# Cryptic diversity and ranavirus infection of a critically endangered Neotropical frog before and after population collapse

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## Abstract

Mesoamerican amphibian declines in apparently pristine and protected habitats have been severe, especially at elevations above 500 m sea level and have been linked to emerging diseases and a changing climate. The *Craugastor punctariolus* species series of direct developing frogs is endemic to the region and used to be comprised of 33 species, seven of which have known populations at present. One of these, *Craugastor ranoides*, endemic to southern Nicaragua and Costa Rica, was historically found in cloud forest sites of Área de Conservación Guanacaste (ACG) in north-west Costa Rica and extended into dry forest sites 20 km distant. Here *C. ranoides* declined and disappeared from high elevation sites between the late 1980s and early 1990s, but populations persisted in the lowland dry forest. We compared the genetic richness and ranavirus infection status of *C. ranoides* from extant dry forest populations to historic museum specimens of now extinct ACG cloud forest populations using DNA sequence diversity at two mitochondrial loci and molecular screening for ranavirus. Extant dry forest populations of *C. ranoides* formed a monophyletic group which included historic specimens sampled at cloud forest sites. However, the extirpated ACG cloud forest population contained additional diversity: samples formed a divergent clade with unknown spatial distribution. Ranavirus was detected in both current and museum samples of *C. ranoides* and sequences from a 267-nucleotide region of the major capsid protein gene shared 100% sequence identity with one another and with *Frog virus 3*. Our findings document cryptic diversity within an endangered species that has demonstrated no recovery in cloud forests and raises questions about *Ranavirus* as a potential driver of amphibian decline in this system. The presence of the same *C. ranoides* clade within present day and historical samples suggests a potential for effective translocation and repopulation of extirpated cloud forest populations.

**Keywords:** Amphibian declines, ranavirosis, *Frog virus 3*, *Craugastor ranoides*, Costa Rica, cryptic diversity

# 1. Introduction

Global, rapid and severe amphibian declines have occurred over the last three decades, gaining additional notoriety because even populations in protected and relatively pristine habitats have been affected (Stuart *et al.*, 2004). These amphibian declines are particularly conspicuous in the Neotropics (Stuart *et al.*, 2004); in Costa Rica alone, the cloud forest amphibian fauna nearly disappeared between the mid-1980s and early 1990s, including the golden toad and harlequin frog (Bolaños, 2002; Lips, 1998; Pounds, Fogden & Campbell, 1999; Pounds *et al.*, 1997).

The most severe amphibian population declines have occurred in high elevation, moist, montane habitats in which the chytrid fungus *Batrachochytrium dendrobatidis* can thrive (Lips *et al.*, 2006; Piotrowski, Annis & Longcore, 2004). *Batrachochytrium dendrobatidis* causes the infectious disease chytridiomycosis and has been associated with amphibian declines globally (Lips *et al.*, 2006; Longcore, Pessier & Nichols, 1999; Vredenburg *et al.*, 2010).

The Neotropics are home to half of all described species of frogs (Duellman, 1999; Young *et al.*, 2001). However, an estimated 5-10% of the Central American amphibian fauna remains undescribed through classical morphological means (Young *et al.*, 2001), and genetic characterisation is expected to reveal additional cryptic diversity even within seemingly well-known species (Vieites *et al.*, 2009). This presents a problem for effective biodiversity conservation in tropical forests, since it is unclear what is being lost, and is a barrier to a more complete understanding of host-pathogen interactions within persisting amphibian populations (Crawford, Bermingham & Carolina, 2007; Crawford, Lips & Bermingham, 2010).

It is evident that there has been an extreme loss of amphibian populations throughout Central America, dramatically affecting biodiversity, however some recent rediscoveries give hope that not all will be lost in the long run (Young *et al.*, 2001; Puschendorf *et al*., 2011; Garcia Rodriguez *et al*., 2012). Understanding which amphibians were lost and why should provide insight into effective conservation strategies. In an analysis of the lineage diversity of a Panamanian amphibian community before and after a mass-mortality event, five of 30 extirpated species were undescribed at the time of their disappearance (Crawford *et al.*, 2010). In comparison, cloud forest amphibians in Costa Rica, including the Golden Toad and Harlequin frog, nearly disappeared between the mid-1980s and early 1990s, (Bolaños, 2002; Lips, 1998; Pounds *et al.*, 1999; 1997). Unfortunately, few systematic collections were undertaken during this period, so very little is known about potential cryptic amphibian species richness and diversity that may have vanished during the Costa Rican amphibian declines. Similarly, not much is known regarding the cause of these declines, though *B. dendrobatidis* is presumed to have played a significant role (Lips *et al.*, 2006; Pounds *et al.*, 2006).

