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Disease Ecology of Two Emerging Amphibian Pathogens in Costa Rica

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DISEASE ECOLOGY OF TWO EMERGING AMPHIBIAN PATHOGENS IN COSTA RICA

by

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At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee.

Work submitted for this research degree at the University of Plymouth has not formed part of any other degree either at the University of Plymouth or at another establishment.

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Abstract

Disease ecology of two emerging amphibian pathogens in Costa Rica

Felicity Wynne

The emergence of infectious diseases is increasing globally, whilst biodiversity is being lost at an unprecedented rate. The amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) has driven the extinction of more species than any other known pathogen. This loss was particularly severe among Neotropical amphibians, with several infamous extinctions from Costa Rica. Although chytrid has been attributed to all enigmatic population declines in Costa Rica, its presence has only been investigated from a few populations. Alongside chytrid, another pathogen, ranavirus (*Ranavirus* spp.), is considered a significant threat to amphibian populations. Very little is known about ranaviruses outside of temperate regions, but its recent confirmation from Costa Rica has provided an opportunity to investigate both pathogens amongst declining amphibian populations. The aim of this thesis was to improve the knowledgebase of chytrid and ranaviruses in the tropics, using Costa Rica as a model system, investigate cryptic diversity among local amphibian hosts and to optimise and determine suitable diagnostics that could be used to aid ranavirus research globally. The Área de Conservación Guanacaste (ACG), northern Costa Rica was chosen as my main study location for the investigation of hosts and pathogens. The ACG is comprised of high habitat diversity that is representative of multiple tropical habitats. Samples from the ACG were used to compare four ranavirus assays to determine appropriate diagnostics. Museum specimens collected between 1976 and 1989 were used to examine how long ranaviruses have been present in northern and central Costa Rica and whether they were present during outbreaks of chytrid. The presence of ranavirus was also

examined amongst highly threatened amphibian populations in tropical regions of Australia. I identified six candidate species of amphibian, indicating current inventories of Neotropical amphibians to be incomplete. Among the populations of these candidate species, ranavirus and chytrid are present. Chytrid was commonly found in wet forest habitats, whereas ranavirus was detected among all habitat types. However, ranavirus prevalence increased with temperature. Both pathogens were non-randomly distributed among host taxonomic families and ranavirus was detected in Costa Rican reptile species, representing the first detection of this pathogen from this group in Central America. Comparisons of ranavirus diagnostics indicated some assays to exhibit poor sensitivity and generate false positives. Using this information and a highly sensitive assay I detected ranavirus from populations sampled from prior to and during amphibian declines. Infected individuals included extinct and severely declined species. I also detected this pathogen from the order Gymnophiona, which, to my knowledge, has not been found infected with ranavirus previously. Additionally, I detected ranavirus from highly threatened Australian amphibian populations. Future research priorities should include the description of candidate species identified here using morphometrics, bioacoustics and nuclear DNA. If confirmed as true species, their conservation status should be assessed. The results of pathogen surveys among Costa Rican populations will allow researchers to identify areas and taxa more likely to be infected within the tropics. My findings from comparing ranavirus assays will facilitate the generation of highly accurate results, hence improve estimates of distribution and impacts of ranavirus. My thesis has improved the knowledge of both hosts and ranaviruses in Costa Rica, but also in tropical systems more generally, and will help guide future researchers with the effective detection of ranaviruses from these under-studied systems.

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Abbreviations

16S/16S rRNA	16S ribosomal ribonucleic acid
ACG	Área de Conservación Guanacaste
AIC	Akaike information criterion
ATV	*Ambystoma tigrinum* virus
BIV	Bohle iridovirus
bp	Base pair(s)
CAC	Cacao
CAR	Caribe
CI	Confidence interval
CMTV	Common midwife toad virus
CO1	Cytochrome c oxidase subunit 1
DNA	Deoxyribonucleic acid
EHNV	Epizootic haematopoietic necrosis virus
FV3	Frog virus 3
GTR	General time reversible model
HKY85	The Hasegawa, Kishino and Yano 1985 model
JC	Jukes and Cantor model
K2P	Kimura 2-parameter distance
MA	Maritza
MCMC	Markov chain Monte Carlo
MCP	Major capsid protein
mtDNA	Mitochondrial deoxyribonucleic acid
MU	Murciélago
PCR	Polymerase chain reaction

PI	Pitilla
qPCR	Quantitative polymerase chain reaction
RC	Rio Calera
SNP	Single nucleotide polymorphism
SR	Santa Rosa

Chapter 1 Introduction

The planet is currently experiencing anthropogenic changes that are so severe the world has recently entered a new distinct geologic time period: the Anthropocene (Crutzen & Stoermer 2000, Waters et al. 2016). The Anthropocene is characterised by climate change, high levels of modern-material wastes ('technofossils'), deforestation, the spread of invasive species and the rapid extinction of biodiversity (Waters et al. 2016, Zalasiewicz et al. 2016, Dellasala et al. 2018). Two thirds of wildlife populations are predicted to be lost from 1970 to 2020 (World Wildlife Fund et al. 2016). So extreme, this loss of biodiversity has been labelled the 'sixth mass extinction' and it has been driven by human overpopulation and overconsumption (Ceballos et al. 2017). Tropical forests, especially those of Central and South America, are among the most biologically diverse ecosystems on Earth (Gentry 1992). Simultaneously, tropical forests have experienced the greatest habitat losses (Hansen et al. 2013). As a result, a 41% decline in tropical forest species was observed between 1970 and 2009 (World Wildlife Fund et al. 2016).

Neotropical herpetofaunal species richness

The Neotropical Realm (hereafter 'Neotropics') is one of Earth's eight land biogeographic realms, which are based on distributional patterns of similar organisms (Udvardy 1975). It represents a tropical region on the American continent, extending from the southern USA and central Mexico to southern Brazil (Udvardy 1975). Species richness is known to increase from the

Earth's poles to the equator, hence is extremely high within tropical regions, such as the Neotropics (Hillebrand 2004). This trend is reflected by the native herpetofaunal species of this region (Wiens 2007).

Patterns of species richness are driven by biogeographic and evolutionary processes such as speciation, extinction and dispersal (Ricklefs 1987, 2004). In particular, Central America has experienced an unusually high number of geomorphic and climatic processes (Gardner et al. 1987), which have shaped Neotropical herpetofaunal diversity. Multiple colonisation events of South American species into modern Central America have been proposed (Heinicke et al. 2007, Pinto-Sánchez et al. 2012). The most famous arose from the formation of Central America to its current state, as a bridge between North and South America (Simpson 1940, Coates & Obando 1996). The formation of the Isthmus of Panama, about 3.5 million years ago (Bacon et al. 2015), drove dispersal of taxa from both regions into the same areas and multiple ecological niches thus became available to these species, driving differentiation (Morgan 2005, Kirby et al. 2008). Additionally, volcanic activity and uplift on the Central American isthmus altered the region's topography, creating new habitats for speciation (Gardner et al. 1987). Simultaneously, global climatic oscillations cooled lowland environments, creating dispersal corridors for montane species adapted to cooler habitats (Whitmore & Prance 1987). This allowed such species to migrate between highland areas and become isolated from one another when the climate warmed (Savage 2002). In northern Costa Rica, volcanism lead to the reduction of trade winds received by the area, drastically drying humid forests, driving further speciation (Savage 2002).

