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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).

46 Given that the majority of species remain undescribed, endeavours to explain and catalogue
47 biodiversity are inevitable to both understanding and preventing extinctions (Pimm et al.
48 2014). For amphibians especially, being the most threatened group of vertebrates (Stuart et al.
49 2004), identifying cryptic diversity is fundamental to their conservation. Habitat loss,
50 fragmentation, climate change and disease epidemics have produced a global decline in
51 amphibian populations (Baillie et al. 2004, Stuart et al. 2004). Losses reflect patterns of
52 ecological preference, range and taxonomic association, with montane stream dwelling
53 species most affected (Stuart et al. 2004). It is also probable that the number of amphibian
54 species is highly underestimated (Fouquet et al. 2007a, Vieites et al. 2009).
55 Whereas some species are presumed to be widely distributed, those within a cryptic complex
56 may have smaller ranges or different ecological requirements (Stuart et al. 2006), meaning
57 failure to recognize these taxa can leave them susceptible to mismanagement. However, when
58 genetic differentiation is established, it can unveil previously unknown units of diversity and
59 endemism (Bickford et al. 2007) that may subsequently warrant protection or species status
60 (Whitfield et al. 2016).
61
62
63 High levels of genetic diversity in Costa Rican and Panamanian frog populations is well
64 recognized (Crawford 2003), as are cryptic species (Wang et al. 2008). *Lithobates*
65 *warszewitschii* (Ranidae) (Schmidt, 1857) is a proposed candidate species - a provisional
66 designation pending further systematic investigation (Vieites et al. 2009). Crawford et al.
67 (2010) (Supporting information) showed that within the amphibian community at El Copé
68 (Omar Torrijos National Park), Panama, *L. warszewitschii* displayed 14.7% pairwise
69 divergence between conspecifics at the CO1 locus. This is an unusually high degree of
70 polymorphism for a single species in sympatry (Crawford 2003, Vences et al. 2005),
71 providing additional evidence this taxon likely contains candidate cryptic lineages (Mallet
72 2008). Paz et al. (2015) compared El Copé with allopatric populations from Brewster
73 (Chagres National Park), revealing 11% pairwise divergence. Consequently, breeding
74 strategy, dispersal and landscape resistance may help explain this variation between both
75 sites.
76
77 *Lithobates warszewitschii* occurs from Honduras to Panama and has been recorded at
78 elevations up to 1740 meters above sea level (m asl). They are fairly common, diurnal and
79 generally abundant frogs in forests near streams where they breed (Savage 2002). In Costa
80 Rica, population declines occurred in montane areas such as Tapantí, Montverde and Braulio
81 Carrillo (Bolaños 2002, Puschendorf et al. 2006). Post-decline it was found to be rare in San
82 Vito (Santos-Barrera et al. 2007) and vanished but found again at San Ramón (IUCN 2015).
83 *Lithobates warszewitschii* was also found to be abundant at mid-elevation sites in Guayacan
84 (Kubicki 2008), Corcovado, Ciudad Colón and Tinamastes (IUCN 2015). A population
85 decline also occurred at lowland site La Selva (Whitfield et al. 2007), however, it is not
86 generally abundant at lower elevations (IUCN 2015). Pre-decline it was one of the most
87 abundant tadpoles encountered in streams at El Copé, Panama, (Ranvestel et al. 2004), but
88 was later extirpated following the emergence of a virulent pathogen (Crawford et al. 2010). In
89 Nicaragua, it was found to be abundant in Rio San Juan (Sunyer et al. 2009) and numbers
90 were increasing at Quebracho (Barquero et al. 2010) post decline, although Nicaragua's
91 decline history is much more nebulous than Costa Rica's. No data was found for Honduras,
92 and additional research is needed to ascertain population sizes, distributions, trends and
93 threats throughout its full range (IUCN 2015).
94
95 In this study we expand the research on cryptic diversity within *L. warszewitschii,* based on
96 published sequence data from two localities in Panama (Crawford et al. 2010, Paz et al. 2015)
97 and samples collected from the Área de Conservación Guanacaste (ACG) in northwestern