In addition to understanding amphibian diversity, establishing drivers behind declines is important for predicting future losses and proactively conserving remaining populations. Emerging infectious disease have now been associated with the majority of these amphibian declines between the mid 1970's to the early 2000's (Phillips & Puschendorf, 2013). In addition to *Batrachochytrium dendrobatidis* a second pathogen group is receiving increasing scientific attention – *Ranavirus* (family *Iridoviridae*). Ranaviruses have an extremely broad host range that includes amphibians, reptiles, and fish, and has caused the collapse of some amphibian populations in Europe (Gray & Chinchar, 2015; Lesbarrères *et al.*, 2012; Price *et al.*, 2014; Rosa *et al.*, 2017). In the tropics, the information is scarce, for example in the Costa Rican Caribbean rainforest lowlands, despite the detection of either *B. dendrobatidis* or

ranavirus or both pathogens in association with 20 frog species, none exhibited signs of disease (Whitfield *et al.*, 2013). A similar situation has been observed in the tropical Andes of Peru (Warne et al., 2016). This absence of diseased or dying frogs suggests a viable coexistence of amphibians with one, or both, of these potentially pathogenic microorganisms, however long-term trends remain unknown. It is also possible that the intensity of the surveillance programs was insufficient to detect outbreaks of acute disease or dead animals in warm, wet ecosystems where amphibian carcases are scavenged or decompose rapidly (Sugiura *et al.*, 2013).

The *Craugastor punctariolus* species series is one of most endangered amphibian clades in Mesoamerica, with a loss of 26 of the characterised 33 species since the early 1980s (Padial, Grant & Frost, 2014; Stuart *et al.*, 2008). One of the remaining seven morphologically characterised species, *Craugastor ranoides*, was once distributed throughout Costa Rica, including lowlands and premontane slopes between 10 and 1300 m above sea level, and on both the Pacific and Caribbean versants (Savage, 2002). Área de Conservación Guanacaste (ACG), which encompasses 120,000 ha of dry, wet and cloud forest (and 43,000 ha of Pacific Ocean) in northwestern Costa Rica, has historic records of *C. ranoides* describing a distribution that included all the region's major habitat types (Janzen & Hallwachs, 2016; Puschendorf *et al.*, 2005; Sasa & Solórzano, 1995; Zumbado-Ulate, Puschendorf & Chavarría, 2007). However, during the mass amphibian declines *C. ranoides* disappeared (Puschendorf *et al.*, 2005) and was last documented in high abundance in the cloud forest at Volcán Cacao in 1987 when specimens were preserved as part of an expedition from UC Berkley, led by David A. Good and David Canatella.

In 1994, *C. ranoides* was discovered unexpectedly in a few small areas of ACG dry forest (Sasa and Solórzano, 1995) and has since been found in other locations, though exclusively on stream-beds that keep flowing in the dry season (Puschendorf pers. obs.). The

seasonally dry and hot environmental conditions of Costa Rican dry forest contrast strongly with the upper elevation rain and cloud forest habitats that foster the high *B. dendrobatidis* prevalence and lethal infection intensity that have been considered the cause of population declines (Whitfield *et al.*, 2017). These dry forest sites are assumed to have served as a refuge from disease-driven amphibian extinctions (Puschendorf *et al.*, 2009; Zumbado-Ulate, Bolaños, Willink *et al.*, 2011; Zumbado-Ulate *et al.*, 2014). However, relict dry forest populations of *C. ranoides* in ACG are separated from historic cloud forest site, Volcán Cacao, by low hills and deep valleys with no drainages that directly connect them, limiting opportunities for this frog to recolonise the cloud forest through natural dispersal.

Conserving amphibian diversity and population density is essential for maintaining biodiversity and ecosystem stability in the face of climate change. With substantial loss already, effective conservation strategies will likely need to incorporate new technologies and bold, informed approaches. One such strategy may include species reintroductions via translocations, which can be highly effective and avoid some of the perils of captive breeding (Frankham, 2008; Williams & Hoffman, 2009). Such intervention requires a solid knowledge of source population genetic diversity, in the context of historic diversity, and any host-pathogens interactions (Hartley & Sainsbury, 2017). Currently, the full extent of the possible cryptic clade diversity within *C. ranoides*, a formerly widespread "species", remains unknown.

In this study, we aimed to partly describe the historic, cryptic diversity of *C. ranoides* and compare this to the extant dry forest population in order to generate baseline data that could inform future conservation actions. Further to this, using historical and present-day samples, we tested the hypothesis that an emerging infectious pathogen other than *B. dendrobatidis* may have been associated with late 1980s declines by screening for the presence of ranavirus.

## 2. Materials and Methods

### 2.1 Field sites and surveys

Six sites with extant *C. ranoides* populations were sampled in the dry forest of ACG (Fig. 1). These sites included Quebrada Grande at 10.90134 N, 85.77522 W; Río Pedregal at 10.90135 N, 85.75068 W; Río Murciélago at 10.89691 N, 85.73029 W; Quebrada La Danta at 10.86623 N, 85.71600 W; Río Nisperal at 10.8273N, 85.6486W and Río La Calera at 10.86139 N, 85.66409 W. One site at which the species is currently thought to be extinct was also sampled (Volcán Cacao at 10.92403 N, 85.46682 W). Prior to our field sampling in 2014, *C. ranoides* had been sighted at all of these dry forest localities, except Río Nisperal which had never been surveyed before for this species.

Field sampling of *C. ranoides* was undertaken from 27 June 2014 to 01 July 2014 in the dry forest of ACG (Fig. 1). Sampling was always conducted after sunset, as late dusk was previously the most likely time to locate *C. ranoides.* Individuals of *C. ranoides* were identified by their distinctive red and yellow hind leg markings, the slight but characteristic webbing on their hind feet and more rotund bodies and heads in comparison to the common rain frog, *C. fitzingeri*, a congeneric which coexists in the same habitats (Savage, 2002).

