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Cryptic diversity in *Lithobates warszewitschii* (Amphibia, Anura, Ranidae)

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Abstract

Lithobates warzewitschii is a species of ranid frog distributed from southern Honduras to Panama. This species suffered severe population declines at higher elevations (above 500 m asl) from the 1980s to early 1990s, but there is more recent evidence of recovery in parts of its range. Here we advocate for the status of *Lithobates warszewitschii* as a candidate cryptic species complex based on sequence data from mitochondrial genes CO1 and 16S. Using concatenated phylogenies, nucleotide diversity (K2P-π), net between group mean distance (NBGMD) (π_{net}) and species delimitation methods, we further elucidate cryptic diversity within this species. All phylogenies display polyphyletic lineages within Costa Rica and Panama. At both loci, observed genetic polymorphism (K2P-π) is also high within and between geographic populations, surpassing proposed species threshold values for amphibians. Additionally, patterns of phylogeographic structure are complicated for this species, and do not appear to be explained by geographic barriers or isolation by distance. These preliminary findings suggest *L. warszewitschii* is a wide-ranging species complex. Therefore, we propose further investigation within its wider range, and recommend integrative taxonomic assessment is merited to assess species status.

Keywords: Área de Conservación Guanacaste (ACG); barcoding; biodiversity; CO1; phylogenetics; phylogeography; 16S; *Lithobates warszewitschii*

Introduction

Cryptic species are poorly defined and highly heterogeneous. Identification of potential singular, nominal species may be masked when morphological traits are shared within and between sister taxa (Bickford et al. 2007). Evolutionary mechanisms that produce cryptic species are also diverse and may best be explained by recent divergence, niche conservatism and morphological convergence (Fišer et al. 2018). Although considered evidence of incomplete species inventories, or potential sources of bias within biodiversity research (Fišer et al. 2018), crypticism is evidently common (Adams et al. 2014) and extensive among animal phyla (Perez-Ponce de León and Poulin 2016). Species concepts have been a topic of debate since Darwin's *Origin of Species* (Mallet 2008), yet most contemporary biologists conceptually envisage separately evolving segments of metapopulation-level evolutionary lineages (Mayden 1997, de Queiroz 1998, 1999, Hey et al. 2003, Bock 2004, Hey 2006).

Given that the majority of species remain undescribed, endeavours to explain and catalogue biodiversity are inevitable to both understanding and preventing extinctions (Pimm et al. 2014). For amphibians especially, being the most threatened group of vertebrates (Stuart et al. 2004), identifying cryptic diversity is fundamental to their conservation. Habitat loss, fragmentation, climate change and disease epidemics have produced a global decline in amphibian populations (Baillie et al. 2004, Stuart et al. 2004). Losses reflect patterns of ecological preference, range and taxonomic association, with montane stream dwelling species most affected (Stuart et al. 2004). It is also probable that the number of amphibian species is highly underestimated (Fouquet et al. 2007a, Vieites et al. 2009).
Whereas some species are presumed to be widely distributed, those within a cryptic complex may have smaller ranges or different ecological requirements (Stuart et al. 2006), meaning failure to recognize these taxa can leave them susceptible to mismanagement. However, when genetic differentiation is established, it can unveil previously unknown units of diversity and endemism (Bickford et al. 2007) that may subsequently warrant protection or species status (Whitfield et al. 2016).

High levels of genetic diversity in Costa Rican and Panamanian frog populations is well recognized (Crawford 2003), as are cryptic species (Wang et al. 2008). *Lithobates warszewitschii* (Ranidae) (Schmidt, 1857) is a proposed candidate species - a provisional designation pending further systematic investigation (Vieites et al. 2009). Crawford et al. (2010) (Supporting information) showed that within the amphibian community at El Copé (Omar Torrijos National Park), Panama, *L. warszewitschii* displayed 14.7% pairwise divergence between conspecifics at the CO1 locus. This is an unusually high degree of polymorphism for a single species in sympatry (Crawford 2003, Vences et al. 2005), providing additional evidence this taxon likely contains candidate cryptic lineages (Mallet 2008). Paz et al. (2015) compared El Copé with allopatric populations from Brewster (Chagres National Park), revealing 11% pairwise divergence. Consequently, breeding strategy, dispersal and landscape resistance may help explain this variation between both sites.

Lithobates warszewitschii occurs from Honduras to Panama and has been recorded at elevations up to 1740 meters above sea level (m asl). They are fairly common, diurnal and generally abundant frogs in forests near streams where they breed (Savage 2002). In Costa Rica, population declines occurred in montane areas such as Tapantí, Montverde and Braulio Carrillo (Bolaños 2002, Puschendorf et al. 2006). Post-decline it was found to be rare in San Vito (Santos-Barrera et al. 2007) and vanished but found again at San Ramón (IUCN 2015). *Lithobates warszewitschii* was also found to be abundant at mid-elevation sites in Guayacan (Kubicki 2008), Corcovado, Ciudad Colón and Tinamastes (IUCN 2015). A population decline also occurred at lowland site La Selva (Whitfield et al. 2007), however, it is not generally abundant at lower elevations (IUCN 2015). Pre-decline it was one of the most abundant tadpoles encountered in streams at El Copé, Panama, (Ranvestel et al. 2004), but was later extirpated following the emergence of a virulent pathogen (Crawford et al. 2010). In Nicaragua, it was found to be abundant in Rio San Juan (Sunyer et al. 2009) and numbers were increasing at Quebracho (Barquero et al. 2010) post decline, although Nicaragua's decline history is much more nebulous than Costa Rica's. No data was found for Honduras, and additional research is needed to ascertain population sizes, distributions, trends and threats throughout its full range (IUCN 2015).

In this study we expand the research on cryptic diversity within *L. warszewitschii,* based on published sequence data from two localities in Panama (Crawford et al. 2010, Paz et al. 2015) and samples collected from the Área de Conservación Guanacaste (ACG) in northwestern

Costa Rica. Using phylogenetic data, species delimitation methods and nucleotide diversity within CO1 and 16S loci we make inferences about phylogeographic structure and proposed candidate status across its wider range.

Methods

Field sampling

Lithobates warszewitschii were sampled from five field sites within the Área de Conservación Guanacaste (ACG), Costa Rica: Pitilla, San Gerardo, Maritza, Cacao and Caribe (Figure 1) between June 2015 – August 2017 (Table 1). Streams and surrounding forest are preferred habitat for *L. warszewitschii* (Savage 2002), and sampling was conducted within these habitats. Each individual was captured, housed separately in moist bags (Beaupre et al. 2004), identified based on morphology (Savage et al. 2002, Leenders 2016), and toe-clipped (Perry et al. 2011). Individuals were then released back at the point of capture.

A total of 34 samples were collected from ACG and obtained from genbank, but only 29 had both CO1 and 16S available and therefore used in this analysis. All data for *L. warszewitschii* samples collected in Panamanian sites El Copé and Brewster were obtained from other studies (Crawford et al. 2010, Paz et al. 2015).

Lab work

In order to extract DNA from tissue samples a standard ammonium acetate protocol was used (Nicholls et al. 2000). The Cytochrome c oxidase subunit I (CO1) and 16S ribosomal RNA (16S) mitochondrial genes were targeted for amplification by PCR. 16S primers (16Sar-L +16Sbr-H) and reaction protocols were adapted from (Kessing et al 2004). Multiple primers were used in the CO1 reactions to maximize the number of successful PCR products. CO1 primers (dgLCO-1490 + dgHCO-2198) and reaction protocols were adapted from Meyer et al. (2005) and CO1 primers (Chmf4 + Chmr4; Che et al. 2012) followed reaction protocols by Ivanova et al. (2008).

Extracted DNA from a subset of samples was sent to the Canadian Centre of DNA barcoding for PCR amplification and sequencing. These samples used CO1 primers (C_VF1LFt1 + C_VF1LRt1) in PCR reactions (Ivanova et al. 2007). The remaining samples were amplified in-house. Thermocycler (*Techne Prime Gradient*) programmes differed depending on the primer and reaction used. CO1 (dgLCO-1490 + dgHCO-2198) and 16S (16Sar-L + 16Sbr-H) reactions were run using the protocol outlined by Crawford et al. (2010). Primer set (COI, Chmf4 + Chmr4) followed thermocycler profiles by (Ivanova et al. 2008). Two percent agar gels were used for electrophoresis with 1% TAE (Smith et al. 2008). Gels were visualized using an *ImageQuant LAS4000* and *Nanodrop 2000* quantification was performed on each successful PCR product visualized at the correct length, prior to dilution.

Bioinformatics

Concatenated gene alignments were used in the phylogenetic analyses. GENEIOUS v11.0.5 (Kearse et al. 2012) bioinformatics software was used to assemble forward and reverse sequences from returned CO1 and 16S chromatographs. Forward and reverse (compliment) sequences were aligned using Geneious' alignment (Global alignment with free end Gaps; Cost matrix = 65% similarity (5.0/-4.0); Gap open penalty = 12; Extension penalty = 3). Sequences were trimmed at the 3' and 5' ends where low quality base calls were present. Consensus sequences were produced for each sample, ranging from 609-658 base pairs (bp) in length for CO1 and 578-601bp for 16S. For both CO1 and 16S, a BLAST search (Altschul

et al. 1990) was conducted using a consensus sequence derived from all Costa Rican sequences. Additional *Lithobates* species sequence data were downloaded to represent an ingroup for *L. warszewitschii* based on previous phylogenetic studies (e.g., Hillis and Wilcox 2005, Frost et al. 2006, Che et al. 2007, Huang et al. 2016): *Lithobates clamitans* (Latreille, 1801), *Lithobates catesbeiana* (Shaw, 1802), *Lithobates maculata* (Brocchi, 1877), *Lithobates palmipes* (Spix, 1824), *Lithobates septentrionalis* (Baird, 1854), *Lithobates sylvatica* (LeConte, 1825), *Lithobates vaillanti* (Brocchi, 1877), *Rana maoershanensis* (Lu et al. 2007) was used as an outgroup (Zhou et al. 2017). All sequences were archived in Genbank (Benson et al. 2012; Table 2). All relevant sequences for each gene were then Geneious aligned (Maddison 1997). Only individuals which had sequence data for both genes were included in the concatenated alignment for the phylogenetic analyses. *Lithobates clamitans*, *L. maculata*, *L. septentrionalis* and *L. vaillanti* were represented by different individuals on 16S and CO1 phylogenetic analyses.