98 Costa Rica. Using phylogenetic data, species delimitation methods and nucleotide diversity
99 within CO1 and 16S loci we make inferences about phylogeographic structure and proposed
100 candidate status across its wider range.
101
102   **Methods**
103
104 *Field sampling*
105 *Lithobates warszewitschii* were sampled from five field sites within the Área de
106 Conservación Guanacaste (ACG), Costa Rica: Pitilla, San Gerardo, Maritza, Cacao and
107 Caribe (Figure 1) between June 2015 – August 2017 (Table 1). Streams and surrounding
108 forest are preferred habitat for *L. warszewitschii* (Savage 2002), and sampling was conducted
109 within these habitats. Each individual was captured, housed separately in moist bags
110 (Beaupre et al. 2004), identified based on morphology (Savage et al. 2002, Leenders 2016),
111 and toe-clipped (Perry et al. 2011). Individuals were then released back at the point of
112 capture.
113
114 A total of 34 samples were collected from ACG and obtained from genbank, but only 29 had
115 both CO1 and 16S available and therefore used in this analysis. All data for *L. warszewitschii*
116 samples collected in Panamanian sites El Copé and Brewster were obtained from other
117 studies (Crawford et al. 2010, Paz et al. 2015).
118
119 *Lab work*
120 In order to extract DNA from tissue samples a standard ammonium acetate protocol was used
121 (Nicholls et al. 2000). The Cytochrome c oxidase subunit I (CO1) and 16S ribosomal RNA
122 (16S) mitochondrial genes were targeted for amplification by PCR. 16S primers (16Sar-L
123 +16Sbr-H) and reaction protocols were adapted from (Kessing et al 2004). Multiple primers
124 were used in the CO1 reactions to maximize the number of successful PCR products. CO1
125 primers (dgLCO-1490 + dgHCO-2198) and reaction protocols were adapted from Meyer et
126 al. (2005) and CO1 primers (Chmf4 + Chmr4; Che et al. 2012) followed reaction protocols
127 by Ivanova et al. (2008).
128
129 Extracted DNA from a subset of samples was sent to the Canadian Centre of DNA barcoding
130 for PCR amplification and sequencing. These samples used CO1 primers (C_VF1LFt1 +
131 C_VF1LRt1) in PCR reactions (Ivanova et al. 2007). The remaining samples were amplified
132 in-house. Thermocycler (*Techne Prime Gradient*) programmes differed depending on the
133 primer and reaction used. CO1 (dgLCO-1490 + dgHCO-2198) and 16S (16Sar-L + 16Sbr-H)
134 reactions were run using the protocol outlined by Crawford et al. (2010). Primer set (COI,
135 Chmf4 + Chmr4) followed thermocycler profiles by (Ivanova et al. 2008). Two percent agar
136 gels were used for electrophoresis with 1% TAE (Smith et al. 2008). Gels were visualized
137 using an *ImageQuant LAS4000* and *Nanodrop 2000* quantification was performed on each
138 successful PCR product visualized at the correct length, prior to dilution.
139
140
141 *Bioinformatics*
142 Concatenated gene alignments were used in the phylogenetic analyses. GENEIOUS v11.0.5
143 (Kearse et al. 2012) bioinformatics software was used to assemble forward and reverse
144 sequences from returned CO1 and 16S chromatographs. Forward and reverse (compliment)
145 sequences were aligned using Geneious' alignment (Global alignment with free end Gaps;
146 Cost matrix = 65% similarity (5.0/-4.0); Gap open penalty = 12; Extension penalty = 3).
147 Sequences were trimmed at the 3' and 5' ends where low quality base calls were present.
148 Consensus sequences were produced for each sample, ranging from 609-658 base pairs (bp)
149 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 (π) and cryptic speciation within and between sites.