Volcán Cacao cloud forest was also surveyed in 2005, 2012, 2014, and 2016. Each of these surveys represents one night in which at least 4-6 hours were spent surveying two streams and one transect, Río San Josecito, Quebrada Arenales and Sendero Derrumbe heading to the

summit of Volcan Cacao, sites in which historical specimens of this species had been collected. In 2017, in an attempt to search more extensively for these missing frogs, four students from Plymouth University under the guidance of RP spent a total of 60 person-hours surveying these historic collection sites at Volcán Cacao.

### 2.2 Sample collection

During the dry forest 2014 surveys, a maximum of six individuals were sexed, aged (male, female, juvenile) and toe-tipped at each locality then returned to their site of capture. Toepads were stored individually in 95% ethanol for subsequent DNA extraction. In total, twenty-five individuals were sampled in the dry forest, and tissues were obtained from four individuals collected in 1987 from the vicinity of Estación Cacao on Volcán Cacao (cloud forest; 20 km east of the nearest dry forest site at 900-1300 m elevation; Fig.1). All frogs sampled, extant and museum specimens, were identified as *C. ranoides* based on morphology.

### 2.3 Laboratory procedures

DNA was extracted from *C. ranoides* toepads using an ammonium acetate extraction protocol (Nicholls *et al.*, 2000). CO1 and 16S mitochondrial genes were amplified using the same primers and cycle settings as Crawford et al. (2010): CO1 using forward (F) 5'-GGTCAACAAATCATAAAGAYATYGG-3' and reverse (R) 5'–TAAACTTCAGGGTGACCAAARAAYCA-3'primers, and 16S using F 5'–CGCCTGTTTATCAAAAACAT-3'and R 5'–CCGGTCTGAACTCAGATCACGT-3 primers. PCR reactions for mitochondrial targets were carried out in a Techne Prime Thermal Cycler. We used total PCR reaction volumes of 20µL, including 2µL of the template DNA. The reaction mix included 2µL Qiagen 10x buffer (containing 15mM MgCl2; QIAGEN Group 2011), 1µL dNTPs (10mM), 0.4 µL $Mg^{2+}$ (25mM), 0.2µL Taq DNA polymerase (5 units/uL, from Qiagen PCR core kit), 0.6µL of the respective forward and reverse primers (at 10µM stock concentration) and 12.7µL nuclease-free water. Amplification was confirmed by

running the PCR products on 1% agarose gels for 20 minutes at 100V using Tris-acetate-EDTA buffer. Gel electrophoresis runs were exposed and visualised on the ImageQuant LAS4000. PCR products were sent to Macrogen Inc. for Sanger sequencing from both the forward and reverse primers.

**2.4 Experimental design and Bioinformatics**

Only individuals for which both CO1 and 16S sequences were generated were included in phylogenetic analyses. Geneious version 8.1 was used to first trim each read at the 3' and 5' ends where low quality base calls were present (maximum probability limit of 0.01 – equating to ≥ 1% chance of the call being an error), then to assemble forward and reverse reads of the 16S and CO1 regions into their respective contigs using the Geneious alignment tool (Kearse *et al.*, 2012). Any disagreements in calls between the forward and reverse sequences were checked manually against chromatograms in order to create final consensus sequences for each gene and each individual sampled.

We compiled all candidate *C. punctariolus* species series sequences available from GenBank that had both CO1 and 16S sequences available for the same individual (Supplementary Table 1) and aligned them to sequences from our samples. We used TOPALI (Milne *et al.*, 2009) to select the best-fit substitution model for our data based on the Bayesian information criterion (BIC). We used MrBayes version 3.2.6 (Ronquist & Huelsenbeck, 2003) through the Geneious plugin to estimate a Bayesian consensus tree from the alignment of each gene separately (two trees) and using the concatenated alignment generated by joining the 16S and CO1 alignments end to end (without partitioning by loci). We ran MrBayes as follows for all three trees: a Hasegawa–Kishino–Yano (HKY85) substitution model was used with gamma distributed rate variation (four rate categories), chain length of 15 million generations, burn-in length of 1.5 million, subsampling frequency of one tree each 10,000 generations, and default settings otherwise. A clade comprising *Craugastor opimus* and *Craugastor*

*megacephalus* was used as an outgroup to root the tree (Crawford & Smith, 2005). Kimura 2-parameter (K2P) corrected average pairwise distance ($\pi$) was used to generate net divergence values in MEGA v7.0.26 using known and potential clades as identified by our tree (Kumar, Stecher & Tamura, 2016; Nei & Li, 1979). A minimum spanning haplotype network for *C. ranoides* based on the concatenated sequences of both CO1 and 16S was constructed in Popart (Leigh & Bryant, 2015).

### 2.5 Ranavirus molecular diagnostics and partial genetic characterisation

We screened all samples of historic and extant *C. ranoides* individuals for ranaviruses using a nested PCR comprised of PCRs with outer primers ('4' and '5') from Mao, Hedrick & Chinchar (1997) and inner primers ('MCP-IF' and 'MCP-IR') described by Meng *et al.* (2013). Total reaction volumes were 8 μL, consisting of outer primers at 0.05 μM or inner primers at 0.4 μM (Meng *et al.*, 2013), 4 μL 2X DreamTaq Green PCR Master Mix (Thermo Scientific, Massachusetts), 1.2 μL of nuclease-free water and 2 μL of template DNA (the second PCR used the product from the first PCR diluted one in ten in nuclease-free water as a template). Both PCRs were run using a touchdown program with the following cycling conditions: 23 cycles of 95°C for 30s, a 30s primer annealing step starting at 62°C and decreasing by 0.5°C per cycle, and a 72°C for 30s elongation step, followed by 25 cycles of 30 s at each of 95°C, 50°C, and 72°C, before a final seven-minute 72°C elongation step. PCR products were visualised on 2% agarose gels, and amplicons with visible bands were sent for Sanger sequencing as above.