Although inventories of Neotropical herpetofauna are numerous, they remain incomplete (e.g. Young et al. 2001, Fouquet et al. 2007, Crawford et al. 2010, Lyra et al. 2016, Waddell et al. 2018). A high degree of amphibian biodiversity is thought to be represented by morphologically cryptic species, which remain hidden under a single name (Bickford et al. 2007). Whilst morphology is often fundamental to species descriptions (Padial et al. 2010), molecular methods can be used to generate higher resolution genetic data (e.g. Camargo et al. 2006, Ron et al. 2006, Angulo & Icochea 2010, Funk et al. 2012, Sheridan & Stuart 2018, Liu et al. 2018). Thus, such methods are being increasingly used, and have rapidly increased the rate of species description (Hanken 1999).

The uncovering of cryptic diversity can lead to the identification of biodiversity hotspots and allow for conservation protection of these newly found units of diversity (Angulo & Icochea 2010). Cryptic species are often concealed within widespread complexes assumed under a single name (Angulo & Icochea 2010). Therefore, what appears as a single non-threatened species may instead be a complex of geographically restricted and threatened constituents (Angulo & Icochea 2010). Hence, effective biodiversity conservation is dependent upon the resolution and description of cryptic taxa (Mace 2004).

Declines of Neotropical herpetofauna

Current and unprecedented worldwide wildlife declines are exemplified by the global amphibian population crisis (Blaustein & Wake 1990, Stuart et al. 2004, Wake & Vredenburg 2008, World Wildlife Fund et al. 2016, IUCN

2018), for which current extinctions exceed the background rate by at least 200 times (McCallum 2007). Forty per cent of assessed amphibian species are threatened with extinction (IUCN 2018). Amphibian declines appeared to occur simultaneously and were particularly severe in Australia and the Neotropics (Laurance et al. 1996, Lips 1998, Lips et al. 2005a, b). In these areas, populations of highly abundant species disappeared within a matter of months (Lips et al. 2006). Stuart et al. (2008) highlighted the severity of Neotropical amphibian population crashes when they found that 93% of the then 130 amphibian species, which were thought to be extinct, were endemic to this region. However, as amphibian diversity within the Neotropics has not been fully assessed, and is often cryptic, the true extent of biodiversity losses may have been vastly underestimated (Crawford et al. 2010).

Amphibian declines have been observed in the Neotropics since the 1980s and have always been exceptionally severe, with seemingly healthy populations in pristine and protected areas suddenly disappearing within a period of a few years (Blaustein & Wake 1990, Pounds & Crump 1994, Lips 1998, Bosch et al. 2001, Rovito et al. 2009). For example, surveys conducted between 1990 and 1994 at high elevations of Costa Rica's Cordillera de Tilaŕan, revealed that 40% of amphibian fauna went missing (Pounds et al. 1999). The most iconic of these missing species is the golden toad (*Incilius periglenes*), which became the 'poster child' for amphibian declines. This species was once abundant in a small localised area of high-altitude forest in Monteverde, Costa Rica (Savage et al. 2008). However, it declined from over 1500 breeding individuals in 1987 to 10 individuals in 1988, a single observed individual in 1989, and the species has not been

sighted since (Crump et al. 1992, Pounds & Crump 1994). The disappearance of the golden toad was initially attributed to drier climatic conditions (Crump et al. 1992). Few tadpoles were reported to metamorphose in 1987 due to breeding pools drying up and in 1988 no eggs or tadpoles could be found (Crump et al. 1992). It was suggested the toads could re-emerge when optimal breeding conditions resumed (Crump et al. 1992). However, when conditions did improve, the frogs did not return (Pounds & Crump 1994). Meanwhile, harlequin frogs of the genus *Atelopus* were also experiencing enigmatic declines (Pounds & Crump 1994, La Marca et al. 2005, Pounds et al. 2006). Contrary to the restricted range of the golden toad, this is a widespread South American genus comprised of at least 118 species which have suffered severe population crashes (La Marca et al. 2005). Eighty one percent of *Atelopus* species have declined and 67% of these declined species are presumed extinct (La Marca et al. 2005, Pounds et al. 2006).

Reptile population declines have been likened to the severity of amphibian declines (Gibbons et al. 2000), but recent research has indicated that a lower proportion (19%) of reptilian populations are threatened with extinction (Böhm et al. 2013). However, similarly to amphibian declines, reptile declines have been especially severe in tropical areas where species richness is extraordinarily high (Stuart et al. 2004, Böhm et al. 2013). Both reptile and amphibian declines have been linked to a variety of factors, with habitat loss and degradation posing the highest threat (IUCN 2008, Böhm et al. 2013, World Wildlife Fund et al. 2016). However, for amphibians many declines have been sudden and of an enigmatic nature, usually affecting populations

within pristine habitats (Lips 1998, Stuart et al. 2004, IUCN 2008). Due to their unexplained nature, many populations were not well characterised prior to declines. Thus, only a few catastrophic amphibian declines are well documented from the Neotropics (Pounds & Crump 1994, Pounds et al. 1997, 1999, Lips 1998, Lips et al. 2003a, 2006, La Marca et al. 2005), but even fewer records of simultaneous reptile declines exist (Pounds et al. 1999, Whitfield et al. 2007, Sinervo et al. 2010). These enigmatic declines of amphibians and reptiles have since been linked to climate change (Pounds & Crump 1994, Pounds et al. 1997, 1999, 2006) and, for amphibians, the emergence of disease (Berger et al. 1998, Lips, et al. 2003b, 2006).

Emerging infectious diseases drive wildlife declines

Globally, human, domestic and wildlife populations are facing increasing and unprecedented levels of disease emergence, which is of great conservation, economical and public health concern (Daszak et al. 2000, Jones et al. 2008). Emerging infectious diseases may be categorised as either those which are endemic, but have increased in incidence, or those which have been introduced into a naïve host population (Jones et al. 2008). Anthropogenic or natural processes of ecosystem alteration and movement of pathogens or vectors, alongside mutational changes in pathogens can drive emergence (Daszak et al. 2000, Williams et al. 2002). For example, deforestation in Brazil has been linked to increased incidents of malaria among local people (Olson et al. 2010). The introduction of the mosquito (*Culex quinquefasciatus*) vector to Hawaii by European sailors drove the emergence of avian malaria (*Plasmodium relictum*) and pox (*Poxvirus avium*)

within immunologically naïve bird populations (Warner 1968). The development of drug resistant strains of tuberculosis (*Mycobacterium tuberculosis*) is driving significant human mortality (Porter & McAdam 1994).