Results

203
204 *Phylogenetic comparison*
205 Concatenated phylogenetic trees reconstructed using Bayesian inference (Figure 2) and
206 Maximum likelihood (Figure 3) methods, show similar topology of three major clades within
207 the focal species. Geographic samples from ACG and Brewster formed well-supported
208 independent monophyletic groups. However, samples from El Copé presented a polyphyletic
209 structure. Four out of five individuals (KRL 1496, KRL 1508, KRL 1540, KRL 1567) formed
210 an independent clade, sister to the ACG clade, whereas sample KRL 0823 formed a clade
211 with samples from Brewster – revealing the presence of two taxa at El Copé. Subsequently,
212 three clades are recognized: ACG and El Copé, containing samples exclusively from these
213 areas, and Brewster (including sample KRL 0823 from El Copé). Single gene trees showed a
214 similar topology to the concatenated ones (Supplementary figures 1 and 2).

215
216 CO1 operational taxonomic units (OTUs) delimitation *results*
217 CO1 species delimitation in GENEIOUS yielded three OTUs (Table 3). Focal clades ACG,
218 Brewster (+KRL0823), and El Copé (KRL 1496, KRL 1508, KRL 1540, KRL 1567) had P
219 values <0.05, indicating they are not conforming to the expected Wright-Fisher criteria.
220 According to this assumption and the data present, all clades were taxonomically distinct.
221 ABGD analysis supported these three distinct OTUs as well (p= 0.0359, supplementary table
222 1).

223
224 *CO1 and 16S nucleotide diversity*
225 K2P-π at the CO1 and 16S loci showed a mean value of 7.2% and 3.4%, respectively, within
226 all *L. warszewitschii* samples (Table 4). Samples from El Copé had the highest intra-group
227 mean distance at 6.3% and 3.2%, respectively, whereas samples from ACG had 0.4% and
228 0.3% and within Brewster 0.1% and 0.2%, respectively. Mean intraspecific distances between
229 ACG and Brewster samples (COI/16S) were the highest at 15.7%/7.2% (Supplementary
230 Table 2). Samples from ACG and El Copé shared the lowest distance at 10.7%/6.2%, and the
231 intermediate distance was 13.8%/6.7% between Brewster and El Copé samples. Interspecific
232 comparisons within the genus resulted in lower interspecific distances among recognized
233 species (COI/16S), such as: *L. clamitans* and *L. catesbeiana* (5.7%/2%), *L. septentrionalis*
234 and *L. clamitans* (8.3%/3.1%), *L. septentrionalis* and *L. catesbeiana* (8.6%/2.2%).

235
236
237
238 *CO1 and 16S Net between group mean distance (NBGMD) (*π_{net}*)*
239 At the CO1 and 16S loci the largest NBGMD (π_{net}) was 15.4% and 6.9%, respectively,
240 between ACG and Brewster samples (Supplementary Tables 2 and 3). Samples from ACG
241 and El Copé shared the lowest distance at 7.3% and 4.5%, respectively, and the intermediate
242 distance was 10.6% and 5%, respectively, between El Copé and Brewster samples. Most
243 intraspecific distances between the geographic groups within *L. warszewitschii*, surpassed the
244 interspecific values between recognized species within the genus (COI/16S), such as: *L.*
245 *catesbeiana* and *L. clamitans* (5.7%/2%), *L. clamitans* and *L. septentrionalis* (8.3%/3.1%), *L.*
246 *catesbeiana* and *L. septentrionalis* (8.6%/2.2%).