Separate Bayesian consensus trees for the CO1 and 16S alignments were estimated independently using MR BAYES v3.2.6 (Ronquist et al. 2013) to ensure they do not conflict with each other. After establishing that there were no conflicts, columns with gaps were removed from the two individual alignments, which were then concatenated end to end with PhyUtility v.2.7.1 (Smith et al., 2008). This concatenated alignment was then used to construct trees using a Bayesian framework (Mr. Bayes with default settings used for Markov chain Monte Carlo (MCMC) analysis—1,000,000 generations, 4 chains, 2 runs, a sample frequency of 500, and a 25% burn-in) and a maximum likelihood framework (RAxML; Stamatakis 2014); 20 maximum-likelihood trees generated on distinct starting trees, 1000 bootstrap replicates calculated and annotated on the best maximum-likelihood tree). The alignment was partitioned by gene, meaning model parameters were unlinked across the partition, to account for the different evolutionary histories of the COI and 16S genes. The General Time Reversible (GTR) model of substitution (Tavaré 1986) was used for all trees in order to be consistent between the Bayesian and maximum likelihood approaches since GTR is the model implemented in RAxML. Rate variation among sites was modelled as a discrete gamma distribution with four rate categories. Trees were rooted on the outgroup (*R. maoershanensis)* and visualised in FigTree v1. 4. 2 (Rambaut 2014).

Species boundaries were assessed in two ways. The first using the GENEIOUS plugin SPECIES DELIMITATION (Masters et al. 2011), which calculates the probability of reciprocal monophylly against the null model of random coalescence (Rosenberg 2007) for single panmictic populations (Rodrigo et al. 2008) and presents the probability for correct identification for putative species, given the data (Ross et al. 2008). Groups with P (Randomly Distinct) values of 0.05 – 1, represent branching events that would be expected under a coalescent model in a Wright-Fisher population and a strict molecular clock (Rodrigo et al. 2008, Masters et al. 2011). The second method used the Automatic Barcode Gap Discovery for primary species delimitation (ABGD; Puillandre et al. 2012) via a web interface (http://wwwabi.snv.jussieu.fr/public/abgd/). A maximum of ten, and minimum of two samples per geographic locality of the focal species were used as required for the minimum estimation of genetic divergence (Hickerson et al. 2007), a minimum of one sample was considered adequate for interspecific analysis (Aliabadian et al. 2009). Where possible, the same individuals were used in the analyses of both genes. Intraspecific and interspecific genetic distances were also calculated and analysed. Average, K2P-corrected (Kimura 1980) pairwise distance (K2P-π) and net between group mean distance (NBGMD) (π_{net}) (Nei and Li 1979) were calculated in MEGA v6 (Tamura et al. 2013) to assess nucleotide diversity ($\boldsymbol{\pi}$) and cryptic speciation within and between sites.

Results

Phylogenetic comparison

Concatenated phylogenetic trees reconstructed using Bayesian inference (Figure 2) and Maximum likelihood (Figure 3) methods, show similar topology of three major clades within the focal species. Geographic samples from ACG and Brewster formed well-supported independent monophyletic groups. However, samples from El Copé presented a polyphyletic structure. Four out of five individuals (KRL 1496, KRL 1508, KRL 1540, KRL 1567) formed an independent clade, sister to the ACG clade, whereas sample KRL 0823 formed a clade with samples from Brewster – revealing the presence of two taxa at El Copé. Subsequently, three clades are recognized: ACG and El Copé, containing samples exclusively from these areas, and Brewster (including sample KRL 0823 from El Copé). Single gene trees showed a similar topology to the concatenated ones (Supplementary figures 1 and 2).

CO1 operational taxonomic units (OTUs) delimitation *results*

CO1 species delimitation in GENEIOUS yielded three OTUs (Table 3). Focal clades ACG, Brewster (+KRL0823), and El Copé (KRL 1496, KRL 1508, KRL 1540, KRL 1567) had P values <0.05, indicating they are not conforming to the expected Wright-Fisher criteria. According to this assumption and the data present, all clades were taxonomically distinct. ABGD analysis supported these three distinct OTUs as well (p= 0.0359, supplementary table 1).

CO1 and 16S nucleotide diversity

K2P-π at the CO1 and 16S loci showed a mean value of 7.2% and 3.4%, respectively, within all *L. warszewitschii* samples (Table 4). Samples from El Copé had the highest intra-group mean distance at 6.3% and 3.2%, respectively, whereas samples from ACG had 0.4% and 0.3% and within Brewster 0.1% and 0.2%, respectively. Mean intraspecific distances between ACG and Brewster samples (COI/16S) were the highest at 15.7%/7.2% (Supplementary Table 2). Samples from ACG and El Copé shared the lowest distance at 10.7%/6.2%, and the intermediate distance was 13.8%/6.7% between Brewster and El Copé samples. Interspecific comparisons within the genus resulted in lower interspecific distances among recognized species (COI/16S), such as: *L. clamitans* and *L. catesbeiana* (5.7%/2%), *L. septentrionalis* and *L. clamitans* (8.3%/3.1%), *L. septentrionalis* and *L. catesbeiana* (8.6%/2.2%).

CO1 and 16S Net between group mean distance (NBGMD) (π_{net})

At the CO1 and 16S loci the largest NBGMD (π_{net}) was 15.4% and 6.9%, respectively, between ACG and Brewster samples (Supplementary Tables 2 and 3). Samples from ACG and El Copé shared the lowest distance at 7.3% and 4.5%, respectively, and the intermediate distance was 10.6% and 5%, respectively, between El Copé and Brewster samples. Most intraspecific distances between the geographic groups within *L. warszewitschii*, surpassed the interspecific values between recognized species within the genus (COI/16S), such as: *L. catesbeiana* and *L. clamitans* (5.7%/2%), *L. clamitans* and *L. septentrionalis* (8.3%/3.1%), *L. catesbeiana* and *L. septentrionalis* (8.6%/2.2%).

Discussion

The concatenated phylogenetic trees consistently outlined three distinct clades within *Lithobates warszewitschii* supported by high posterior probabilities, bootstrap values and

taxonomic distinctness at the CO1 locus. No field sites within the ACG exhibited any well-defined cladistic structure, indicating it is a larger panmictic population. The individuals from El Copé were polyphyletic, revealing the presence of two OTUs at this site. Geographic groups within *L. warszewitschii* also exhibited greater genetic distances than many other recognized species pairs within the genus, suggesting cryptic species may be present.

In the analyses of nucleotide diversity and NBGMD, isolation by distance (IBD) (Wright 1943) does not explain all patterns of genetic variation, as samples from ACG and El Copé are most closely related in all scenarios. Additionally, the range of 16S (K2P- π) distance values within El Copé reached the highest for any geographic group at both loci. Thus, there is evidence that IBD contributes towards greater polymorphism in the most isolated allopatric populations, but other intrinsic (dispersal capability) and extrinsic (environmental and ecological) factors may explain large variation within and between finer geographic scales.

Isolation by distance may be the main driver of divergence or speciation among conspecific populations (Slatkin 1993) in allopatry (Vences and Wake 2007), other drivers include, low vagility due to limitations of physiology (Balinsky 1981, Navas and Otani 2007) and dispersal (Blaustein et al. 1994). However, recurrent hybridization, secondary contact or overlap with sister species can decrease this genetic distance correlation (Fouquet et al. 2007b). If populations follow a simple pattern of IBD, they may be considered with some probability, conspecific (Fouquet et al. 2007a). Conversely, where large variations in genetic distance cannot be explained by this concept, it is likely that cryptic speciation is present.

Lithobates warszewitschii is widely distributed throughout Central America, and the possibility of vicariance may explain mechanisms for genetic divergence. The Talamanca mountain range divides the Pacific and Atlantic versants at ~2000m altitude (Savage 1982). Many of the Isthmian fauna disperse through the Caribbean lowlands but have disjunct distribution along Costa Rica's Pacific southwest (McDiarmid and Savage 2005) that historically contained more dry forest. Crawford et al. (2007) hypothesized that the presence of a filter barrier (Remington 1968), caused by extreme topography and narrowing of the rainforest corridor in Panama's Bocas del Toro province induced the deepest phylogeographical split between northern and southern populations of *Craugastor* rainforest species. For *Craugastor fitzingeri* (Schmidt, 1857), a generalist species, these effects were much less accentuated and its phylogenetic structure may be attributed to a more recent range expansion. For *L. warszewitschii*, gene flow is still possible, even if regional dry forests were transformed into savannah during the Pleistocene glacial maxima (Piperno and Pearsall, 1998), patches of gallery forest that allowed reproduction in freshwater could permit dispersal westward into Costa Rica.

Although vicariance does divide sister species (Avise et al. 1987), it fails to form a general explanation for divergence in the tropics (Antonelli et al. 2010). Barriers such as mountains do not impede gene flow directly, but promote ecological gradients (Janzen 1967). An alternative explanation for the phylogeographic structure within *L. warszewitschii* could be peripatric (Mayr 1954) or dichopatric (Bush 1994) speciation – a common mode of evolution in amphibians (Vences and Wake, 2007).