Traditional epidemiological models predict that highly virulent pathogens have the capacity to rapidly reduce host population densities below a threshold value that is required to maintain transmission, resulting in pathogen extinction (Anderson & May 1978, May & Anderson 1978, 1979). Therefore, pathogens should be unable to directly cause host extinctions. However, transmission is theoretically able to persist beyond this threshold if it is density-independent. Such transmission may arise from pathogens with low host specificity, which are able to persist in one or more species (a biotic reservoir) or the environment (an abiotic reservoir), whilst continuing to be transmitted to susceptible species (Rosà et al. 2003). Although real-life examples of disease-induced extinctions are rare, one convincing case is the sudden enigmatic extinctions of potentially hundreds of amphibian species, which have been associated with outbreaks of a highly virulent and non-host-specific chytrid fungus (*Batrachochytrium dendrobatidis*, hereafter 'chytrid', Berger et al*.* 1998, Farrer et al*.* 2011).

Chytrid is the aetiologic agent of the disease chytridiomycosis (Berger et al. 1998, Martel et al. 2013). It is one of two currently recognised species within the phylum Chytridiomycota known to parasitise vertebrates, specifically amphibians (Berger et al. 1998, Martel et al. 2013). Chytrid was first identified in the late 1990s from Central American and Australian amphibians. Despite the two decade gap between its discovery and initial enigmatic amphibian declines, many of these declines have since been

attributed or assumed to be due to chytridiomycosis (Skerratt et al. 2007). Chytridiomycosis is considered to be emerging due to the sudden increase in outbreak frequency observed over the last five decades (Berger et al. 1998, Skerratt et al. 2007). One of the most compelling lines of evidence for chytrid-driven declines is from Panama, where the emergence of chytrid was followed by mass mortalities of infected amphibians, resulting in population declines (Lips et al. 2006). Other evidence includes the fulfilment of Koch's postulates from Central American, Australian and species from other areas experiencing declines (Berger et al. 1998, Longcore et al. 1999, Davidson et al. 2003), and its detection from other severely declining populations (Lips et al. 2003b). Chytrid now occurs on every continent with amphibians, but current evidence indicates it to have originated in East Asia (O'Hanlon et al. 2018). For the most part, the widespread emergence of chytrid has been attributed to its spread from the international pet, laboratory, food and zoo trades (Picco & Collins 2008, Catenazzi et al. 2010). However, other routes of transmission include conservation actions (Walker et al. 2008), and possibly migration of wildlife (Johnson & Speare 2005, Garmyn et al. 2012).

Post-metamorphic amphibians are more susceptible to severe chytridiomycosis, where hyperkeratosis (skin thickening), hyperplasia (cell proliferation) and sloughing occur (Berger et al. 2005). As the host's electrolytic balance is disrupted the critical pathogen intensity threshold is reached and cardiac arrest and death often ensue (Berger et al. 2005, Voyles et al. 2007, 2009, Campbell et al. 2012). However, not all species or individuals infected with chytrid develop chytridiomycosis, and certain taxa

appear more susceptible to disease than others (e.g. Woodhams et al. 2007; Olson et al. 2013).

In addition to the emergence of the chytrid fungus another group of pathogens, ranaviruses, pose a significant threat to wildlife (Daszak et al. 1999). The genus *Ranavirus* is one of five genera of the family *Iridoviridae*, and contains multiple genetically distinct viral species which infect fish, reptiles and amphibians (Chinchar 2002). Ranaviruses have been associated with mass die-offs and population declines of amphibians in the Americas, Asia, Australia and Europe (Moody & Owens 1994, Jancovich et al. 1997, Bollinger et al. 1999, Greer et al. 2005, Une et al. 2009, Teacher et al. 2010, Kik et al. 2011, Price et al. 2014, Stark et al. 2014, Rosa et al. 2017). However, research has tended to focus on ranaviruses within temperate regions, so very little is known about ranaviruses from tropical regions. Examples of outbreaks include elevated levels of population mortality associated with ranavirus from Saskatchewan, Canada and in Arizona, USA, where *Ambystoma tigrinum* virus (ATV) has been linked to tiger salamander (*Ambystoma tigrinum*) die-offs (Jancovich et al. 1997, Bollinger et al. 1999). Localised median population declines of 81% from UK common frogs (*Rana temporaria*) have been associated with frog virus 3-like (FV3-like) ranavirus infections (Teacher et al. 2010). In Iberia, mortality events and declines involving multiple amphibian and a single reptile host (one dead individual) species have been associated with the emergence of common midwife toad virus (CMTV)-like ranaviruses (Price et al. 2014, Rosa et al. 2017). In Portugal, ranavirus disease emergence followed altitudinally-restricted chytrid-associated population declines, affecting multiple amphibian hosts,

life-stages and altitudinal ranges (Rosa et al. 2017). Thus, hosts appear highly susceptible in some cases, with disease transmission possibly independent of host density through biotic and abiotic reservoirs, vectors and host behaviour (Miller et al. 2011, Earl & Gray 2014, Kimble et al. 2015). Ranaviruses are thought to be emerging, with supporting evidence including a lack of coevolutionary history found between host and virus for ATV (Storfer et al. 2007), a link between spread and human actions (Schloegel et al. 2009, Price et al. 2014, 2016, Rijks et al. 2016) and an apparent recent increase in geographic distribution (Duffus et al. 2015; though sampling efforts have also drastically increased and technology advanced).

When ranavirosis is present, signs include lethargy, oedema, haemorrhaging, ulceration and mortality is through cellular and organ necrosis (Cunningham et al. 1996, Hyatt et al. 1998). Similarly to chytrid, not all ranavirus infections seem to result in disease, and certain taxa appear more susceptible to infection (Hoverman et al. 2011, Hoverman et al. 2012a).

Ranavirus research from declining Central American amphibian populations is severely lacking. An iridovirid was initially suspected from a cane toad (*Rhinella marina*) in the Área de Conservación Guanacaste, north-west Costa Rica, in the early 1990s (Speare et al. 1991). Ranavirus was not detected in Monteverde, north-west Costa Rica, and Las Cruces Biological Station, south west Costa Rica, during surveys undertaken in 2005 (Picco & Collins 2007). However, it was detected from samples collected from La Selva Biological Station, north-east Costa Rica, between 2006 and 2008 (Whitfield et al. 2012). In 2011, a die-off event of 55 tadpoles from Ometepe Island, south Nicaragua, was associated with ranavirus (Stark et al. 2014).

These later surveys (Whitfield et al. 2012, 2013, Stark et al. 2014) have provided some information on ranavirus host ranges in Central America, but data regarding host and geographic ranges are still lacking. Additionally, information on the drivers and ecology of this Central American pathogen are entirely unknown but should be examined to inform potential conservation actions and disease management.

Pathogen detection

In order to conduct effective disease monitoring, risk assessments, disease management (e.g. culling) and conservation actions (e.g. translocations, *ex situ* populations) estimates of pathogen presence, prevalence and dynamics must be accurate (Jennelle et al. 2007, McClintock et al. 2010). Hence, sampling and diagnostic strategies used for inferring disease presence need to be reliable.