247
248
249 **Discussion**
250
251 The concatenated phylogenetic trees consistently outlined three distinct clades within
252 *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

305 Copé, with large genetic distances in proportion to their geographical distance. A possible
306 explanation for this pattern could be a secondary contact during the post glacial maxima
307 (Schneider 1993) or selection for different ecological roles, such as within habitat or resource
308 use (Alizon et al. 2008). It is true that *L. warszewitschii*'s colouration, habitat use, elevation
309 range and distribution vary (Savage 2002, Leenders 2016). Thus, high intraspecific diversity
310 may be attributed to ecological specialization (Schluter 2000) in allopatry or coexistence of
311 sister species in sympatry, such as in El Copé. For example, even if broad colouration of this
312 species is genuine, frogs use non-morphological signals such as advertisement calls, cuticular
313 hydrocarbons and other pheromones in mating systems and species recognition (Bickford et
314 al. 2007), meaning they often remain inconspicuous. Divergent or cryptic species should
315 therefore be considered a hypothesis of separately evolving entities (Hey et al. 2003, de
316 Quieroz 2007, Fiser et al. 2018) and species status further scrutinized through integrative
317 taxonomic methods (Padial et al. 2010).
318
319 Polyphyly can be used as indication of undescribed species in a lineage (Fouquet et al.
320 2007a). However, its presence complicates the classification of species in phylogenies as it
321 may represent transitional stages in the evolution of taxa (Hörandl and Stuessy 2010, Xiang
322 et al. 2012). Cryptic species often show morphological, ecological or genetic differentiation
323 and usually a degree of reproductive isolation, which may occur through phenotypic
324 plasticity or single locus polymorphisms. Hybridization may persist, leaving traces of
325 introgression, speciation or hybrid vigour. Alternatively, fusion may be resisted by
326 disruptive/divergent selection or postzygotic isolation (Sasa et al. 1998). This continuum is
327 evident across large geographic ranges to highly localized, providing explanations for the
328 evolutionary transitions of ecological races to species (Mallet 2008). Consequently, in *L.
329 warszewitschii*, patterns of polyphyly, relatedness between ACG and El Copé samples, or