Paz et al. (2015) used a trait-based phylogeographic approach to model environmental and ecological variables in Panamanian frog populations. Indirect development encouraged greater dispersal and species with large ranges had lower genetic divergence - a characteristic associated with generalists (Duminil et al. 2007). Despite being oviparous and wide-ranging, *L. warszewitschii* scored highest when modelling landscape resistance (resistance to dispersal caused by environmental conditions) and was highly divergent between Brewster and El

Copé, with large genetic distances in proportion to their geographical distance. A possible explanation for this pattern could be a secondary contact during the post glacial maxima (Schneider 1993) or selection for different ecological roles, such as within habitat or resource use (Alizon et al. 2008). It is true that *L. warszewitschii*'s colouration, habitat use, elevation range and distribution vary (Savage 2002, Leenders 2016). Thus, high intraspecific diversity may be attributed to ecological specialization (Schluter 2000) in allopatry or coexistence of sister species in sympatry, such as in El Copé. For example, even if broad colouration of this species is genuine, frogs use non-morphological signals such as advertisement calls, cuticular hydrocarbons and other pheromones in mating systems and species recognition (Bickford et al. 2007), meaning they often remain inconspicuous. Divergent or cryptic species should therefore be considered a hypothesis of separately evolving entities (Hey et al. 2003, de Quieroz 2007, Fiser et al. 2018) and species status further scrutinized through integrative taxonomic methods (Padial et al. 2010).

Polyphyly can be used as indication of undescribed species in a lineage (Fouquet et al. 2007a). However, its presence complicates the classification of species in phylogenies as it may represent transitional stages in the evolution of taxa (Hörandl and Stuessy 2010, Xiang et al. 2012). Cryptic species often show morphological, ecological or genetic differentiation and usually a degree of reproductive isolation, which may occur through phenotypic plasticity or single locus polymorphisms. Hybridization may persist, leaving traces of introgression, speciation or hybrid vigour. Alternatively, fusion may be resisted by disruptive/divergent selection or postzygotic isolation (Sasa et al. 1998). This continuum is evident across large geographic ranges to highly localized, providing explanations for the evolutionary transitions of ecological races to species (Mallet 2008). Consequently, in *L. warszewitschii*, patterns of polyphyly, relatedness between ACG and El Copé samples, or large pairwise ranges in sympatry may reflect occasional or historical gene flow from migrants, hybridization, introgression, retention of ancestral polymorphisms or incomplete lineage sorting when using mitochondrial genes (Moritz and Cicero 2004). Alternatively, the presence of two sympatric OTUs at El Copé, may reflect human-induced introduction. Because of these scenarios, nuclear DNA is also recommended in subsequent evolutionary and taxonomic studies (Vences et al. 2005).

At both CO1 and 16S loci, K2P-π mean (Meyer and Paulay 2005) intraspecific ingroup values overlapped with interspecific species values, surpassing proposed general thresholds: 8% at CO1 and 2% 16S (Crawford et al. 2010), 10% CO1, 5% 16S (Vences et al. 2005) and for neotropical amphibians at 16S (>3%) (Fouquet et al. 2007a). This indicates a wider ranging cryptic complex is present, and advocates for the use of both genes in comparative amphibian phylogenetics (Vences et al. 2005). Ultimately, concatenated genes may yield the best phylogenies (Gadagkar et al. 2005), however, interspecific comparisons are limited in this study due to having one individual representing each congeneric species, and an incomplete taxonomy that can hamper results (Meyer and Paulay 2005).

Conclusion

The type specimen of *Lithobates warszewitschii* originated from Volcán Chiriqui, western Panama (Schmidt 1857, Savage 1970), a locality near the Costa Rican border at almost equal distance between ACG and Brewster. Whilst the topotype locality was not sampled, all clades in this study may represent cryptic species. We have extended the research on cryptic diversity within *L. warszewitschii* by revealing an additional clade from ACG, and propose this clade is a candidate cryptic species that warrants further taxonomic investigation. Determination of evolutionary mechanisms are beyond the scope of this study, but an additional paraphyletic lineage from Costa Rica suggests it is probably a wide-ranging

species complex, a likely scenario for many neotropical amphibians. Population trends in Costa Rica and Panama reflect both historical factors and recent habitat destruction, declines and introduced disease. Further sampling within Costa Rica, Nicaragua and Honduras is likely to yield more cryptic diversity, and extirpation of a candidate lineage within El Copé (Crawford et al. 2010) highlights the importance of DNA barcoding in rapid, preliminary species identification. Such assessments are necessary to inform biodiversity estimates, taxonomic progress and conservation of amphibian species. Phylogeographic structure in *L. warszewitschii* highlights the difficulty in explaining mechanisms of speciation in Mesoamerican amphibian fauna. Evolutionary theory, supported by morphological, ecological, physiological and multiple genetic methods are necessary to evaluate divergent processes in this group, and in achieving species status of sister taxa in this complex.

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References

Adams M, Raadik TA, Burridge CP, Georges A (2014) Global biodiversity assessment and hyper-cryptic species complexes: more than one species of elephant in the room? Systematic Biology 63(4): 518-533. https://doi.org/10.1093/sysbio/syu017

Aliabadian M, Kaboli M, Nijman V, Vences M (2009) Molecular identification of birds: performance of distance-based DNA barcoding in three genes to delimit parapatric species. PLoS One 4(1): e4119. https://doi.org/10.1371/journal.pone.0004119

Alizon S, Kucera M, Jansen VA (2008) Competition between cryptic species explains variations in rates of lineage evolution. Proceedings of the National Academy of Sciences 105(34): 12382-12386. https://doi.org/10.1073/pnas.0805039105

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. Journal of Molecular Biology 215(3): 403-410. https://doi.org/10.1016/S0022-2836(05)80360-2

Antonelli A, Quijada-Mascareñas A, Crawford AJ, Bates JM, Velazco PM, Wüster W (2010) Molecular studies and phylogeography of Amazonian tetrapods and their relation to geological and climatic models. Amazonia, landscape and species evolution: a look into the past, 386-404. https://doi.org/10.1002/9781444306408ch24

Avise JC, Arnold J, Ball RM, Bermingham E, Lamb T, Neigel JE, Reeb CA, Saunders NC (1987) Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Annual Review of Ecology and Systematics 18(1): 489-522. https://doi.org/10.1146/annurev.es.18.110187.002421

Baillie J, Hilton-Taylor C, Stuart SN (2004) 2004 IUCN red list of threatened species: a global species assessment. IUCN, Cambridge. https://portals.iucn.org/library/sites/library/files/documents/rl-2004-001.pdf

Balinsky JB, (1981) Adaptation of nitrogen metabolism to hyperosmotic environment in Amphibia. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology 215(3): 335-350. https://doi.org/10.1002/jez.1402150311

Barquero MD, Salazar-Saavedra M, Sandoval L, Brenes D, Martínez F, Figueroa A (2010) Composition and species richness of herpetofauna in two isolated regions of southern Nicaragua. Herpetology Notes 3: 341-352. https://www.herpnet.org

Baird SF (1854) Descriptions of new genera and species of North American frogs. Proceedings of the Academy of Natural Sciences of Philadelphia 7: 59–62.

Beaupre SJ, Jacobson ER, Lillywhite HB, Zamudio K (2004) Guidelines for the use of live amphibians and reptiles in field and laboratory research. American Society of Ichthyologists and Herpetologists. Miami, Florida.

Benson DA, Cavanaugh M, Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW (2012) GenBank. Nucleic Acids eRsearch 4: 36-42. https://doi.org/10.1093/nar/gks1195

Bickford D, Lohman DJ, Sodhi NS, Ng PK, Meier R, Winker K, Ingram KK, Das I (2007) Cryptic species as a window on diversity and conservation. Trends in ecology & evolution 22(3): 148-155. https://doi.org/10.1016/j.tree.2006.11.004

Blaustein AR, Wake DB, Sousa WP (1994) Amphibian declines: judging stability, persistence, and susceptibility of populations to local and global extinctions. Conservation Biology 8(1): 60-71. https://doi.org/10.1046/j.1523-1739.1994.08010060.x

Bock WJ (2004) Species: the concept, category and taxon. Journal of Zoological Systematics and Evolutionary Research 42(3): 178-190. https://doi.org/10.1111/j.1439-0469.2004.00276.x

Bolaños F (2002) Anfibios en retirada. Ambientico 107: 12-3.

Brocchi P (1877) Sur quelques batraciens raniformes et bufoniformes de l'Amérique Centrale. Bulletin de la Société Philomathique de Paris. 7(1): 175–197.

Bush GL (1994) Sympatric speciation in animals: new wine in old bottles. Trends in Ecology & Evolution 9(8): 285-288. https://doi.org/10.1016/0169-5347(94)90031-0.

Chakrabarty P, Warren M, Page L (2013) GenSeq: An updated nomenclature and ranking for genetic sequences from type and non-type sources. ZooKeys 346: 29-41. https://doi.org/10.3897/zookeys.346.5753

Chambers EA, Hebert PD (2016) Assessing DNA barcodes for species identification in North American reptiles and amphibians in natural history collections. Plos One 11(4): e0154363. https://doi.org/10.1371/journal.pone.0154363

Che J, Chen HM, Yang JX, Jin JQ, Jiang KE, Yuan ZY, Murphy RW, Zhang YP (2012) Universal COI primers for DNA barcoding amphibians. Molecular Ecology Resources 12(2): 247-258. https://doi.org/10.1111/j.1755-0998.2011.03090.x

Che J, Pang J, Zhao H, Wu GF, Zhao EM, Zhang YP (2007) Phylogeny of Raninae (Anura: Ranidae) inferred from mitochondrial and nuclear sequences. Molecular Phylogenetics and Evolution 43(1): 1-13. https://doi.org/10.1016/j.ympev.2006.11.032

Cope ED (1894) Third addition to a knowledge of the Batrachia and Reptilia of Costa Rica. Proceedings of the Academy of Natural Sciences of Philadelphia 46: 194–206.