## 3. Results

The 2014 surveys documented *C. ranoides* at each site where it had previously been observed post-decline and recorded its presence at a new location on the Río Nisperal (Fig. 1;

(Puschendorf *et al.*, 2005; Zumbado-Ulate *et al.*, 2007). *Craugastor ranoides* was not found at high elevation sites on Volcán Cacao.

Both the haplotype network and the phylogenetic tree, inferred from concatenated CO1 and 16S aligned data, show that all samples of *C. ranoides* collected within the dry forest (n = 15), across six independent streams and rivers, cluster in one well supported clade (posterior probability = 1) comprising three well defined haplotypes (Fig. 2). The presence of a small sub-group within this dry-forest clade (posterior probability = 0.99) revealed some mitochondrial gene diversity (0.126% CO1 within group divergence).

Two of the historic museum samples from the cloud forest in Volcán Cacao (MVZ 207286 and MVZ 207289) fall within the dry forest clade (clade 1; Fig. 2). However, the other two historic cloud forest samples (MVZ 207278 and MVZ 207285) form a separate, well-supported 'Cacao' clade (posterior probability = 1, Fig. 2) composed of a single haplotype, which clustered with a Panamanian species, *Craugastor evanesco*. Net divergence between the Cacao *C. ranoides* clade and *C. evanesco* is 2.5–4.1% at CO1 and 1.4–1.8% at 16S (Table 1).

Both current ACG dry forest and historic ACG cloud forest *C. ranoides* samples tested positive for ranavirus. Out of the 25 samples collected in 2014, one sample (RP56) from Quebrada Grande was positive for ranavirus. Similarly, one of the four historic samples (MVZ 207285) was positive for the pathogen. The two generated sequences (264-267 bp) showed 100% sequence identity with *Frog virus 3* (FV3, GenBank accession AY548484), the type species of the genus *Ranavirus.*

## 4. Discussion

The persistence of *C. ranoides* at all sites with earlier observations and the discovery of a new subpopulation in Quebrada Nisperal suggest that this species may be more widespread across the ACG dry forest than previously thought. Similarly, the presence of multiple genetic clades within the morphologically characterised *C. ranoides* suggests a previously unknown cryptic diversity, indicative of a species complex. Furthermore, the presence of the FV3 ranavirus associated with historic and present-day *C. ranoides* samples suggests a potential involvement of this pathogen in the 1980s declines and possible subsequent co-existence within surviving populations. Overall, this provides optimism that similar dry forest habitats may harbour undiscovered populations of the species-complex *C. ranoides*, that ranavirus may not always be pathogenic, and that frog reintroductions are potentially viable.

The *C. punctariolus* species series of frogs has undergone severe, multi-species declines, but recent rediscoveries, such as documented here for *C. ranoides,* suggest new searches for missing species may be fruitful. Since 2011, two populations of *Craugastor taurus* have been rediscovered in south-eastern Costa Rica after 17 years without sightings (Chaves, Zumbado-Ulate, García-Rodríguez *et al*., 2014) whilst individual frogs of two highland species, *Craugastor escoces* (Jiménez & Alvarado, 2017) and *Craugastor angelicus* (Kubicki, 2016), were rediscovered in 2016, suggesting that some species may persist at extremely low densities. Therefore, it remains possible that *C. ranoides* could still be present at Volcán Cacao and similar areas but at very low densities that have so far evaded detection.

Our genetic characterisation of the relict dry forest population of *C. ranoides* revealed some variation between sites. This is likely due to the area's rugged terrain and relatively high mountain range (Santa Elena's mountains reach 700 m asl) with several deep valleys, despite the short linear distance between the different sampling localities. Whilst the isolated, independent river systems are likely to have enforced a degree of historic isolation on this stream dwelling species of frog, the relatively low genetic diversity indicates that individuals

sampled across the six dry forest sites belong to the same species and that these populations were historically connected. In contrast, the genotypes of samples from the cloud forest point to a more complex evolutionary history.

The historic sampling revealed that two divergent populations of *C. ranoides* coexisted at Volcán Cacao. One of these populations is grouped in the same clade as the rediscovered dry forest populations and suggests a degree of connectivity between them. In contrast, the divergent Volcán Cacao population is not known to persist at any location and is assumed to be extirpated with unknown historical distribution. Although it was not the aim of this study to create a phylogeny of the *C. punctariolus* species series, these results highlight the ongoing taxonomic complexities within this group of critically endangered frogs, especially when species descriptions are based on morphology alone. Our molecular data revealed cryptic lineage richness within *C. ranoides*, however the levels of mtDNA divergence between the dry forest and Volcán Cacao populations are within the levels of genetic diversity observed in sympatric samples of conspecific amphibians where cases of low interspecific divergence are usually around 5-7% in COI and 3-5% in 16S (Chambers & Hebert, 2016; Crawford, 2003; Vences *et al.*, 2005). Alternatively, our DNA sequence data might suggest that the *C. ranoides* could be conspecific with *C. evanesco*, thus greatly extending the known geographic distribution of the latter from central Panama to northern Costa Rica. However, further systematic investigations are necessary before accepting the idea that *C. ranoides* is composed of more than one species (Vieites *et al.*, 2009), or that *C. ranoides* and *C. evanesco* are indeed the same species. A comprehensive revision of this group, with data from additional localities across isthmian Central America, is needed to reassess the taxonomic status of many of these species.