330 large pairwise ranges in sympatry may reflect occasional or historical gene flow from
331 migrants, hybridization, introgression, retention of ancestral polymorphisms or incomplete
332 lineage sorting when using mitochondrial genes (Moritz and Cicero 2004). Alternatively, the
333 presence of two sympatric OTUs at El Copé, may reflect human-induced introduction.
334 Because of these scenarios, nuclear DNA is also recommended in subsequent evolutionary
335 and taxonomic studies (Vences et al. 2005).
336
337 At both CO1 and 16S loci, K2P-π mean (Meyer and Paulay 2005) intraspecific ingroup
338 values overlapped with interspecific species values, surpassing proposed general thresholds:
339 8% at CO1 and 2% 16S (Crawford et al. 2010), 10% CO1, 5% 16S (Vences et al. 2005) and
340 for neotropical amphibians at 16S (>3%) (Fouquet et al. 2007a). This indicates a wider
341 ranging cryptic complex is present, and advocates for the use of both genes in comparative
342 amphibian phylogenetics (Vences et al. 2005). Ultimately, concatenated genes may yield the
343 best phylogenies (Gadagkar et al. 2005), however, interspecific comparisons are limited in
344 this study due to having one individual representing each congeneric species, and an
345 incomplete taxonomy that can hamper results (Meyer and Paulay 2005).
346
347 ## **Conclusion**
348
349 The type specimen of *Lithobates warszewitschii* originated from Volcán Chiriqui, western
350 Panama (Schmidt 1857, Savage 1970), a locality near the Costa Rican border at almost equal
351 distance between ACG and Brewster. Whilst the topotype locality was not sampled, all clades
352 in this study may represent cryptic species. We have extended the research on cryptic
353 diversity within *L. warszewitschii* by revealing an additional clade from ACG, and propose
354 this clade is a candidate cryptic species that warrants further taxonomic investigation.
355 Determination of evolutionary mechanisms are beyond the scope of this study, but an
356 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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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.
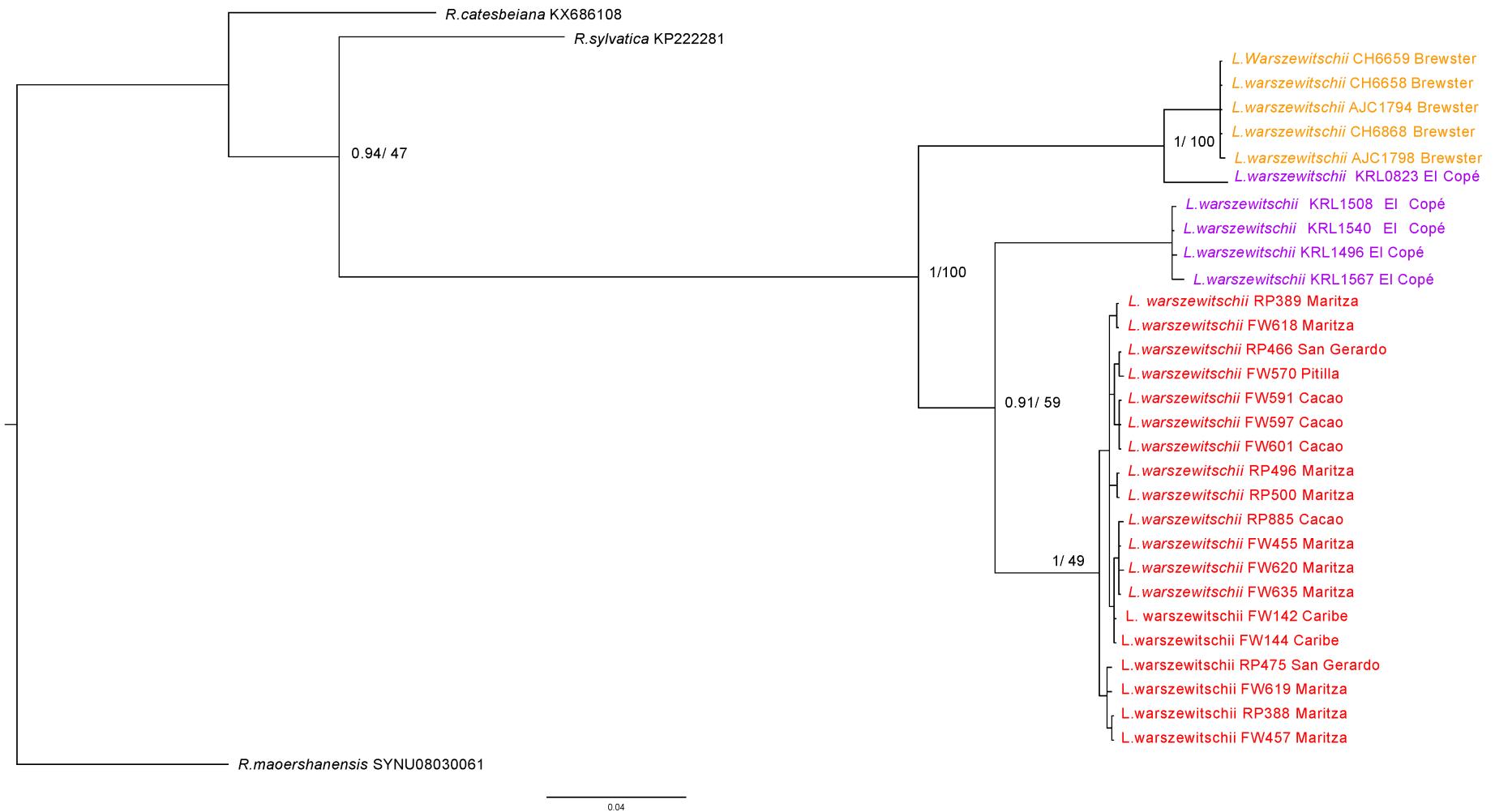
Figure 2. Phylogenetic reconstruction of *Lithobates warszewitschii* relationships between Costa Rican and Panamanian populations using concatenated alignments of CO1 and 16S. Node support values (posterior probabilities) and percentages calculated from 1000 bootstrap

