns of call differentiation match

these expectations because the allopatric *E. guayaco* and *E. montubio* have nearly indistinguishable calls while the sympatric *E. guayaco* and *E. randi* show markedly different call rates (Fig. 5; Ron et al., 2005).

Engystomops randi shows a disjunct distribution with two small ranges separated by an unoccupied region 80 km long (Fig. 5). In our phylogeny, four populations clustered into two clades corresponding to each of the two ranges (Figs. 2 and 3). Support for each clade is high (bootstrap = 100) suggesting limited gene flow. However, small sample size, low patristic distances (10–14), and limited differentiation in advertisement call and external morphology (SRR, unpublished) are inconclusive regarding the taxonomic status of these populations. Nuclear markers will be useful to explore this question.

4.2. Impact of molecular systematics on estimates of amphibian diversity

There is an urgent need to describe the biodiversity of tropical regions because of their extreme species richness and the rapid destruction rate of their natural habitats. The amphibian fauna of the Neotropics is an example of the prominence of the tropics in biodiversity richness given that roughly one half of all recognized amphibian species lives in Central or South America (Duellman, 1999). Moreover, the number of Neotropical amphibians that await description may be high considering that the rate of discovery of amphibians exceeds that of any other vertebrate group, and a majority of the newly described species is from tropical regions (Cannatella and Hillis, 2004). To our knowledge, no attempts have been made to estimate the number of undescribed amphibian species.

Although only a few intraspecific and species-level molecular phylogenies are available for Neotropical amphibians, the available data suggest that a significant number of species have either been overlooked by morphology-based taxonomic reviews or have not been sampled at all. A list of some of those molecular analyses, including numbers of undescribed species discovered, is shown in Table 2. The numbers of undescribed species are based on each study authors' explicit decisions. In two studies where the authors did not make decisions to define species limits (*Bufo marinus* and 30-chromosome *Hyla*), we applied the Wiens and Penkrot (2002) criteria to delimit species. In both cases, cryptic species were found according to criterion *c* (Fig. 1 in Wiens and Penkrot, 2002).

The data show 35 undescribed species discovered in 7 studies, a 28% increase to the 123 described species included (Table 2). This percentage is even higher (39%) for studies where taxon sampling has been more intensive (i.e., studies that included more than 50% of the described species). Given that there are approximately 2800 described species in the Neotropics (Amphibia Web, 2005) and assuming, conservatively, that the proportion of undescribed amphibians lies between 0.28 and 0.39, then the number of species awaiting description should lie between 784 and 1092, a figure comparable to the total described amphibian diversity of Africa.

Table 2
Numbers of described and undescribed species of Neotropical amphibians in molecular systematics studies

	Described species included (b)	Total number of described species in target group (a)	Proportion sampled (b/a)	Undescribed species discovered	Increase %	Source
1. *Bufo marinus*	1	1	1.00	2	200	Slade and Moritz (1998)
2. Andean *Gastrotheca* Ecuador	6	6	1.00	3	50	Duellman and Hillis (1987)
3. *Engystomops*	5	5	1.00	At least 4	80	This publication; Cannatella et al. (1998)
4. Sierrana	34	42	0.81	8	23	Hillis and Wilcox (2005)
5. 30-chromosome *Hyla*	12	38	0.32	1	8	Chek et al. (2001)
6. Dendrobatidae	62	210	0.29	15	24	Santos et al. (2003); Vences et al. (2003)
7. *Pseudoeurycea bellii* complex	3	3	1.00	2	67	Parra-Olea et al. (2005)
Subtotal for b/a >0.5 (studies 1–4 in this column)	49	57	0.86	19	39	—
Overall total	123	305	0.40	35	28	—

Quantity (a) refers to the number of extant described species when the study was performed; (b) refers to the number included in the study. Note that there is a large proportion of undescribed species discovered. Figures were extracted from the ingroup taxa only. To be conservative, the species listed as discovered in *Engystomops* only include *E. montubio*, *E. randi*, *E. guayaco*, and *E.* sp. B.

This estimate is conservative because the proportion of discovered species should increase with taxon sampling (sampling was exhaustive only in *Engystomops* and *Sierrana*). Taxon sampling is frequently constrained by tissue availability. Therefore, studies with restricted sampling often include predominantly species of easy access, available in the pet trade and/or distributed in habitats of easy reach (e.g., poison-arrow frogs, genus *Dendrobates*) that, because of their accessibility and conspicuousness, are already described. Additionally, many groups included in Table 2 are composed primarily of "weedy" species, common in human-disturbed areas and therefore more likely present in scientific collections. Molecular phylogenies of species occurring in forested habitats that are difficult to access (e.g., some *Eleutherodactylus* and centrolenids) are likely to include larger proportions of unknown species.

Appendix A. Phylogenetic classification of *Engystomops*

Engystomops, Jiménez de la Espada 1872 (converted clade name). Definition: clade stemming from the most recent common ancestor of *E. petersi* Jiménez de la Espada 1872, and *E. pustulatus* (Shreve, 1941). Content: all species in the definition, as well as *E. freibergi* (Donoso-Barros, 1969), and all species in Duovox (see below). Type species: *E. petersi* Jiménez de la Espada 1872. Comments: Lynch (1970) stated that *P. moreirae* may belong to *Engystomops* (= "*P. pustulosus* group"). Haddad and Pombal (1998) and Nascimento et al. (2005) included *P. moreirae* in the *P. signifer* species group.

A. Duovox, new clade name. Definition: clade stemming from the most recent common ancestor of *E. pustulatus* (Shreve, 1941), and *E. randi* (Ron et al., 2004). Etymology: from the Latin *duo*, meaning "duet" and *vox*, meaning "voice", in reference to their breeding aggregations that often include males from two species from this clade, calling next to each other. Content: species in the definition, as well as *E. coloradorum* (Cannatella and Duellman, 1984), *E. guayaco* (Ron et al., 2005), *E. montubio* (Ron et al., 2004), and two undescribed species from SW Ecuador and NW Peru (*E.* sp. B and *E.* sp. D in Fig. 3).

1. Brevivox, new clade name. Definition: clade stemming from the most common ancestor of *E. coloradorum* (Cannatella and Duellman, 1984), *E. guayaco* (Ron et al., 2005), *E. montubio* (Ron et al., 2004), and *E.* randi (Ron et al., 2004). Etymology: from the Latin *brevis*, meaning "short" and *vox*, meaning "voice"; it refers to both the short duration of their calls and their small body size compared to other members of *Engystomops*. Content: species in the definition.

2. Vivavox, new clade name. Definition: clade stemming from the most recent common ancestor of *E.*

pustulatus (Shreve, 1941) and the undescribed species from Puyango, Ecuador (*E.* sp. D in Fig. 3). Etymology: from the Latin *viva voce*, meaning "aloud"; it refers to the loudness of their advertisement calls. Content: species in the definition plus *E.* sp. B (Fig. 3).

B. Edentulus, new clade name. Definition: clade stemming from the most recent common ancestor of *E. pustulosus* (Cope, 1864) and *E. petersi* Jiménez de la Espada 1872. Etymology: from the Latin *edentulus*, meaning "toothless"; it refers to the absence of maxillary and premaxillary teeth, a synapomorphy for the clade (Cannatella et al., 1998). Content: species in the definition, as well as *E. freibergi* (Donoso-Barros, 1969).

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