A suite of diagnostic methods should be used to identify disease, but the use of molecular methods in detecting infection is increasing, replacing conventional techniques such as histology as the primary means of pathogen detection (e.g. Boyle et al. 2004, Kriger et al. 2007). Although molecular assays cannot be used to explicitly identify disease, they can be used to rapidly detect the presence of a pathogen, which may or may not be viable and cause disease (Kriger et al. 2007). Molecular techniques are objective, have broader applications and often offer greater sensitivity and specificity (Hyatt et al. 2007, Lorch et al. 2010, Skerratt et al. 2011), although false-positives and false-negative rates need to be generated. Nucleic acid-based molecular techniques such as conventional polymerase chain reaction

(PCR), quantitative (q) PCR and sequencing are often used in pathogen detection. Sequencing typically follows the generation of an amplicon from PCR and is highly specific. As such, it is often recommended for pathogen identification (OIE, 2017) and can be used to investigate the pathogen's taxonomy and genetics. However, the PCR assay itself is slower, requires a gel electrophoresis step, and can be less sensitive and specific than qPCR, especially 'probe-based' qPCR assays which include fluorescent-labelled target-specific probes (Hyatt et al. 2007). But the equipment and reagents required to undertake qPCR, particularly probe-based methods, are often more expensive than those required for conventional PCR (e.g. Kriger et al. 2006).

In general, diagnostic methods may vary considerably to one another but should be standardised according to the best available techniques, and when multiple diagnostic assays are in use each should be assessed for their comparative limitations in detection ability. For example, for amphibians histology was once commonly used to detect chytrid infection, but qPCR methods were found to outperform histology in their sensitivity (Boyle et al., 2004; Hyatt et al., 2007), so this method was identified as the best available.

Thesis aims

The broad aim of this thesis was to explore the ecology of two amphibian pathogens from under-studied systems. Amphibians and reptiles have not been thoroughly studied from the area I chose to base my research in, the Área de Conservación Guanacaste (ACG). Because of this, my first aim was to characterise the amphibian hosts that would be used for subsequent

pathogen testing and analyses. The ACG also contains a high diversity of habitat types, making it ideal for investigating pathogen ecology. Thus, I decided to investigate drivers of both chytrid and ranavirus infection and to determine the distribution of these pathogens among hosts and tested habitats. As almost nothing is known about ranaviruses in Costa Rica, I aimed to investigate the viral diversity among my samples, to explore how long this pathogen has been present in the country, and to find out whether the pathogen could have been involved in amphibian declines. I further investigated ranaviruses from tropical regions, by testing for their presence in other tropical areas outside of Costa Rica. In order to effectively meet these aims, I determined appropriate methods for the detection of ranaviruses from the systems I worked with. My findings are then linked to environmental changes, such as climate change and conservation recommendations are made.

Chapter 2 Preliminary analysis of cryptic diversity among northern and central Costa Rican amphibian populations

Abstract

Accurate species descriptions are required to set effective conservation priorities. Cryptic species are common among amphibians, especially in the Neotropics. Yet, despite the high conservation concern expressed for Central American amphibians, many have yet to be examined for cryptic diversity using molecular tools. Costa Rican amphibians have undergone declines that may have left such undescribed species threatened, with no effective conservation plan in place. Here, I undertook a preliminary analysis of amphibian diversity in Costa Rica and other areas of the Neotropics, using mtDNA to highlight species that may merit further investigation. Mitochondrial 16S ribosomal RNA and cytochrome oxidase subunit 1 concatenated and single-gene phylogenies were examined for cladistic structure within units currently considered to be unique species. Interspecific, intraspecific and net between group mean distances were calculated, as per the phylogenies, for all candidate species. Species boundaries at or above 2% at 16S and 8% at CO1 K2P-corrected between groups distance and a species delimitation analysis were used to support these results. In total, I uncovered six candidate species found nested within four wide-ranging currently described species. Isolation by distance has likely driven at least some of these divergences due to broad distances between them. However, the presence of other mechanisms of speciation, such as historic climatic and habitat changes, are apparent. Further analyses including the use of nuclear DNA,

morphometric and bioacoustics data are necessary to test if these candidates are truly distinct species.

Introduction

At least 42% of amphibian populations are undergoing declines (IUCN 2017). Effective biodiversity conservation is reliant upon the accurate description of distinct species (Mace 2004), yet it is thought that considerable amphibian diversity remains hidden within single taxa described from morphology alone (Bickford et al. 2007). This has become increasingly apparent over the last 20 years with the advancement of molecular techniques uncovering numerous candidate species per year (Hanken 1999). Since 1985 the number of amphibian species has increased by 60%; in 2017 alone, 168 new species were described (AmphibiaWeb 2018).

Geographic distributions of species complexes can be widespread with apparent high densities of individuals, yet the constituent cryptic units within such complexes may exist as restricted, potentially threatened populations (Ravaoarimanana et al. 2004). The irretrievable loss of evolutionarily distinct units represents a greater loss of biodiversity than the extirpation of populations nested within a larger, genetically similar species (Isaac et al. 2007). Thus differentiating cryptic units is essential if conservationists expect to undertake effective prioritised conservation. To tackle this, the evolutionarily significant units (ESU) concept was proposed to provide conservation protection to such units that are not currently encompassed within their own distinct taxonomy (Ryder 1986). ESUs are used as a way of distinguishing important diversity within species and can be recognised using

morphology, distribution, life history and genetics (Ryder 1986). Genetic markers are commonly used in distinguishing ESUs as they can be used to effectively identify populations with little or no gene flow between them (Fraser & Bernatchez 2001).

In the Neotropics, studies investigating cryptic amphibian diversity have uncovered numerous undescribed species, with the number of candidate species sometimes exceeding the original number of described species (Funk et al. 2012). These discoveries have also occasionally occurred after the proposed species has become extinct. For example, in El Copé, Panama, amphibian diversity loss over an eight-year period resulting from chytrid emergence has been quantified (Crawford et al. 2010). This assessment indicated 41% of amphibian diversity to have been lost, including 5 undescribed species. This study has explicitly shown species extinctions to have occurred prior to their recognition as ESUs with high conservation importance.

In nearby Costa Rica, amphibian diversity is thought to be of a similar high level (Crawford 2003), with many ESUs yet to be discovered and described (Young et al. 2001, Savage 2002). However, unlike in Panama, data from systematic sampling of Costa Rican amphibians prior to declines is less readily available. Costa Rican amphibians disappeared much earlier than those in Panama, at a time when the significance of amphibian declines was not fully recognised (Bolaños 2002, Lips et al. 2008, Phillips & Puschendorf 2013). Therefore, the understanding of the cryptic diversity lost as a result of Costa Rican amphibian declines is lacking. Additionally, few studies have investigated the diversity of current populations, for which the opportunity of

preservation still exists. This is especially important as amphibian population declines have left many populations highly threatened. Within these, ESUs could exist, resulting in the division of known populations into even smaller groups that require urgent conservation action.

As part of a preliminary study of genetic diversity among Costa Rican amphibia, I compared sequence divergence between two mitochondrial genes from amphibians sampled from areas that underwent extreme declines, including population extinctions. Samples collected from the present day and prior to and during amphibian declines in the 1980s were used. I tested for cryptic diversity and defined ESUs that may warrant species status and hypothesised that cryptic amphibian diversity exists within Costa Rica and can be rapidly revealed using simple, low-cost genetic approaches.