This study also underlines the value of knowing the clade-based taxonomy of a species prior to moving animals as part of conservation actions. The Chinese giant

salamander, a species that has been farmed intensively, may represent the best example from the Amphibia where the evolutionary history of the species has been ignored during a series of intentional and unintentional reintroductions (Cunningham *et al.*, 2016). This giant salamander seems to be a complex of cryptic species that is able to hybridise, as will the members of many species complexes as they gradually diverge into different clades (Yan *et al.*, 2018). The broad genetic mixing may eradicate the original wild clades and lead to their extinction by genetic homogenisation (Marie, Bernatchez & Garant, 2010).

In the case of *C. ranoides*, it is clear that the dry forest clade is confined to a relatively small area in the dry forest of ACG, with no direct drainage connecting to the nearby mountains, making a re-invasion into its former range extremely unlikely. Added to that, the last El Niño of 2014-2016 produced the strongest drought on the pacific coast of Costa Rica since 1937 (Alvarado Gamboa, 2015). If this pattern of stronger El Niños continues, it will put these dry forest populations at risk as *C. ranoides* requires year-round flowing water for their survival. These dry forest frogs could be used as a source for translocation to the cloud forest highlands. They could for example be translocated into open lowland streams near the foothills of the volcanoes where they could escape the cold and moist environment where chytrid is likely to cause outbreaks. While potentially viable and necessary, a detailed, knowledge-based plan needs to be developed before a translocation is attempted (Germano & Bishop, 2009).

Both current ACG dry forest and historic ACG cloud forest *C. ranoides* samples tested positive for *Frog virus 3* (FV3), a type of *Ranavirus* that is a frequent cause of mass mortality incidents in amphibians and reptiles in North America and is thought to have been translocated internationally (Price *et al.*, 2017; 2016). Ranavirus was the likely cause of an amphibian die-off at Volcán Maderas, on the island of Ometepe in Nicaragua in 2011 (Stark *et al.*, 2014), a site only 55 km from ACG. In Costa Rica, FV3 has been found associated

with a variety of species of amphibians at La Selva Biological Station, a lowland Caribbean site, but no dead and dying amphibians have been recorded there during these studies (Whitfield *et al.*, 2013; 2012). Despite the absence of observations of mortality in amphibian and reptile populations at La Selva there has been shown to be a long trend of decline between 1970 and 2005 (Whitfield *et al.*, 2007), similar to observations of lowland populations on the Pacific coast of Costa Rica (Ryan *et al.*, 2014).

The presence of FV3 on a *C. ranoides* individual in 1987 from Volcán Cacao supports the conclusion of a study published in 1991 that icosahedral viral particles observed using microscopy in the blood of *Rhinella marina* collected in Guanacaste, Costa Rica, belonged to the family *Iridoviridae* (Speare, Freeland & Bolton, 1991). The finding also confirms that ranavirus was present in Costa Rica during country-wide amphibian declines at high elevation sites (Pounds *et al.*, 1997). These severe, regional amphibian declines were linked to the presence of *B. dendrobatidis*, and consequent chytridiomycosis, but the presence of ranavirus in this system raises the possibility that the causes of these population crashes were more complex and could have involved more than one pathogen. Cloud forest and montane anoles declined in addition to amphibians such as the golden toad and harlequin toad in Monteverde (Pounds *et al.*, 1999). These reptile declines are often overlooked, but they are an additional reason to further consider the role of ranaviruses in driving declines since these viruses have broad host ranges and can have severe impacts on reptiles (e.g. Kimble *et al.*, 2017).

The interaction between the two pathogens may be important in determining disease outcomes for amphibian populations. In high elevation sites in the Serra da Estrela, Portugal, chytrid caused very severe declines of a single anuran species between 2009 and 2011 following an assumed introduction (Rosa *et al.*, 2013). There were no signs of ranavirus disease in any of the amphibian populations in the region during this time but incidents of

ranavirus disease and mortality were observed in multiple species (anurans and caudates) in late 2011, with subsequent – and sometimes dramatic – declines in densities within the next three years (Rosa *et al.*, 2017). In contrast to these temperate systems the historic and long-term impacts of ranavirus infection in neotropical ecosystems remain unknown and further investigation is certainly warranted to assess the pathogenicity, host range and potential impact.

The disappearance of amphibian diversity in Costa Rica is poorly characterised and understood. We have used the recent discoveries of populations of a previously widespread frog along with archived specimens to reveal a loss of historic diversity and the presence and persistence of an important pathogen. Our results further understanding of historical diversity and taxonomy, generate new hypotheses about the causes of declines, and provide valuable baseline data that can be used to plan conservation actions for a critically endangered frog.