Crawford AJ (2003) Huge populations and old species of Costa Rican and Panamanian dirt frogs inferred from mitochondrial and nuclear gene sequences. Molecular Ecology 12(10): 2525-2540. https://doi.org/doi.org/10.1046/j.1365-294X.2003.01910.x

Crawford AJ, Bermingham E, Carolina PS (2007) The role of tropical dry forest as a long-term barrier to dispersal: a comparative phylogeographical analysis of dry forest tolerant and intolerant frogs. Molecular Ecology 16(22): 4789-4807. https://doi.org/10.1111/j.1365-294X.2007.03524.x

Crawford AJ, Lips KR, Bermingham E (2010) Epidemic disease decimates amphibian abundance, species diversity, and evolutionary history in the highlands of central Panama. Proceedings of the National Academy of Sciences 107(31): 13777-13782. https://doi.org/10.1073/pnas.0914115107

de Queiroz K (1998) The general lineage concept of species, species criteria, and the process of speciation. In: Howard DJ, Berlocher SH (Eds), Endless Forms: Species and Speciation. Oxford University Press, Oxford, 57-75.

de Queiroz K (1999) The general lineage concept of species and the defining properties of the species category. Wilson RA (Eds) Species: New Interdisciplinary Essays. MIT Press, Cambridge, 49-49.

de Queiroz K (2007) Species concepts and species delimitation. Systematic biology 56(6): 879-886.

Duminil J, Fineschi S, Hampe A, Jordano P, Salvini D, Vendramin GG, Petit RJ (2007) Can population genetic structure be predicted from life-history traits? The American Naturalist 169(5): 662-672. https://doi.org/10.1086/513490

Fišer C, Robinson CT, Malard F (2018) Cryptic species as a window into the paradigm shift of the species concept. Molecular Ecology. https://doi.org/10.1111/mec.14486

Fouquet A, Gilles A, Vences M, Marty C, Blanc M, Gemmell NJ (2007a) Underestimation of species richness in Neotropical frogs revealed by mtDNA analyses. PLoS One 2(10): e1109. https://doi.org/10.1371/journal.pone.0001109

Fouquet A, Vences M, Salducci MD, Meyer A, Marty C, Blanc M, Gilles A (2007b)

Revealing cryptic diversity using molecular phylogenetics and phylogeography in frogs of the Scinax ruber and Rhinella margaritifera species groups. Molecular Phylogenetics and Evolution 43(2): 567-582. https://doi.org/10.1016/j.ympev.2006.12.006

Frost DR, Grant T, Faivovich J, Bain RH, Haas A, Haddad CF, De Sa RO, Channing A, Wilkinson M, Donnellan SC, Raxworthy CJ (2006) The amphibian tree of life. Bulletin of the American Museum of Natural History: 1-291. https://doi.org/10.1206/0003-0090(2006)297[0001:TATOL]2.0.CO;2

Gadagkar SR, Rosenberg MS, Kumar S (2005) Inferring species phylogenies from multiple genes: concatenated sequence tree versus consensus gene tree. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution 304(1): 64-74. https://doi.org/10.1002/jez.b.21026

Hammond SA, Warren RL, Vandervalk BP, Kucuk E, Khan H, Gibb EA, Pandoh P, Kirk H, Zhao Y, Jones M, Mungall AJ (2017) The North American bullfrog draft genome provides insight into hormonal regulation of long noncoding RNA. Nature Communications 8(1): 1433. https://doi.org/10.1038/s41467-017-01316-7.

Hey J (2006) On the failure of modern species concepts. Trends in Ecology & Evolution 21(8): 447-450. https://doi.org/10.1016/j.tree.2006.05.011

Hey J, Waples RS, Arnold ML, Butlin RK, Harrison RG (2003) Understanding and confronting species uncertainty in biology and conservation. Trends in Ecology & Evolution 18(11): 597-603. https://doi.org/10.1016/j.tree.2003.08.014

Hickerson MJ, Stahl E, Takebayashi N (2007) msBayes: pipeline for testing comparative phylogeographic histories using hierarchical approximate Bayesian computation. BMC Bioinformatics 8(1): 268. https://doi.org/10.1186/1471-2105-8-268

Hilje B, Aide TM (2012) Recovery of amphibian species richness and composition in a chronosequence of secondary forests, northeastern Costa Rica. Biological Conservation 146(1): 170-176. https://doi.org/10.1016/j.biocon.2011.12.007

Hillis DM, Wilcox TP (2005) Phylogeny of the New World true frogs (Rana). Molecular Phylogenetics and Evolution 34(2): 299-314. https://doi.org/10.1016/j.ympev.2004.10.007

Hörandl E, Stuessy TF (2010) Paraphyletic groups as natural units of biological classification. Taxon 59(6): 1641-1653. https://doi.org/10.2307/41059863

Huang Z, Yang C, Ke D (2016) DNA barcoding and molecular phylogeny in Ranidae. Mitochondrial DNA Part A 27(6): 4003-4007. https://doi.org/10.3109/19401736.2014.989522

Isidoro-Ayza M, Lorch JM, Grear DA, Winzeler M, Calhoun DL, Barichivich WJ (2017) Pathogenic lineage of Perkinsea associated with mass mortality of frogs across the United States. Scientific Reports 7(1): 10288. https://doi.org/10.1038/s41598-017-10456-1

IUCN SSC Amphibian Specialist Group (2015) Lithobates warszewitschii. The IUCN Red List of Threatened Species 2015: e.T58749A54353071. http://dx.doi.org/10.2305/IUCN.UK.2015-4.RLTS.T58749A54353071.en.

Ivanova NV, Fazekas AJ, Hebert PD (2008) Semi-automated, membrane-based protocol for DNA isolation from plants. Plant Molecular Biology Reporter 26(3): 186. https://doi.org/10.1007/s11105-008-0029-4

Ivanova NV, Zemlak T, Hanner R, Hebert PD (2007) Universal primer cocktails for fish DNA barcoding. Molecular Ecology Notes 7: 544-548.

https://doi:10.1111/j.1471-8286.2007.01748.x

Janzen DH (1967) Why mountain passes are higher in the tropics. The American Naturalist 101(919): 233-249. https://doi.org/10.1086/282487

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28(12): 1647-1649. https://doi.org/10.1093/bioinformatics/bts199.

Kessing B, Crrom H, Martin A, McIntosh C, Mcmillan WO, Palumbi S (2004) The Simple Fool's Guide to PCR, version 1.0. Department of Zoology, University of Hawaii, Honolulu, 24 pp

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16(2): 111-120. https://doi.org/10.1007/BF01731581

Kubicki B (2008) Amphibian diversity in Guayacán, Limón province, Costa Rica. Brenesia 69: 35-42. https://crarc5.files.wordpress.com/2012/07/amphib-of- guayacan.pdf

Latreille PA (1801) Histoire Naturelle des Reptiles, avec Figures dissinées d'après Nature. Volume 2. Paris: Deterville.

Le Conte JE (1825) Remarks on the American species of the genera Hyla and Rana. Annals of the Lyceum of Natural History of New-York 1: 278–282.

Leenders T (2016) Amphibians of Costa Rica: A Field Guide. Cornell University Press, 484-485 pp.

Lu YY, Li PP, Jiang DB (2007) A new species of Rana (Anura, Ranidae) from China. Acta Zootaxonomica Sinica 32(4): 792-801. http://bionames.org/

Lyra ML, Haddad CF, Azeredo-Espin AML (2017) Meeting the challenge of DNA barcoding Neotropical amphibians: polymerase chain reaction optimization and new COI primers. Molecular Ecology Resources 17(5): 966-980. https://doi.org/10.1111/1755-0998.12648

Maddison WP (1997) Gene trees in species trees. Systematic Biology 46(3): 523-536. https://doi.org/10.1093/sysbio/46.3.523

Mallet J (2008) Hybridization, ecological races and the nature of species: empirical evidence for the ease of speciation. Philosophical Transactions of the Royal Society B: Biological Sciences 363(1506): 2971-2986. https://doi.org/10.1098/rstb.2008.0081

Masters BC, Fan V, Ross HA (2011) Species delimitation–a geneious plugin for the exploration of species boundaries. Molecular Ecology Resources 11(1): 154-157. https://doi.org/10.1111/j.1755-0998.2010.02896.x

Mayden RL (1997) A hierarchy of species concepts: the denouement in the saga of the species problem. In: Claridge MF, Dawah HA, Wilson MR (Eds) Species: The units of diversity. Chapman & Hall, London, 381-423.

Mayr E (1942) Systematics and the origin of species, from the viewpoint of a zoologist. Harvard University Press, New York.

Mayr E (1954) Change of genetic environment and evolution. In: Huxley J, Hardy AC, Ford EB (Eds) Evolution as a Process, Unwin Brothers, London. 157–180. http://krishikosh.egranth.ac.in/bitstream/1/22987/1/IVRI%20OB%201897.pdf

McDiarmid RW, Savage JM (2005) The herpetofauna of the Rincón area, Península de Osa, Costa Rica, a Central American lowland evergreen forest site. In: Donnelly MA, Crother BI, Guyer C, Wake MH, White ME (Eds) Ecology and Evolution in the Tropics. University of Chicago Press, Chicago, 366-427.