Methods

Laboratory analyses

Amphibian toe-clips were taken from anurans of the Área de Conservación Guanacaste (ACG), north-west Costa Rica, between 2015 and 2017 and a subset containing 161 of these was used in this study. In addition, internal anuran and Gymnophionan tissues were donated from the Museum of Vertebrate Zoology, UC Berkley. The latter tissues were taken from 309 historic museum specimens collected from central to northern Costa Rica between 1976 and 1989. Both recent and historic samples were collected for two additional studies and collection methods are therefore expanded upon in Chapters 3 and 5 of this thesis. I extracted DNA from amphibian toe-clips

and internal tissues using a standard ammonium acetate protocol (Nicholls et al. 2000).

Conventional PCR assays from Crawford et al. (2010) that targeted a region of the mitochondrial (mt) 16S ribosomal RNA gene (16S) were performed at the University of Plymouth. Each reaction consisted of 1.8μL DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Massachusetts, USA), 10μM of both forward (5'– CGC CTG TTT ATC AAA AAC AT –3') and reverse primer (5'– CCG GTC TGA ACT CAG ATC ACG T –3'), 1μL template DNA and were made up to 10μL using molecular grade water. Thermocycler conditions were used for 40 cycles and included a 94°C denaturation step for 30 seconds, a 49°C annealing step for 30 seconds and a 72°C 40 second extension step, which increased by 1 second per cycle. Amplicon size for these primers is between 500-650bp and amplification was confirmed by running PCR products on a 2% agarose gel. All amplicons were sent to Macrogen Inc for Sanger sequencing of both strands. For mitochondrial cytochrome oxidase subunit 1 (CO1) amplification and sequencing, DNA extractions were sent to the Canadian Centre for DNA Barcoding for PCR and sequencing (Ivanova et al. 2008).

Bioinformatics

Sequence files (ABI) were uploaded to Geneious V8 (https://www.geneious.com, Kearse et al. 2012) for processing. Sequences were trimmed manually at the 3' and 5' ends when low quality base calls were present. Sequence reads from forward and (reverse-complemented) reverse strands were then aligned and consensus sequences were

generated and base call discrepancies were checked and corrected manually against chromatograms. The Basic Local Alignment Search Tool (Nucleotide BLAST+2.8.0; Altschul et al. 1990; https://www.ncbi.nlm.nih.gov/BLAST) was used with default settings for each contig to check sequence identities. In order to compare my samples to those from other individuals, samples of each species sampled here were imported from GenBank (Benson et al. 2005), when available.

16S and CO1 concatenated phylogenies were generated using the MrBayes (Huelsenbeck & Ronquist 2001) Geneious Plugin. The Hasegawa, Kishino and Yano 1985 (HKY85) model + gamma distribution (+G) (Hasegawa et al. 1985) was identified as the best-fit, determined by Akaike Information Criterion (AIC), model of molecular evolution using TOPALi v2 (Milne et al. 2009). MrBayes analyses were run for six million generations with a Markov chain Monte Carlo (MCMC) subsampling frequency of 10,000 and burn in length of 1.5 million. Single gene 16S and CO1 maximum likelihood-based phylogenies were generated using the FastTree (Price et al. 2010) plugin in Geneious. For all anuran phylogenies the Mexican burrowing toad *Rhinophrynus dorsalis* was used as an outgroup due to its comparative high evolutionary distinctiveness (Pyron & Wiens 2011). Only 16S sequences were available from samples of caecilians, therefore a separate phylogeny was generated for these individuals. This separate 16S phylogeny for caecilians was generated using the Bayesian tree methods described above, with the exception of TOPALI v2 indicating the general time reversible (GTR) model + G (Tavaré 1986) to be used instead of HKY85+G. The Costa Rican caecilian, *Oscaecilia ochrocephala*, was used as an outgroup due to its

genetic distinctiveness (Pyron & Wiens 2011). Well supported clades were defined by a threshold of >70% for FastTree support values and >0.95 posterior probability for the Bayesian phylogeny (Hillis & Bull 1993, Greenbaum et al. 2011).

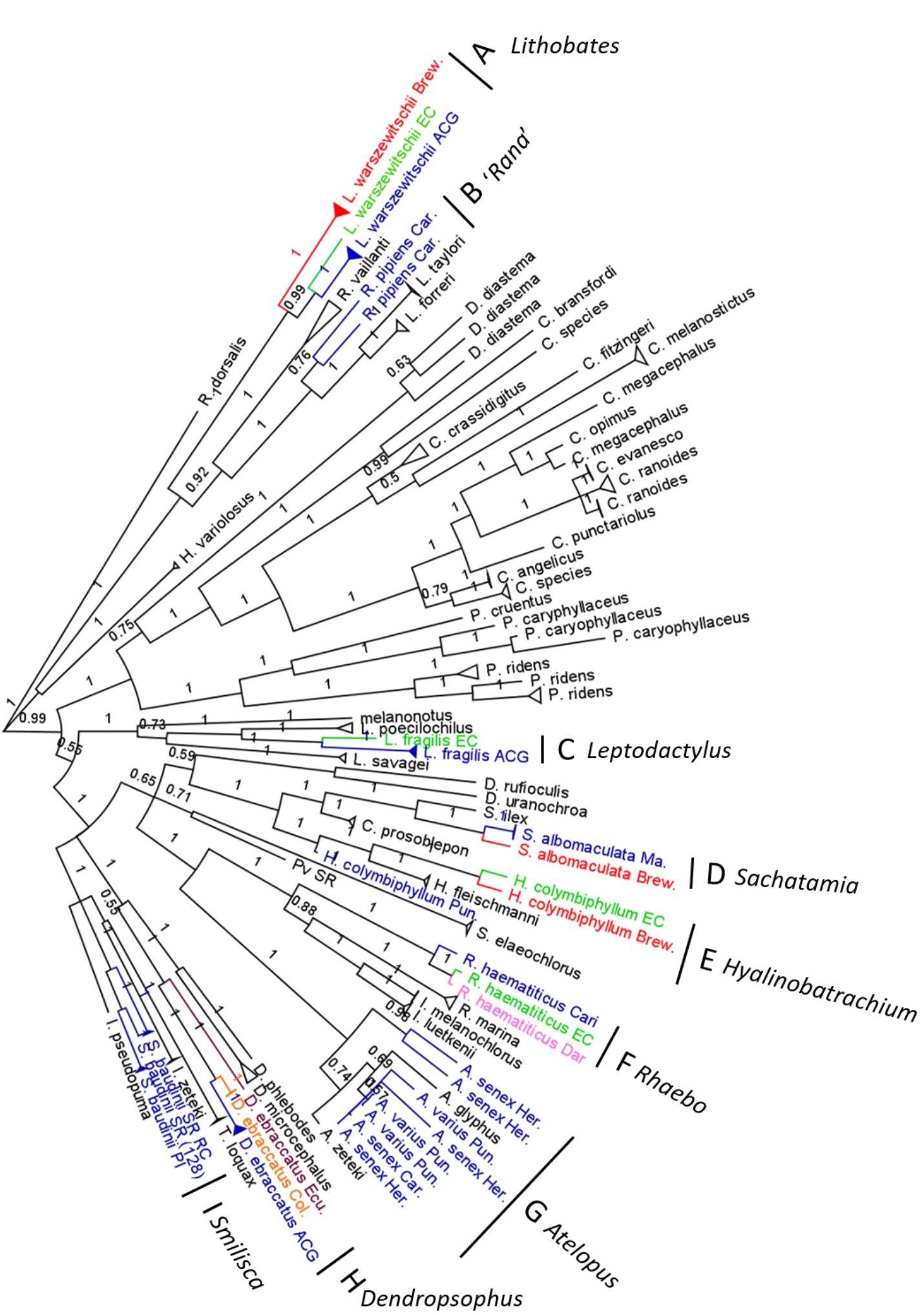
Between group ('interspecific') Kimura two parameter (K2P)-corrected (Kimura 1980) genetic distances were calculated between sister taxa using MEGA7 (Kumar et al. 2016). I defined a candidate species as those showing a between group K2P-corrected divergence of more than 8% in CO1 and more than 2% at 16S, as in Crawford et al. (2010). Within group ('intraspecific') K2P-corrected genetic distances were calculated in MEGA7 and used to indicate groups with high diversity. Additionally, net between-group mean (NBGM) distances were calculated in MEGA7. The Species Delimitation Plugin (Masters et al. 2011) in Geneious was used to delineate candidate species using the *P(Randomly Distinct*) statistic. This statistic is able to mitigate the effects of stochastic gene coalescence processes, which may cause phylogenies to display distinct cladistic structure when sequences are collected from panmictic (random mating of individuals within a population) populations (Masters et al. 2011). *P(Randomly Distinct) v*alues <0.05 indicate cladistic distinctiveness as unlikely to be attributed to stochastic gene coalescence (Masters et al. 2011).