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## Ethics Statement

Sampling was carried out under the following permits: ACG permit number ACG-PI-036-2014 and CONAGEBIO Permit number R-036-2013-OT-CONAGEBIO. We aimed to minimise potential stress to all sampled individuals but in particular gravid *C. ranoides* females, who were entirely excluded from sampling because of their demographic and conservation importance.

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## Legends for Figures and Tables

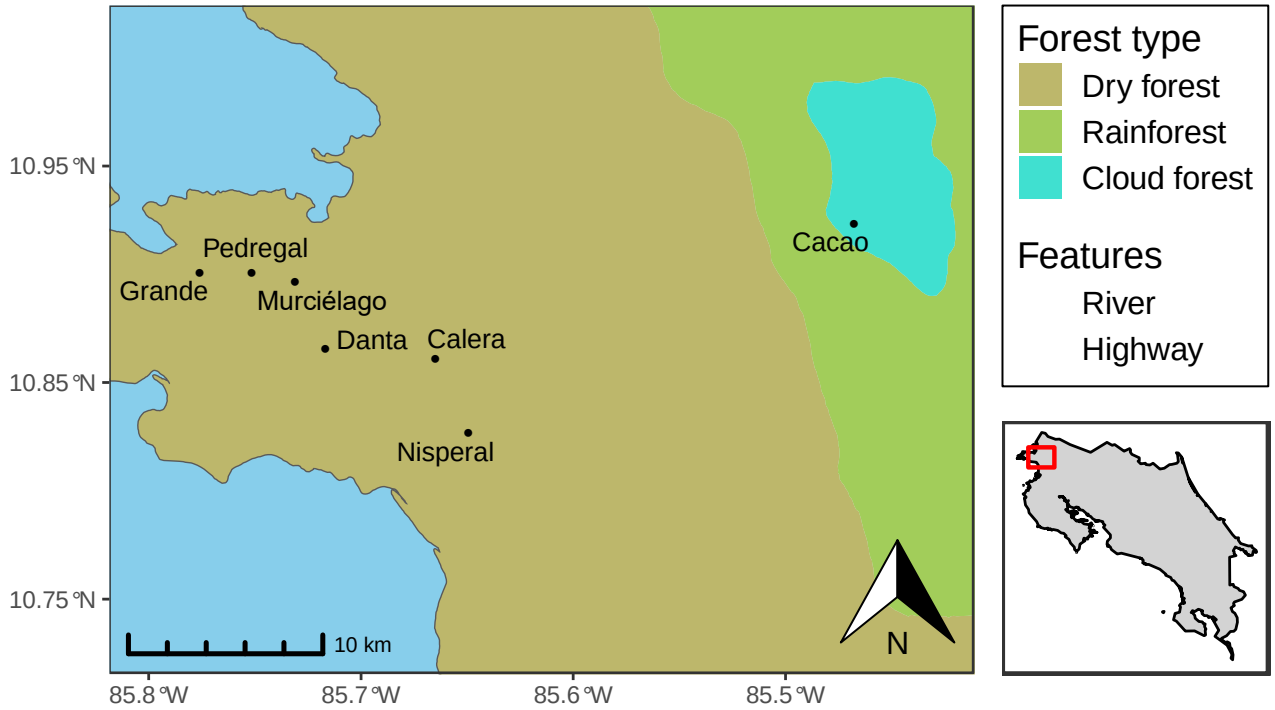
**Figure 1.** Study area and sampling sites for *Craugastor ranoides* across Área de Conservación de Guanacaste (ACG), Guanacaste, Costa Rica. Quebrad Grande (Grande); Río Pedregal (Pedregal); Río Murciélago (Murciélago); Quebrada La Danta (Danta), Río Nisperal (Nisperal); Río La Calera (Calera); Volcán Cacao (Cacao). Inset map shows the study area (red box) in the context of the Costa Rican border.

**Figure 2.** Diversity among extant and historic samples of *Craugastor ranoides* from the Área de Conservación de Guanacaste (ACG). a) A Bayesian phylogeny constructed from concatenated alignments of 16S rRNA and CO1 genes of *C. ranoides* samples from ACG and their sister taxa. The tree was rooted using the *C. opimus* and *C. megacephalus* clade as an outgroup. Mr Bayes v3.2.6 was run without partitioning by loci using the HKY85 substitution model with rate variation modelled by a gamma distribution with four rate categories. Support values at nodes are posterior probabilities. B) Minimum spanning haplotype network of *Craugastor ranoides* constructed in Popart. Green shading denotes historic samples collected at Volcán Cacao; beige shading denotes extant samples collected from the seasonally dry tropical forest.

**Table 1.** Estimates of Net Evolutionary Divergence with K2P correction between Groups of CO1 (bold), and 16S sequences based on our phylogeny (Fig. 1). The number of amino acid substitutions per site from estimation of net average between groups of sequences are shown. Analyses were conducted using the Poisson correction model (Zuckerkandl & Pauling, 1965).

The analysis involved 24 amino acid sequences. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.*, 2016).

**Supplementary Table 1.** Species name, voucher number, locality information, ranavirus infection status and GenBank accession numbers for the 16S and CO1 mitochondrial gene markers. Voucher number acronyms: University of Costa Rica (UCR), United States National Museum (USNM; now National Museum of Natural History, Smithsonian Institution), Museum of Vertebrate Zoology at Berkeley (MVZ), Robert Puschendorf field voucher (RP). Country acronyms: Costa Rica (CR), Panama (PA). NA indicates that either a sample was not tested for ranavirus infection, the test was negative, or no sequences were generated.