Meyer CP, Geller J, Paulay G (2005) Fine scale endemism on coral reefs: archipelagic differentiation in turbinid gastropods. Evolution 59: 133. https://doi:10.1554/04-194

Meyer CP, Paulay G (2005) DNA barcoding: error rates based on comprehensive sampling. PLoS Biology 3(12): e422. https://doi.org/10.1371/journal.pbio.0030422

Moritz C, Cicero C (2004) DNA barcoding: promise and pitfalls. PLoS Biology 2(10): e354. https://doi.org/10.1371/journal.pbio.0020354

Mulder KP, Cortazar-Chinarro M, Harris DJ, Crottini A, Grant EHC, Fleischer RC, Savage AE (2017) Evolutionary dynamics of an expressed MHC class IIβ locus in the Ranidae (Anura) uncovered by genome walking and high-throughput amplicon sequencing. Developmental & Comparative Immunology 76: 177-188. https://doi.org/10.1016/j.dci.2017.05.022

Navas CA, Otani L (2007) Physiology, environmental change, and anuran conservation. Phyllomedusa. Journal of Herpetology 6(2): 83-103. https://doi.org/10.11606/issn.2316- 9079.v6i2p83-103

Nei M, Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proceedings of the National Academy of Sciences 76(10): 5269-5273. https://doi.org/10.1073/pnas.76.10.5269

Ni N, Yu D, Storey KB, Zheng R, Zhang J (2016) The complete mitochondrial genome of Lithobates sylvaticus (Anura: Ranidae). Mitochondrial DNA Part A 27(4): 2460-2461. https://doi.org/10.3109/19401736.2015.1033697

Nicholls JA, Double MC, Rowell DM, Magrath RD (2000) The evolution of cooperative and pair breeding in thornbills Acanthiza (Pardalotidae). Journal of Avian Biology 31(2): 165-176. https://doi.org/10.1034/j.1600-048X.2000.310208.x

Padial JM, Miralles A, De la Riva I, Vences M (2010) The integrative future of taxonomy. Frontiers in Zoology 7(1):16. https://doi.org/10.1186/1742-9994-7-16

Paz A, Ibáñez R, Lips KR, Crawford AJ (2015) Testing the role of ecology and life history in structuring genetic variation across a landscape: a trait-based phylogeographic approach. Molecular Ecology 24(14): 3723-3737. https://doi.org/10.1111/mec.13275

Perez – Ponce de León GPP, Poulin R (2016) Taxonomic distribution of cryptic diversity among metazoans: not so homogeneous after all. Biology Letters 12(8): 20160371. https://doi.org/10.1098/rsbl.2016.0371

Perry G, Wallace MC, Perry D, Curzer H, Muhlberger P (2011) Toe clipping of amphibians and reptiles: science, ethics, and the law. Journal of Herpetology 45(4): 547-555. https://doi.org/10.1670/11-037.1

Pimm SL, Jenkins CN, Abell R, Brooks TM, Gittleman JL, Joppa LN, Raven PH, Roberts CM, Sexton JO (2014) The biodiversity of species and their rates of extinction, distribution, and protection. Science 344(6187): 1246752. https://doi.org/10.1126/science.1246752

Piperno DR, Pearsall DM (1998) The origins of agriculture in the lowland Neotropics. Academic Press, San Diego.

Puillandre, N, A. Lambert, S. Brouillet, S G. Achaz. (2012), ABGD, Automatic Barcode Gap Discovery for primary species delimitation. Molecular Ecology, 21: 1864-1877. doi:10.1111/j.1365-294X.2011.05239.x

Puschendorf R, Bolaños F, Chaves G (2006) The amphibian chytrid fungus along an altitudinal transect before the first reported declines in Costa Rica. Biological Conservation 132(1): 136-142. https://doi.org/10.1016/j.biocon.2006.03.010

Rambaut A (2014) FigTree-v1.4.2. http://tree.bio.ed.ac.uk/software/figtree/

Ranvestel AW, KR Lips, CM Pringle, MR Whiles, RJ Bixby (2004) Neotropical tadpoles influence stream benthos: evidence for the ecological consequences of decline in amphibian populations. Freshwater Biology 49(3): 274-285. https://doi.org/10.1111/j.1365-2427.2004.01184.x

Remington CL (1968) Suture-zones of hybrid interaction between recently joined biotas. In: Dobzhansky T, Hecht MK, Steere WC (Eds) Evolutionary biology. Springer, Boston, 321-428.

Rodrigo A, Bertels F, Heled J, Noder R, Shearman H, Tsai P (2008) The perils of plenty: what are we going to do with all these genes?. Philosophical Transactions of the Royal Society B. Biological Sciences 363(1512): 3893-3902. https://doi.org/10.1098/rstb.2008.0173

Ronquist F, Teslenko M, van der Mark P, Ayres D, Darling A, Höhna S, Larget B, Liu L, Suchard MHuelsenbeck J (2012) MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice Across a Large Model Space. Systematic Biology 61: 539-542. doi: https://doi.org/10.1093/sysbio/sys029

Rosenberg NA (2007) Statistical tests for taxonomic distinctiveness from observations of monophyly. Evolution 61(2): 317-323. https://doi.org/10.1111/j.1558-5646.2007.00023.x

Ross HA, Murugan S, Sibon Li WL (2008) Testing the reliability of genetic methods of species identification via simulation. Systematic Biology 57(2): 216-230. https://doi.org/10.1080/10635150802032990

Santos-Barrera G, Pacheco J, Mendoza-Quijano F, Bolaños F, Cháves G, Daily GC, Ehrlich PR, Ceballos G (2008) Diversity, natural history and conservation of amphibians and reptiles from the San Vito Region, southwestern Costa Rica. Revista de Biología Tropical 56(2): 755-778. https://doi.org/10.15517/rbt.v56i2.5622

Sasa MM, Chippindale PT, Johnson NA (1998) Patterns of postzygotic isolation in frogs. Evolution 52(6): 1811-1820. https://doi.org/10.1111/j.1558-5646.1998.tb02258.x.

Savage JM (1982) The enigma of the Central American herpetofauna: dispersals or vicariance? Annals of the Missouri Botanical Garden 69(3): 464-547. https://doi.org/10.2307/2399082

Savage JM (1970) On the trail of the golden frog: with Warszewicz and Gabb in Central America. Proceedings of the California Academy of Sciences 38(4): 273–288.

Savage JM (2002) The amphibians and reptiles of Costa Rica: a herpetofauna between two continents, between two seas. University of Chicago press. Chicago, 404-405 pp.

Schluter D (2000) Ecological character displacement in adaptive radiation. The American Naturalist 156(S4): S4-S16. https://doi.org/10.1086/303412

Schmidt O (1857) Diagnosen neuer Frösche des zoologischen Cabinets zu Krakau. Sitzungsberichte der Kaiserlichen Akademeie der Wissenschaften, Mathematisch-Naturwissenschaftliche Classe 24: 10-15.

Shaw G (1802) General Zoology or Systematic Natural History. Volume 3, Part 1. Amphibia. London: Thomas Davison.

Slatkin M (1993) Isolation by distance in equilibrium and non-equilibrium populations. Evolution 47(1): 264-279. https://doi.org/10.1111/j.1558-5646.1993.tb01215.x

Smith M, Poyarkarkov NA, Hebert PD (2008) DNA barcoding: CO1 DNA barcoding amphibians: take the chance, meet the challenge. Molecular Ecology Resources 8(2): 235-246. https://doi.org/10.1111/j.1471-8286.2007.01964.x

Smith SA, Dunn CW. Phyutility: a phyloinformatics tool for trees, alignments and molecular data. Bioinformatics. 2008; 24: 715–716. https://doi.org/10.1093/bioinformatics/btm619 PMID: 18227120

Spix JB (1824) Animalia nova sive Species novae Testudinum et Ranarum quas in itinere per Brasiliam annis MDCCCXVII–MDCCCXX jussu et auspiciis Maximiliani Josephi I. Bavariae Regis. München: F. S. Hübschmann.

Stuart BL, Inger RF, Voris HK (2006) High level of cryptic species diversity revealed by sympatric lineages of Southeast Asian forest frogs. Biology Letters 2(3): 470-474. https://doi.org/10.1098/rsbl.2006.0505

Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 2014 May 1;30(9):1312-3. https://doi.org/10.1093/bioinformatics/btu033

Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues AS, Fischman DL, Waller RW (2004) Status and trends of amphibian declines and extinctions worldwide. Science 306(5702): 1783-1786. https://doi.org/10.1126/science.1103538

Sunyer J, Páiz G, Dehling DM, Köhler G (2009) A collection of amphibians from Río San Juan, southeastern Nicaragua. Herpetology Notes 2: 189-202. http://www.herpetologynotes.seh-herpetology.org/

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution, 30(12): 2725-2729. https://doi.org/10.1093/molbev/mst197

Tavaré S (1986) Some probabilistic and statistical problems in the analysis of DNA sequences. Lectures on Mathematics in the Life Sciences 17(2): 57-86.

Vences M, Wake DB (2007) Speciation, species boundaries and phylogeography of amphibians. Amphibian Biology 7: 2613-2671. https://doi.org/10.1.1.694.4387

Vences M, Thomas M, Bonett RM, Vieites DR (2005). Deciphering amphibian diversity through DNA barcoding: chances and challenges. Philosophical Transactions of the Royal Society B 360(1462): 1859-1868. https://doi.org/10.1098/rstb.2005.1717

Vieites DR, Wollenberg KC, Andreone F, Köhler J, Glaw F, Vences M (2009) Vast underestimation of Madagascar's biodiversity evidenced by an integrative amphibian inventory. Proceedings of the National Academy of Sciences 106(20): 8267-8272. https://doi.org/10.1073/pnas.0810821106

Wang IJ, Crawford AJ, Bermingham E (2008) Phylogeography of the Pygmy Rain Frog (Pristimantis ridens) across the lowland wet forests of isthmian Central America. Molecular Phylogenetics and Evolution 47(3): 992-1004. https://doi.org/10.1016/j.ympev.2008.02.021

Whitfield SM, Bell KE, Philippi T, Sasa M, Bolaños F, Chaves G, Savage JM, Donnelly MA (2007) Amphibian and reptile declines over 35 years at La Selva, Costa Rica. Proceedings of the National Academy of Sciences 104(20): 8352-8356. https://doi.org/10.1073/pnas.0611256104

Whitfield SM, Lips KR, Donnelly MA (2016) Amphibian decline and conservation in Central America. Copeia 104(2): 351-379. https://doi.org/10.1643/CH-15-300

Wright S (1943) Isolation by distance. Genetics 28(2): 114-138. https://www.ncbi.nlm.nih.gov/pubmed/17247074

Figures legends and Tables.