Results

A total of 47 species, as defined by visual inspection, and 8 individuals of unassigned taxonomy were used in analyses. Two sets of trees were generated, one for anuran and one for Gymnophionan amphibians. The

Gymnophionan phylogenies were comprised of 13 16S sequences. Anuran phylogenies were generated using 321 sequences of the 16S gene region, 172 sequences of the CO1 gene region and 192 concatenated sequences, for which both CO1 and 16S sequences were generated. CO1 sequencing for historic samples was of low success with sequences generated for 75% of samples, but with lengths between 85-188bp. Where sequence lengths were under 200bp, samples were eliminated from the CO1 phylogenetic estimates, but were still used in concatenated estimates.

The topology of the anuran 16S (Figure S 2.1), CO1 (Figure S 2.2) and concatenated phylogenies (Figure 2.1) were visually compared, and no significant differences were observed. Upon examining the anuran phylogeny, cladistical structure was observed indicating variation within eight groups, and three species showed structure that likely reflected misidentification (Figure 2.1). The results below describe the tree topology and patristic distances for focal anuran and Gymnophionan species. Interspecific, intraspecific and NBGM distances were calculated for each clade. Finally, species delimitation results are presented for clades represented by more than one individual.


A Lithobates
B 'Rana'
C Leptodactylus
D Sachatamia
E Hyalinobatrachium
F Rhaebo
G Atelopus
H Dendropsophus
I Smilisca
L. warszewitschii Brew.
L. warszewitschii EC
L. warszewitschii ACG
R. vaillanti
R. pipiens Car.
R. pipiens Car.
L. taylori
L. forreri
R. dorsalis
H. variolosus
D. diastema
D. diastema
D. diastema
C. bransfordi
C. species
C. crassidigitus
C. fitzingeri
C. melanostictus
C. megacephalus
C. opimus
C. megacephalus
C. evanesco
C. ranoides
C. ranoides
C. punctariolus
C. angelicus
C. species
P. cruentus
P. caryphyllaceus
P. caryophyllaceus
P. caryophyllaceus
P. ridens
P. ridens
P. ridens
melanonotus
L. poecilochilus
L. fragilis EC
L. fragilis ACG
L. savagei
D. rufioculis
D. uranochroa
S. ilex
S. albomaculata Ma.
S. albomaculata Brew.
C. prosoblepon
H. colymbiphyllum EC
H. colymbiphyllum Brew.
H. fleischmanni
H. colymbiphyllum Pun.
Pv SR
S. elaeochlorus
R. haematiticus Cari
R. haematiticus EC
R. haematiticus Dar
R. marina
I. melanochlorus
I. luetkenii
A. senex Her.
A. senex Her.
A. glyphus
A. varius Pun.
A. varius Pun.
A. senex Her.
A. varius Pun.
A. senex Car.
A. senex Her.
A. zeteki
D. phlebodes
D. microcephalus
D. ebraccatus Col.
D. ebraccatus Ecu.
D. ebraccatus ACG
T. loquax
I. zeteki
S. baudinii SR RC
S. baudinii SR (128)
S. baudinii PI
I. pseudopuma
1
0.99
0.92
0.76
0.63
0.99
0.5
0.79
0.75
0.73
0.59
0.55
0.65
0.71
0.88
0.98
0.74
0.69
0.57

◄Figure 2.1. Phylogeny of concatenated CO1 and 16S genes for Neotropical anuran species. Locations are shown in names for samples where geographic cladistics structuring was observed. These include Costa Rica ('CR'); four provinces of Costa Rica: Guanacaste ('Gu'), Cartago ('Car'), Heredia ('Her'), Puntarenas ('Pun'); the ACG ('ACG'); two dry forest sites from the ACG: Santa Rosa ('SR'), Rio Calera ('RC') and three wet forest sites from the ACG: Maritza ('Ma'), Caribe ('Cari'), Pitilla ('PI'). Panamanian locations include: El Cope ('EC'), Brewster ('Brew') and Darien ('Dar'). Locations outside of Panama and Costa Rica include Ecuador ('Ecu') and Colombia ('Col'). Colours correspond to locations shown in Figure 2.2. Species in black letters have either been previously assessed or showed no significant cryptic diversity. Letters correspond to written descriptions below. Support values at nodes are posterior probabilities. Tree constructed using MrBayes (Huelsenbeck & Ronquist 2001) Plugin Geneious V8 (Kearse et al. 2012).