Forest type
Dry forest
Rainforest
Cloud forest
Features
River
Highway
Pedregal
Grande
Murciélago
Danta
Calera
Cacao
Nisperal
10.95°N
10.85°N
10.75°N
85.8°W
85.7°W
85.6°W
85.5°W
10 km
N

1

2 Figure 1.

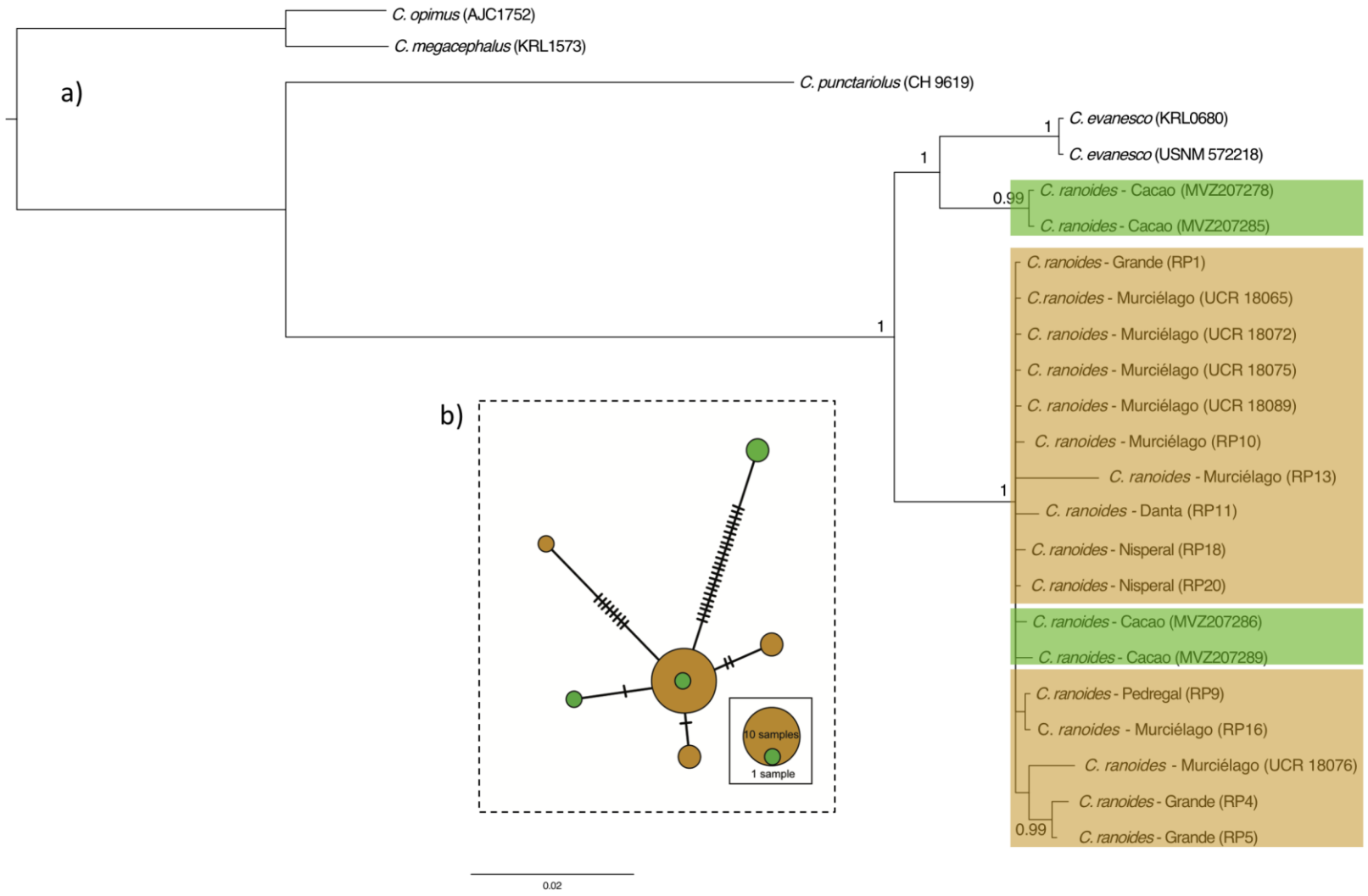

a)
C. opimus (AJC1752)
C. megacephalus (KRL1573)
C. punctariolus (CH 9619)
C. evanesco (KRL0680)
C. evanesco (USNM 572218)
C. ranoides - Cacao (MVZ207278)
C. ranoides - Cacao (MVZ207285)
C. ranoides - Grande (RP1)
C.ranoides - Murciélago (UCR 18065)
C. ranoides - Murciélago (UCR 18072)
C. ranoides - Murciélago (UCR 18075)
C. ranoides - Murciélago (UCR 18089)
C. ranoides - Murciélago (RP10)
C. ranoides - Murciélago (RP13)
C. ranoides - Danta (RP11)
C. ranoides - Nisperal (RP18)
C. ranoides - Nisperal (RP20)
C. ranoides - Cacao (MVZ207286)
C. ranoides - Cacao (MVZ207289)
C. ranoides - Pedregal (RP9)
C. ranoides - Murciélago (RP16)
C. ranoides - Murciélago (UCR 18076)
C. ranoides - Grande (RP4)
C. ranoides - Grande (RP5)
1
1
0.99
1
1
0.99
b)
10 samples
1 sample
0.02

Figure 2.