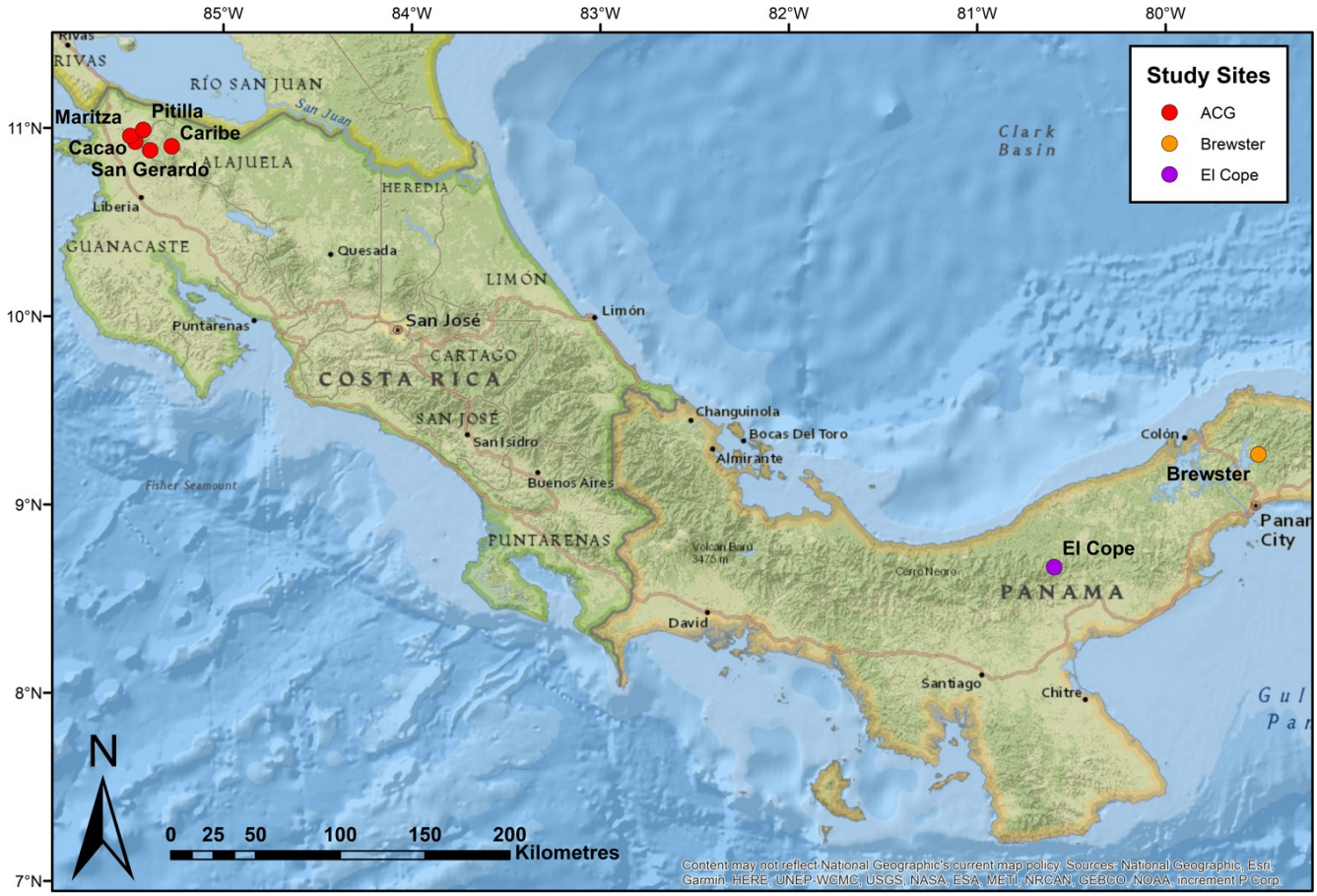


Figure 1. Study sites included in phylogenetic analysis of *L. warszewitschii*. Sites: Cacao, Caribe, Maritza and San Gerardo are within the Área de Conservación Guanacaste (ACG), Costa Rica. Sites El Cope and Brewster are within Panama.

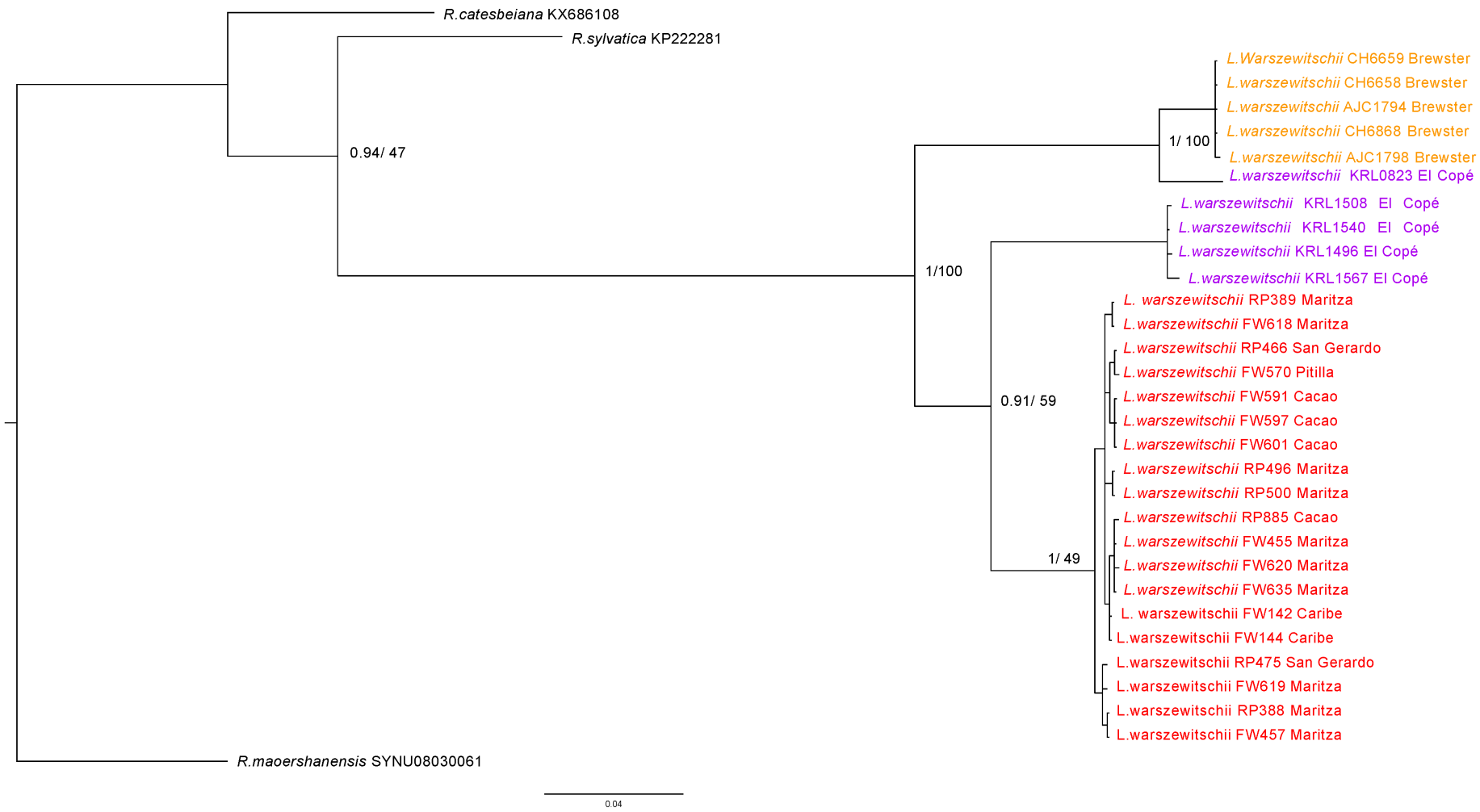


Figure 2. Phylogenetic reconstruction of *Lithobates warszewitschii* relationships between Costa Rican and Panamanian populations using concatenated alignments of CO1 and 16S. Node support values (posterior probabilities) and percentages calculated from 1000 bootstrap

replicates are annotated at nodes. Samples collected in different localities are represented by different colours: individuals from Área de Conservación Guanacaste (ACG; Cacao, Caribe, Maritza, Pitilla and San Gerardo) highlighted in red, individuals from Brewster highlighted in purple, and individuals from El Copé highlighted in orange. Sample information can be found in Table 2. Separate trees were constructed in Mr. Bayes and RAxML using a GTR model of molecular evolution, both with similar topologies, therefore node supports were included in a single tree. Scale of branch lengths is in nucleotide substitutions per site.

Table 1. Information on study sites

Sites	Collection dates	No. tissue samples	Habitat	Longitude	Latitude	Elevation (m)	Reference
Pitilla	August, 2016	1	Rainforest	10.989	-85.426	650-750	Field data - this study
	June, 2017	1					
San Gerardo	August, 2017	2	Rainforest/pasture land	10.881	-85.389	470-640	Field data - this study
Maritza	June, 2015	7	Dry/wet forest	10.956	-85.495	570-610	Field data - this study
	August, 2015	7					
	November, 2016	6					
	July, 2017	3					
	August, 2017	5					
Cacao	November, 2016	4	Rain/cloud forest	10.923	-85.468	980-1130	Field data - this study
	August, 2017	3					
Caribe	June, 2015	4	Rainforest	10.902	-85.275	370	Field data - this study
El Copé	July, 2010	NA	Rainforest	8.667	-80.592	700-750	(KRL 0823) Paz et al. 2015
Brewster	June, 2015	NA	Rainforest	9.265	-79.508	130-810	(CH 6868) Paz et al. 2015

Table 1. Description of sites where populations of *Lithobates warszewitschii* were sampled. Habitat type, georeferences and information sources (field data GPS coordinates, or external sources e.g. other researchers, ACG website or literature) are included.