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

Partition	No. of partitions	Gap width (X)	Prior Intraspecific divergence								
			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)

Group	ACG	Brewster	El Cope	L. catesbeiana	L. clamitans	L. palmipes	L. septentrionalis	L. sylvatica	L. vaillanti	R. maoershanensis
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)

Group	ACG	Brewster	El Cope	L. catesbeiana	L. clamitans	L. palmipes	L. septentrionalis	L. sylvatica	L. vaillanti	R. maoershanensis
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	Brewster	El Cope	L. catesbeian	L. clamitans	L. maculata	L. palmipes	L. septentrior	L. sylvatica	L. vaillanti	L. vibicaria	R. maoershar
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	Brewster	El Cope	L. catesbeian	L. clamitans	L. maculata	L. palmipes	L. septentrior	L. sylvatica	L. vaillanti	L. vibicaria	R. maoershar
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.
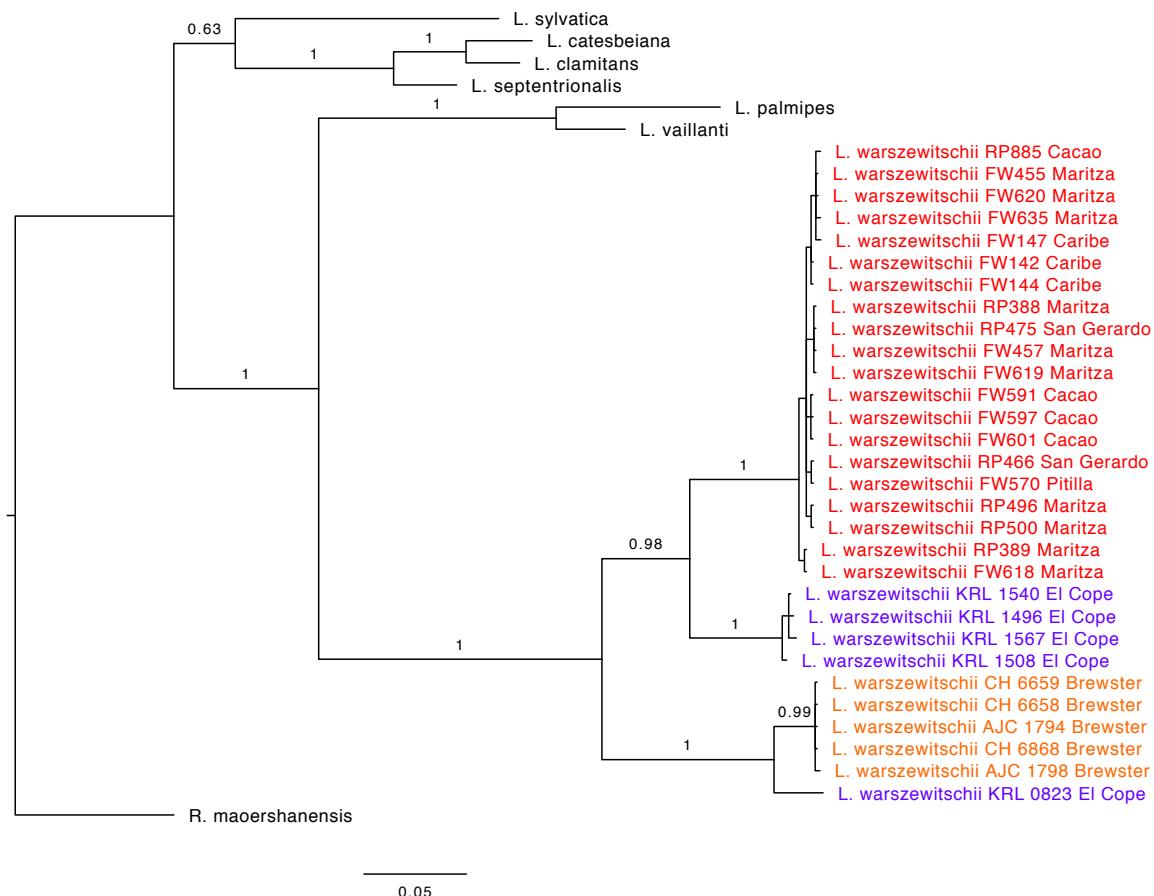
1. CO1 Phylogenetic tree