6 Table 1.

7

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1. outgroup | | **0.1898** | **0.2202** | **0.1794** | **0.2012** |
| 2. *C. punctariolus* | 0.0828 | | **0.2096** | **0.2181** | **0.2007** |
| 3. *C. evanesco* | 0.1013 | 0.0978 | | **0.0401** | **0.0245** |
| 4. *C. ranoides* dry forest lineage | 0.0951 | 0.0947 | 0.0137 | | **0.0292** |
| 5. *C. ranoides* cloud lineage | 0.1076 | 0.1008 | 0.0182 | 0.0206 | |

8

Supplementary Table 1.

| Species | Voucher number | Collection locality | Latitude | Longitude | Ranavirus inf | Genbank rana | Genbank 16S | Genbank CO1 |
|---|---|---|---|---|---|---|---|---|
| *C. evanesco* | KRL0680 | Omar Torrijos National Park, PA | 8.68727 | -80.64397 | NA | NA | FJ784332 | FJ766637 |
| *C. evanesco* | KRL 0804 | Río Blanco, Cocle, 1100 m, PA | | | NA | NA | KC129324 | KC129216 |
| *C. megacepha* | KRL 1573 | Omar Torrijos National Park, PA | 8.68727 | -80.64397 | NA | NA | KR863193 | KR862938 |
| *C. megacepha* | KRL 0618 | Omar Torrijos National Park, PA | 8.68727 | -80.64397 | NA | NA | FJ784323 | FJ766663 |
| *C. opimus* | AJC 1752 | Chagres National Park, PA | 9.41953 | -79.43244 | NA | NA | KR863198 | KR862943 |
| *C. punctariola* | AJC 1135 | Altos del Maria, 930 m, PA | | | NA | NA | KC129325 | KC129217 |
| *C. ranoides* | UCR 18072 | Rio Murcielago, ACG, CR | 10.89691 | -85.73029 | NA | NA | KC129329 | KC129220 |
| *C. ranoides* | UCR 18089 | Rio Murcielago, ACG, CR | 10.89691 | -85.73029 | NA | NA | KC129327 | KC129218 |
| *C. ranoides* | UCR 18065 | Rio Murcielago, ACG, CR | 10.89691 | -85.73029 | NA | NA | KC129219 | KC129219 |
| *C. ranoides* | UCR 18075 | Rio Murcielago, ACG, CR | 10.89691 | -85.73029 | NA | NA | KC129330 | KC129221 |
| *C. ranoides* | UCR 18076 | Rio Murcielago, ACG, CR | 10.89691 | -85.73029 | NA | NA | KC129331 | KC129222 |
| *C. ranoides* | 1 | Quebrada Grande, ACG, CR | 10.90134 | -85.77522 | 0 | NA | MH347338 | MH347337 |
| *C. ranoides* | 4 | Quebrada Grande, ACG, CR | 10.90134 | -85.77522 | 0 | NA | MH368226 | MH368236 |
| *C. ranoides* | 5 | Quebrada Grande, ACG, CR | 10.90134 | -85.77522 | 0 | NA | MH368227 | MH368242 |
| *C. ranoides* | 9 | Rio Pedregal, ACG, CR | 10.90135 | -85.75068 | 0 | NA | MH368233 | MH368235 |
| *C. ranoides* | 10 | Rio Murcielago, ACG, CR | 10.90135 | -85.75068 | 0 | NA | MH368228 | MH368234 |
| *C. ranoides* | 11 | Quebrada Danta, ACG, CR | 10.86623 | -85.71600 | 0 | NA | MH368225 | MH368241 |
| *C. ranoides* | 13 | Rio Murcielago, ACG, CR | 10.89691 | -85.73029 | 0 | NA | MH368229 | MH368244 |
| *C. ranoides* | 16 | Rio Murcielago, ACG, CR | 10.89691 | -85.73029 | 0 | NA | MH368230 | MH368243 |
| *C. ranoides* | 18 | Rio Nisperal, ACG, CR | 10.82730 | -85.64860 | 0 | NA | MH368232 | MH368245 |
| *C. ranoides* | 20 | Rio Nisperal, ACG, CR | 10.82730 | -85.64860 | 0 | NA | MH368231 | MH368246 |
| *C. ranoides* | 56 | Quebrada Grande, ACG, CR | 10.90134 | -85.77522 | 1 | MK348557 | NA | NA |
| *C. ranoides* | MVZ 207278 | Volcan Cacao, ACG, CR | 10.93333 | -85.45000 | 0 | NA | MH368221 | MH368238 |
| *C. ranoides* | MVZ 207285 | Volcan Cacao, ACG, CR | 10.93333 | -85.45000 | 1 | MK348558 | MH368222 | MH368239 |
| *C. ranoides* | MVZ 207286 | Volcan Cacao, ACG, CR | 10.93333 | -85.45000 | 0 | NA | MH368223 | MH368240 |
| *C. andi* | MVZ207255 | Volcan Cacao, ACG, CR | 10.93333 | -85.45000 | 1 | NA | | |
| *C. ranoides* | MVZ 207289 | Volcan Cacao, ACG, CR | 10.93333 | -85.45000 | 0 | NA | MH368224 | MH368237 |

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