Table 2. Genbank (NCBI) Voucher ID & Accession numbers

Species	Study Site	Voucher ID	CO1 Genbank Accession #	16S Genbank Accession #
L. warszewitschii	Maritza	RP 388	MH559513	MH603380
L. warszewitschii	Maritza	RP 389	MH559517	MH603379
L. warszewitschii	Pitilla	RP 435	NA	MH603378
L. warszewitschii	San Gerardo	RP 466	MH559519	MH603377
L. warszewitschii	San Gerardo	RP 475	MH559514	MH603376
L. warszewitschii	Maritza	RP 496	MH559518	MH603375
L. warszewitschii	Maritza	RP 500	MH559515	MH724925
L. warszewitschii	Cacao	RP 878	NA	MH724926
L. warszewitschii	Cacao	RP 885	MH559516	MH724927
L. warszewitschii	Cacao	RP 887	NA	MH724928
L. warszewitschii	Caribe	RP Fw142	MH559500	MH603393
L. warszewitschii	Caribe	RP Fw144	MH559501	MH603392
L. warszewitschii	Caribe	RP Fw147	MH559502	NA
L. warszewitschii	Maritza	RP Fw455	MH559503	MH603391
L. warszewitschii	Maritza	RP Fw457	MH559504	MH603390
L. warszewitschii	Pitilla	RP Fw570	MH559505	MH603389
L. warszewitschii	Cacao	RP Fw591	MH559506	MH603388
L. warszewitschii	Cacao	RP Fw597	MH559507	MH603387
L. warszewitschii	Cacao	RP Fw601	MH559508	MH603386
L. warszewitschii	Cacao	RP Fw616	NA	MH603385
L. warszewitschii	Maritza	RP Fw618	MH559509	MH603384
L. warszewitschii	Maritza	RP Fw619	MH559510	MH603383
L. warszewitschii	Maritza	RP Fw620	MH559511	MH603382
L. warszewitschii	Maritza	RP Fw635	MH559512	MH603381
L. warszewitschii	Brewster	CH 6868	KR863019	KR863275
L. warszewitschii	Brewster	AJC 1794	KR863021	KR863277
L. warszewitschii	Brewster	AJC 1798	KR863026	KR863282
L. warszewitschii	Brewster	CH 6658	KR863027	KR863283
L. warszewitschii	Brewster	CH6659	KR863028	KR863284
L. warszewitschii	El Copé	KRL 0823	FJ766749	FJ84384
L. warszewitschii	El Copé	KRL 1540	FJ766751	FJ84552
L. warszewitschii	El Copé	KRL 1508	KR911913	KR911916
L. warszewitschii	El Copé	KRL 1496	KR911914	KR911917
L. warszewitschii	El Copé	KRL 1567	KR911915	KR911918
L. catesbeiana	*NA*	-	KX686108*	KX686108*
L. clamitans	*NA*	-	EF525879	KY677813
L. maculata	*NA*	-	*NA*	AY779207
L. palmipes	*NA*	CFBHT12435	KU494586	KU495379
L. septentrionalis	*NA*	-	EF525896	AY779200
L. sylvatica	*NA*	-	KP222281*	KP222281*
L. vaillanti	*NA*	-	KY587190	AY779214
R. maoershanensis	*NA*	SYNU08030061	KX1397728	KX1397722

Table 2. Voucher ID and GenBank accession numbers for all individuals and sequences of *Lithobates warszewitschii* used in this study. (*) indicates that gene sequences derived from a whole mitochondrial genome sequence.

Table 3. CO1 Species delimitation results

OTU	Closest OTU	Monophyletic?	Intra Dist	Inter Dist - Closest	Intra/Inter	P ID(Strict)	P ID(Liberal)	Av(MRCA-tips)	P(Randomly Distinct)	Rosenberg's P(AB)
1: ACG	2: El Cope	yes	0.01	0.109	0.08	0.97 (0.91, 1.0)	0.99 (0.96, 1.0)	0.0076	0.05	8.10E-06
2: El Cope	1: ACG	yes	0.01	0.109	0.06	0.83 (0.69, 0.97)	0.97 (0.86, 1.0)	0.0047	0.05	8.10E-06
3: Brewster & KRL 0823	2: El Cope	yes	0.02	0.197	0.08	0.88 (0.75, 1.0)	0.97 (0.87, 1.0)	0.0211	0.05	1.10E-07
4: palmipes	*5: R. vaillanti*	yes	0	0.114	0	0	0.96 (0.83, 1.0)	0	NA	1
5: R. vaillanti	*4: palmipes*	yes	0	0.114	0	0	0.96 (0.83, 1.0)	0	NA	1
6: R. catesbeiana	*7: R. clamitans*	yes	0	0.057	0	0	0.96 (0.83, 1.0)	0	NA	1
7: R. clamitans	*catesbeiana*	yes	0	0.057	0	0	0.96 (0.83, 1.0)	0	NA	1
8: R. septentrion	*7: R. clamitans*	yes	0	0.092	0	0	0.96 (0.83, 1.0)	0	NA	0.33
9: R. sylvatica	*8: R. septentrion*	yes	0	0.238	0	0	0.96 (0.83, 1.0)	0	NA	0.17

Table 3. Species delimitation results of *Lithobates warszewitschii* in Costa Rica and Panama using partial sequences of the CO1 gene. Analysis conducted in Geneious using the Species Delimitation plugin (Masters et al. 2011). Clades defined in phylogenetic analysis: ACG, Brewster (+ sample KRL 0823) and El Cope are all represented as putative species. The table also includes ingroup and outgroup species.

Table 4. Intraspecific nucleotide diversity (**π**) within geographic groups of *L. warszewitschii*

Population	Mean (**π**)	Range (**π**)
	CO1	
ACG	0.004	0 - 0.008
El Copé	0.063	0.002 - 0.154
Brewster	0.001	0 - 0.002
L. warszewitschii	0.072	0 - 0.166
	16S	
ACG	0.003	0 - 0.009
El Copé	0.032	0 - 0.076
Brewster	0.002	0 - 0.006
L. warszewitschii	0.034	0 - 0.079

Table 4. Nucleotide diversity (**π**) within *Lithobates warszewitschii* for the geographic groups ACG, Brewster and El Cope based on pairwise values for CO1 and 16S sequences. Analyses were conducted using the Kimura 2-parameter model (Kimura 1980). The rate variation among sites was modelled with a gamma distribution (shape parameter = 4).

Supplementary Section

Supplementary Table 1. ABGD analysis from CO1 using all species presented in table 2.

			Prior Intraspecific divergence								
Partition	No. of partitions	Gap width (X)	0.059948	0.035938	0.021544	0.012915	0.007743	0.004642	0.002783	0.001668	0.001
Initial partition	10	1	6	11	11	11	11	11	11	11	11
Recursive	10	1	7	11	11	11	11	11	11	21	21

nbr

Supplementary Table 1. The parameters include Gap width (X) = 1, (min) DIST = 0.001 - 0.1 (Max) DIST for P (P = maximum value for intraspecific divergence). Generated through the ABGD user interface website (http://wwwabi.snv.jussieu.fr/public/abgd/).

Supplementary Table 2. Estimates of evolutionary divergence (π), and net evolutionary divergence (π_{net}) over CO1 sequence pairs between groups

						CO1 (K2P-π)				
ACG		0.018	0.012	0.024	0.023	0.025	0.025	0.023	0.023	0.026
Brewster	0.157		0.015	0.024	0.024	0.022	0.025	0.021	0.022	0.027
El Cope	0.107	0.138		0.023	0.022	0.023	0.024	0.021	0.021	0.025
L. catesbeiana	0.264	0.256	0.250		0.010	0.022	0.012	0.021	0.022	0.021
L. clamitans	0.247	0.263	0.251	0.057		0.022	0.012	0.020	0.021	0.020
L. palmipes	0.265	0.224	0.245	0.218	0.219		0.025	0.023	0.014	0.023
L. septentrionalis	0.264	0.267	0.259	0.086	0.083	0.246		0.019	0.022	0.021
L. sylvatica	0.234	0.220	0.228	0.218	0.194	0.239	0.181		0.021	0.022
L. vaillanti	0.234	0.227	0.220	0.230	0.205	0.106	0.226	0.211		0.024
R. maoershanensis	0.239	0.274	0.255	0.199	0.183	0.228	0.199	0.208	0.241	
						CO1 (πnet)				
ACG		0.018	0.010	0.026	0.024	0.025	0.026	0.024	0.023	0.025
Brewster	0.154		0.013	0.025	0.026	0.022	0.026	0.021	0.022	0.027
El Cope	0.073	0.106		0.023	0.023	0.022	0.024	0.020	0.019	0.023
L. catesbeiana	0.262	0.256	0.218		0.010	0.023	0.012	0.023	0.024	0.021
L. clamitans	0.245	0.262	0.219	0.057		0.023	0.012	0.021	0.022	0.020
L. palmipes	0.262	0.224	0.213	0.218	0.219		0.026	0.024	0.015	0.024
L. septentrionalis	0.261	0.267	0.227	0.086	0.083	0.246		0.020	0.024	0.022
L. sylvatica	0.232	0.220	0.196	0.218	0.194	0.239	0.181		0.021	0.022
L. vaillanti	0.232	0.227	0.189	0.230	0.205	0.106	0.226	0.211		0.025
R. maoershanensis	0.237	0.274	0.224	0.199	0.183	0.228	0.199	0.208	0.241	

