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

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**Abstract**

*Lithobates warszewitschii* 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) (πnet) 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 warzewitschii (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. warzewitschii 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 warzewitschii 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 Tapanti, 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 warzewitschii 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. warzewitschii, 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
Consensus sequences were produced for CO1 and 16S loci using Geneious’ alignment software. 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 578-601 bp 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 monophyly 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) (πnet) (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

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 Brewer – 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%).

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 phylogenies (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 Chiriquí, 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.
warzewitschii 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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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       |                    |                              |            |           |               |                                 |
| 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     |                    |                              | 10.956     | -85.495   |               |                                 |
|           | November, 2016   | 6                  |                              | 10.956     | -85.495   | 570-610       |                                 |
|           | July, 2017       | 3                  |                              | 10.956     | -85.495   |               |                                 |
|           | August, 2017     | 5                  |                              | 10.956     | -85.495   | 570-610       |                                 |
| Cacao     | November, 2016   | 4                  | Rain/cloud forest            | 10.923     | -85.468   | 980-1130      | Field data - this study        |
|           | August, 2017     | 3                  |                              | 10.923     | -85.468   |               |                                 |
| 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* | 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                | FI84384                |
| *L. warszewitschii* | El Copé    | KRL 1540   | FJ766751                | FI84552                |
| *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         | -          | N4                      | 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 | P(D)(Strict) | P(D)(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 (\( \pi \)) within geographic groups of *L. warszewitschii*

| Population     | Mean (\( \pi \)) | Range (\( \pi \)) |
|----------------|------------------|-------------------|
|                | CO1              | 16S               |
| ACG            | 0.004            | 0.003             |
| El Copé        | 0.063            | 0.032             |
| Brewster       | 0.001            | 0.002             |
| *L. warszewitschii* | 0.072         | 0.034             |

Table 4. Nucleotide diversity (\( \pi \)) 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.

| 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     | 10                 | 1             | 6        | 11       | 11       | 11       | 11       | 11       | 11       | 11       | 11    |
| Recursive   | 10                 | 1             | 7        | 11       | 11       | 11       | 11       | 11       | 11       | 21       | 21    |

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 ($\pi$), and net evolutionary divergence ($\pi_{net}$) over CO1 sequence pairs between groups.

|                  | CO1 (K2P-$\pi$) | CO1 ($\pi_{net}$) |
|------------------|------------------|------------------|
| ACG              | 0.018 0.012 0.024 0.023 0.025 0.025 0.023 0.023 0.026 | 0.018 0.010 0.026 0.024 0.025 0.025 0.023 0.023 0.025 |
| Brewster         | 0.157 0.015 0.024 0.024 0.022 0.022 0.025 0.021 0.027 | 0.154 0.013 0.025 0.026 0.022 0.022 0.026 0.021 0.027 |
| El Cope          | 0.107 0.138 0.023 0.022 0.023 0.024 0.021 0.021 0.025 | 0.073 0.106 0.023 0.023 0.022 0.024 0.020 0.019 0.023 |
| L. catesbeiana   | 0.264 0.256 0.250 0.010 0.022 0.012 0.021 0.022 0.021 | 0.262 0.256 0.218 0.023 0.023 0.024 0.020 0.019 0.023 |
| L. clamitans     | 0.247 0.263 0.251 0.057 0.022 0.012 0.020 0.021 0.020 | 0.245 0.262 0.219 0.057 0.023 0.012 0.021 0.022 0.020 |
| L. palmipes      | 0.265 0.224 0.245 0.218 0.219 0.205 0.226 0.211 0.211 | 0.262 0.224 0.213 0.218 0.219 0.218 0.219 0.219 0.219 |
| L. septentrionalis | 0.264 0.267 0.259 0.086 0.083 0.246 0.019 0.022 0.021 | 0.261 0.267 0.227 0.086 0.083 0.246 0.020 0.024 0.022 |
| L. sylvatica     | 0.234 0.220 0.228 0.218 0.194 0.239 0.181 0.211 0.211 | 0.232 0.220 0.196 0.218 0.194 0.239 0.181 0.211 0.211 |
| L. vaillanti     | 0.234 0.227 0.220 0.230 0.205 0.106 0.226 0.211 0.211 | 0.232 0.227 0.189 0.230 0.205 0.106 0.226 0.211 0.211 |
| R. maoershanensis | 0.239 0.274 0.255 0.199 0.183 0.228 0.199 0.208 0.241 | 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 ($\pi$). The number of base substitutions per site from estimation of net average between groups of CO1 sequences are shown below (NBGMD / $\pi_{net}$). 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 (\(\pi\)), and net evolutionary divergence (\(\pi_{\text{net}}\)) over 16S sequence pairs between groups.

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

Supplementary Table 3. The number of base substitutions per site from averaging over all 16S sequence pairs between groups are shown above (\(\pi\)). The number of base substitutions per site from estimation of net average between groups of 16S sequences are shown below (NBGMD / \(\pi_{\text{net}}\)). 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. **16S Phylogenetic tree**
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.