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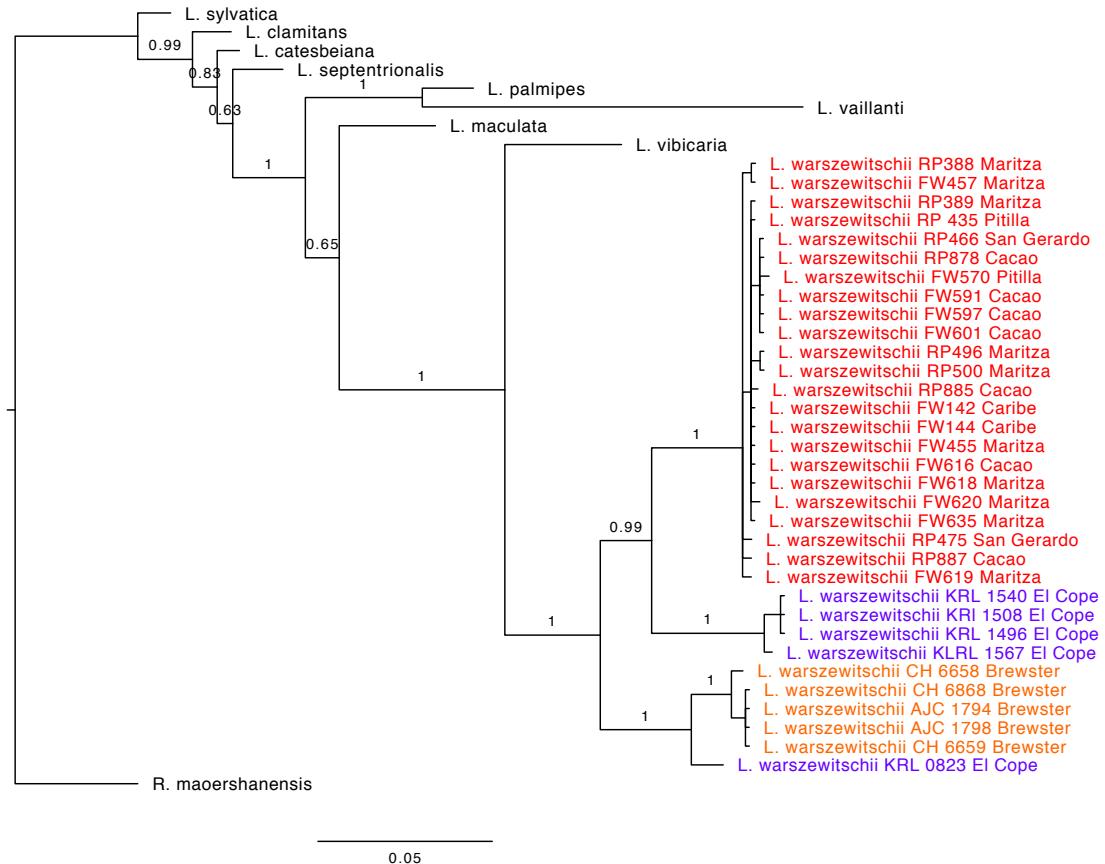
2. <u>16S Phylogenetic tree</u>

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.