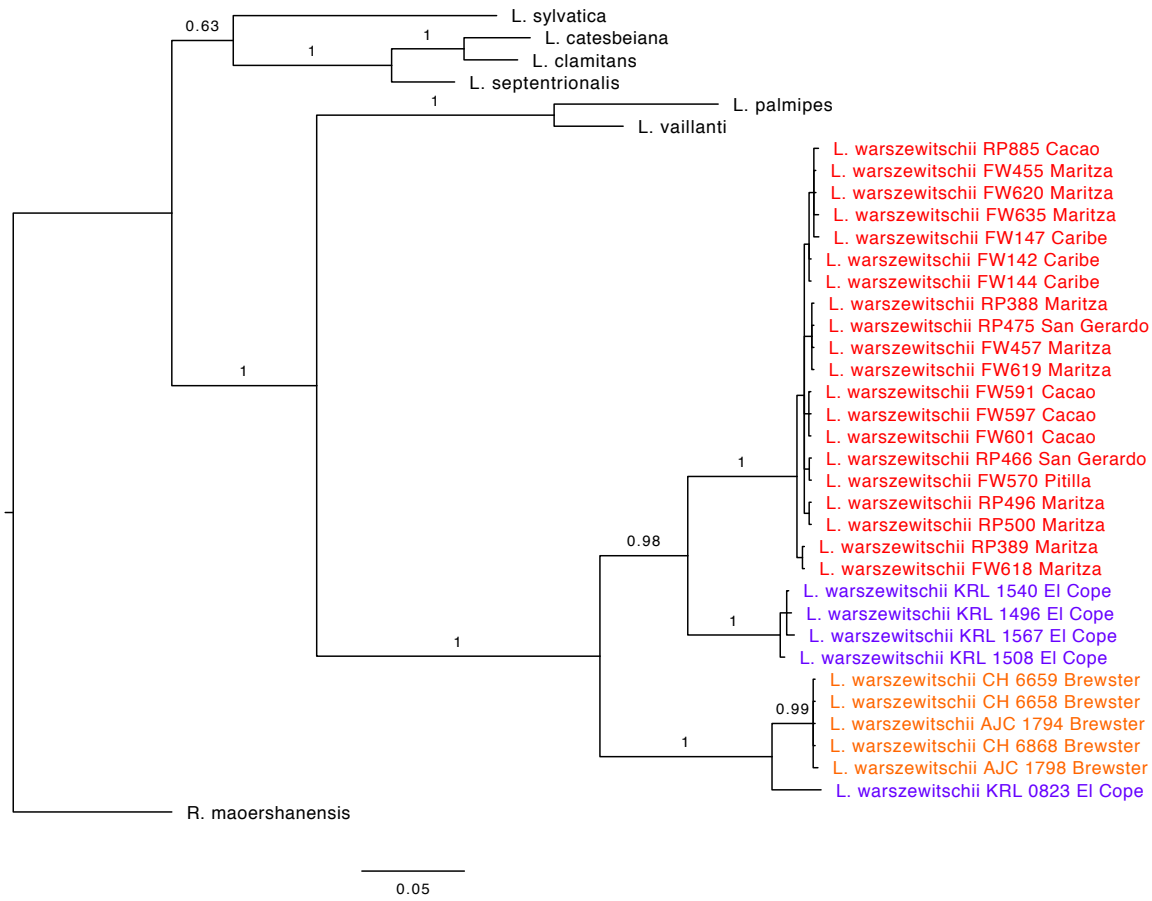
Supplementary Table 2. The number of base substitutions per site from averaging over all CO1 sequence pairs between groups are shown above (π). The number of base substitutions per site from estimation of net average between groups of CO1 sequences are shown below (NBGMD / π_{net}). Standard error estimate(s) are shown above the diagonal. Analyses were conducted using the Kimura 2-parameter model (Kimura 1980). The rate variation among sites was modelled with a gamma distribution (shape parameter = 4). The analysis involved 37 nucleotide sequences. There were a total of 658 positions in the final dataset.

Supplementary Table 3. Estimates of evolutionary divergence (π), and net evolutionary divergence (π_{net}) over 16S sequence pairs between groups

						16S (K2P-π)						
ACG		0.012	0.009	0.015	0.015	0.016	0.018	0.016	0.016	0.024	0.014	0.016
Brewster	0.072		0.010	0.016	0.017	0.017	0.019	0.016	0.017	0.028	0.014	0.019
El Cope	0.062	0.067		0.015	0.015	0.015	0.017	0.015	0.017	0.026	0.013	0.017
L. catesbeian	0.109	0.118	0.122		0.006	0.011	0.013	0.006	0.007	0.019	0.014	0.012
L. clamitans	0.117	0.126	0.131	0.020		0.010	0.013	0.008	0.007	0.020	0.016	0.013
L. maculata	0.124	0.133	0.123	0.061	0.059		0.013	0.011	0.012	0.018	0.016	0.015
L. palmipes	0.136	0.146	0.143	0.081	0.086	0.081		0.015	0.014	0.018	0.018	0.016
L. septentrior	0.120	0.119	0.124	0.022	0.031	0.063	0.091		0.009	0.019	0.015	0.014
L. sylvatica	0.127	0.139	0.139	0.033	0.031	0.071	0.093	0.041		0.021	0.016	0.012
L. vaillanti	0.219	0.258	0.242	0.154	0.174	0.151	0.128	0.156	0.174		0.024	0.020
L. vibicaria	0.094	0.091	0.095	0.104	0.115	0.109	0.126	0.113	0.117	0.210		0.018
R. maoershar	0.145	0.167	0.156	0.079	0.086	0.115	0.113	0.097	0.074	0.193	0.150	
						16S (πnet)						
ACG		0.011	0.008	0.015	0.015	0.016	0.018	0.015	0.016	0.024	0.014	0.017
Brewster	0.069		0.008	0.016	0.017	0.017	0.018	0.015	0.017	0.026	0.014	0.019
El Cope	0.045	0.050		0.014	0.015	0.014	0.017	0.015	0.016	0.025	0.012	0.016
L. catesbeian	0.108	0.117	0.106		0.006	0.011	0.013	0.006	0.007	0.018	0.015	0.012
L. clamitans	0.115	0.124	0.116	0.020		0.010	0.013	0.008	0.007	0.019	0.016	0.013
L. maculata	0.123	0.132	0.108	0.061	0.059		0.014	0.011	0.012	0.018	0.016	0.015
L. palmipes	0.135	0.145	0.128	0.081	0.086	0.081		0.014	0.014	0.018	0.018	0.015
L. septentrior	0.118	0.118	0.109	0.022	0.031	0.063	0.091		0.008	0.018	0.015	0.013
L. sylvatica	0.126	0.138	0.123	0.033	0.031	0.071	0.093	0.041		0.020	0.016	0.011
L. vaillanti	0.217	0.257	0.226	0.154	0.174	0.151	0.128	0.156	0.174		0.023	0.020
L. vibicaria	0.093	0.090	0.079	0.104	0.115	0.109	0.126	0.113	0.117	0.210		0.019
R. maoershar	0.144	0.166	0.140	0.079	0.086	0.115	0.113	0.097	0.074	0.193	0.150	

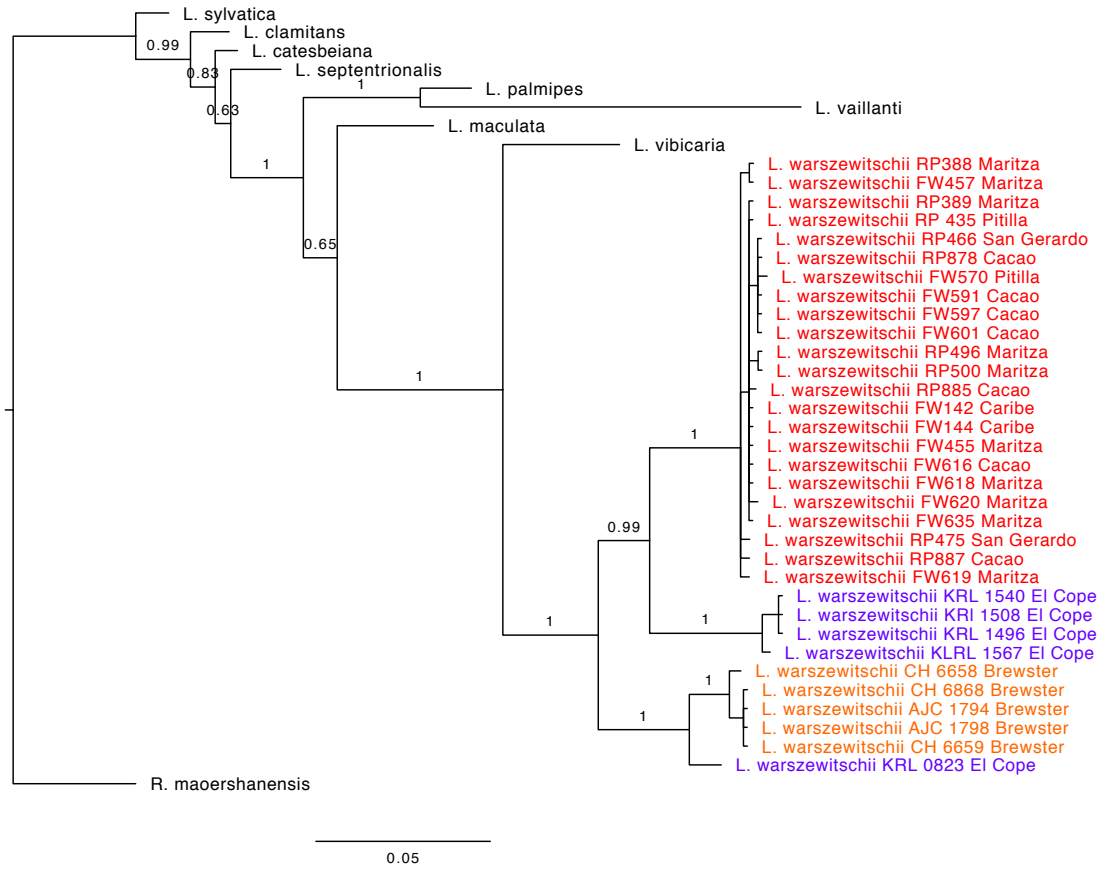
Supplementary Table 3. The number of base substitutions per site from averaging over all 16S sequence pairs between groups are shown above (π). The number of base substitutions per site from estimation of net average between groups of 16S sequences are shown below (NBGMD / π_{net}). Standard error estimate(s) are shown above the diagonal. Analyses were conducted using the Kimura 2-parameter model (Kimura 1980).

The rate variation among sites was modelled with a gamma distribution (shape parameter = 4). The analysis involved 42 nucleotide sequences. There were a total of 601 positions in the final dataset.



1. <u>CO1 Phylogenetic tree</u>

Supplementary figure 1. CO1 phylogenetic tree. Geographic populations ACG (red), Brewster (orange), El Cope (purple) of *L. warszewitschii* are represented. Samples include Genbank voucher ID, NCBI database information for other ingroup/outgroup species can be found in Table. 4. Posterior probability/branch support is also shown.



2. <u>16S Phylogenetic tree</u>

Supplementary figure 2. 16S phylogenetic tree. Geographic populations ACG (red), Brewster (orange), El Cope (purple) of *L. warszewitschii* are represented. Samples include Genbank voucher ID, NCBI database information for other ingroup/outgroup species can be found in Table. 4. Posterior probability/branch support is also shown.