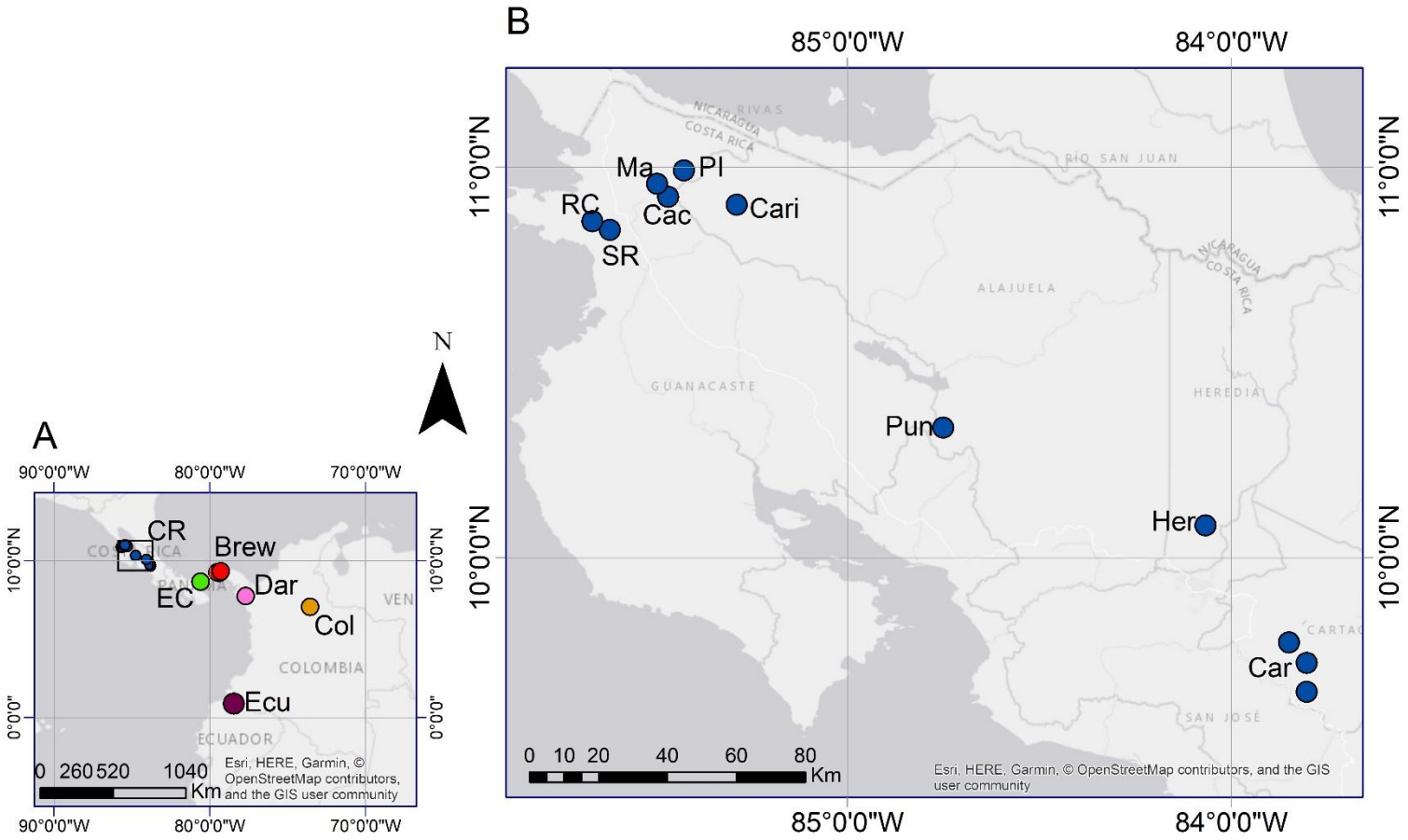


Figure 2.2. A. Distribution of all sites samples were collected from. The extent indicator shows the region displayed in B. B. The distribution of Costa Rican samples. Colour points correspond to those displayed in Figure 2.1. Map constructed in ArcMap 10.5.1.

Anurans

Lithobates warszewitschii (Figure 2.1. A)

Lithobates warszewitschii was consistently divided into three groups representing one Costa Rican and two Panamanian clades (Figure 2.1. A). Splits among Brewster, El Cope and Costa Rican sequences were supported by the high posterior probabilities (>0.99) and high interspecific distances found for both 16S (6.9-11.7%) and CO1 (9.5-16.0%) regions. The Costa Rican individuals were most closely related to individuals from El Cope. Diversity at both 16s (11.7%) and CO1 (16.0%) was greatest between sequences from El Cope and Brewster, and Brewster and Costa Rica (8.9% and 15.2%, for 16s and CO1, respectively). Intraspecific distances were low for 16S (0.0-0.2%) and CO1 (0.2-0.4%). NBGM distances were high for 16S (7.4-12.7%) and CO1 (9.3-15.9%). The highest NBGM distances were between the Costa Rican and Brewster (16S: 12.7%, CO1: 14.9%) clades and controversially, for 16S, Costa Rican and El Cope (11.5%) clades and for CO1 Brewster and El Cope (15.9%) clades. As the El Cope clade was comprised of only one species a *P (Randomly Distinct)* value could not be generated for this clade. However, *P (Randomly Distinct)* values were <0.05 for both Brewster and Costa Rican clades, indicating these to represent a candidate species.

'*Rana pipiens*' (Figure 2.1. B)

'*Rana pipiens*' is a species complex containing *Lithobates taylori* and *L. forreri* in Costa Rica. However, here I found two individuals that formed one well-supported (posterior probability of 1) distinct clade outside of both *L.*

taylori and *L. forreri* clades (Figure 2.1. B). This clade is comprised of two poorly supported clades (posterior probability of 0.76), so I have considered it as one. This clade split prior to the *Lithobates taylori/forreri* split indicating high cladistic distinctiveness. Using the species delimitation plugin I found the closest species to this '*R. pipiens*' clade to be *L. forreri*. *P*(*Randomly Distinct)* values were <0.05 for this '*R. pipiens'*, indicating it to represent a candidate species.

Leptodactylus fragilis (Figure 2.1. C)

Two clades of *Leptodactylus fragilis* are formed, one comprising sequences of Costa Rican origin and another from El Cope, Panama (Figure 2.1. C). For both the concatenated and 16S trees only the El Cope clade was represented by a single sequence only. These clades are highly supported by a posterior probability of 1. The interspecific distance was low for 16S (1.2%) but extremely high for CO1 (17.4%). Intraspecific distances for the Costa Rican clade were low for 16S (0.1%) and CO1 (0.5%). The NBGM distances could not be calculated, as only one individual was present within the El Cope clade.

Sachatamia albomaculata (Figure 2.1.D)

Two well-supported (posterior probability of 1) clades were observed within *Sachatamia albomaculata*, with one containing a single individual from Cerro Azul (considered part of the 'Brewster' group here), Panama, and the other is comprised of two individuals from Costa Rica (Figure 2.1. D). Interspecific distances were low at 16S (0.0%) and fairly high at CO1 (7%). Intraspecific

distances were low for the Costa Rican group at 16S (0.0%) and CO1 (0.0%). NBGM distances could not be calculated.

Hyalinobatrachium colymbiphyllum (Figure 2.1. E)

Three individuals were identified as *H. colymbiphyllum*, yet one individual from Monteverde, Costa Rica, formed a separate distinct, well-supported (posterior probability of 1) clade from the other two, which were from Panama (Figure 2.1. E). This clade appeared to represent a completely different species that splits prior to the *Hyalinobatrachium* group comprised of *H. fleischmanni* and *H. colymbiphyllum*. With the aim of determining which species this sample (149768) belonged to, a more detailed phylogenetic tree (Figure 2.3) was generated, including all known Costa Rican glass frog species. Although, this individual still formed its own well-supported clade, interspecific distances at the 16S region between the focal individual and *H. fleischmanni* were low (1.0%), indicating it to have been misidentified. Sequences were unavailable for many individuals from the CO1 region, so distance analyses were conducted on the 16S region only.

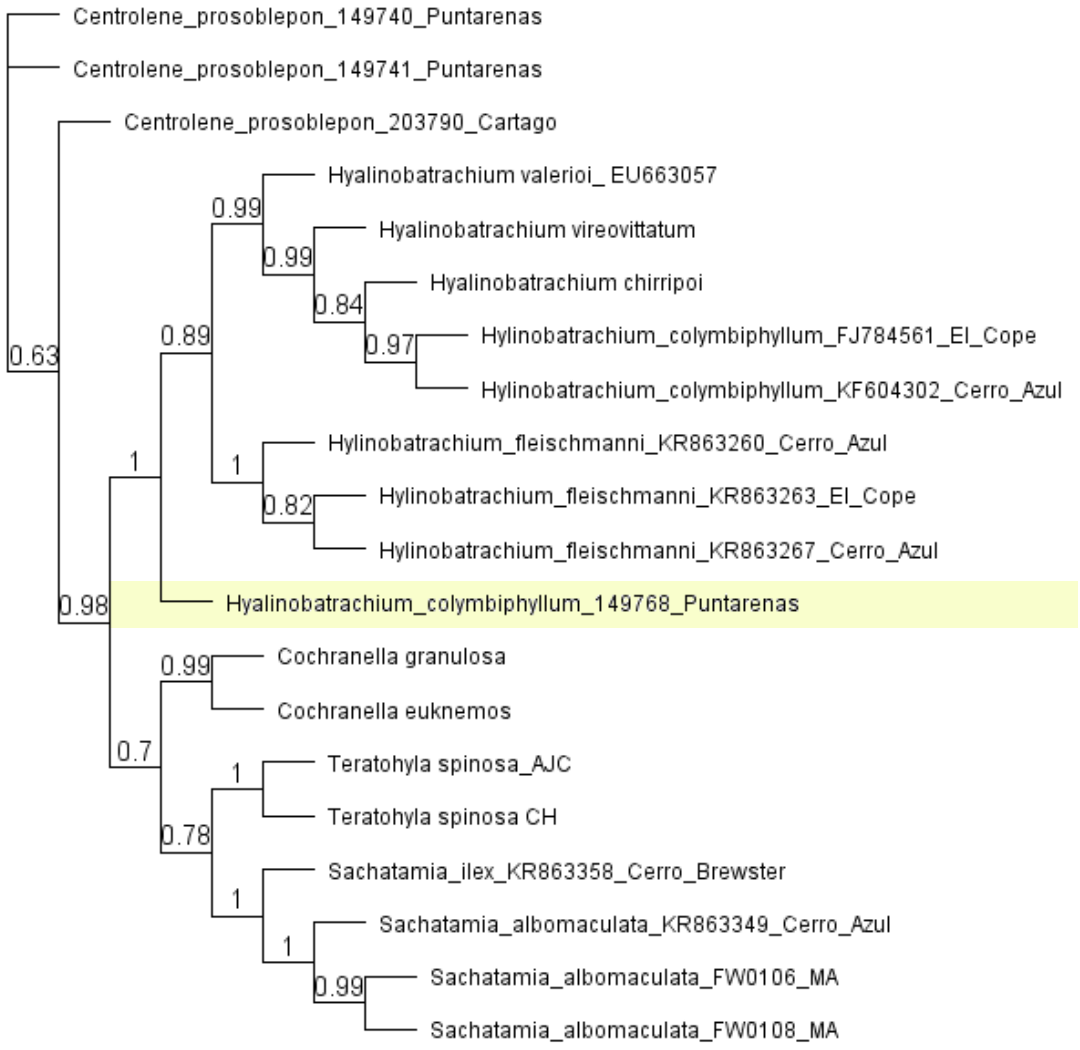


Figure 2.3. *Hyalinobatrachium colymbiphyllum* phylogenetic positioning on a tree including all other Costa Rican glass frog species. Yellow shading indicates potentially misidentified species.

Additionally, the two clades formed by one sequence from Brewster and another from El Cope showed low interspecific diversity at 16S (0.6%) and CO1 (5.3%). Intraspecific and NBGM distances could not be calculated as only one individual was present within each Panamanian clade.

Rhaebo haematiticus (Figure 2.1. F)

Two well supported (posterior probability of 1) clades were shown within *Rhaebo haematiticus* (Figure 2.1. F), including one Costa Rican clade represented by a single individual and one clade containing individuals from El Cope and Brewster in Panama. Interspecific distances were low at the 16S (1.2%) and CO1 regions (5.5%). Intraspecific distances were low for the Panamanian clade at 16S (0.5%) and CO1 (1.1%), and could not be calculated for the Costa Rican clade. The NBGM distances could not be calculated, as only one individual was present within the Costa Rican clade.

Atelopus species (Figure 2.1. G)

Three *Atelopus varius* and three *A. senex* individuals were grouped into one clade consisting of *A. varius, A. senex*, *A. glyphus* and *A. zeteki,* with another group of two well-supported (posterior probability >0.98) clades containing a single *A. senex* individual each (Figure 2.1. G). The grouping of multiple different species indicates some level of misidentification among these samples. CO1 sequences from *Atelopus* individuals were extremely short, thus analyses are for 16S only. The two *A. senex* clades containing a single individual each showed extremely low interspecific variation (0.7%), indicating them to be of the same species. Intraspecific and NBGM distances could not be calculated.

Dendropsophus ebraccatus (Figure 2.1.H)

Three well-defined clades with posterior probability values of 1 were observed within *Dendropsophus ebraccatus* (Figure 2.1. H). These were split

into an Ecuadorian, Colombian and Costa Rican clade. The Ecuadorian clade was comprised of only a single individual so some subsequent statistics could not be reported. Interspecific distances were high for 16S (8.7-15.8%) and CO1 (9.7-24.1%). The highest distances were between the Costa Rican and Ecuadorian clades for 16S (15.8%) and CO1 (24.1%) and the Colombian and Ecuadorian clades for 16S (13.9%) and CO1 (19.0%). Intraspecific distances were low for the Colombian and Costa Rican clades at 16S (0.0% and 0.2%, respectively) and CO1 (0.0% and 0.2%, respectively) clades. NBGM distances were high for 16S (8.7-15.7%) and CO1 (9.6-24.0%), following the same relationship above, with distances between the Ecuadorian and Colombian clades high for 16S (13.9%) and CO1 (19.0%). *P*(*Randomly Distinct)* values were <0.05 for both Colombian and Costa Rican clades, indicating candidate species to be present.

Smilisca baudinii (Figure 2.1. I)

Three well-defined clades were present (posterior probabilities >0.97) within *Smilisca baudinii*, with two dry forest and one wet forest clade observed (Figure 2.1. I). However, one of the dry forest clades was represented by a single individual sampled from Santa Rosa, which was more closely related to individuals sampled from the wet forest, than to individuals from the dry forest. Allopatric K2P-corrected interspecific distances between sister *S. baudinii* showed high divergence using 16S (2.7-4.5%) and CO1 (9.1-16.7%). The largest distances were between the dry forest and wet forest clades (16S: 4.5%, CO1: 16.7%) and between the clade formed by the individual from Santa Rosa and the dry forest clade (16S: 4.1%, CO1: 15.7%). Intraspecific distances could not be calculated for the clade

comprised of a single individual. However, for the larger dry forest clade these were 0.1% and 0.4% for 16S and CO1, respectively. For the wet forest clade, intraspecific distances were 0.1% and 0.2% for 16S and CO1, respectively. NBGM distances were high for 16S (2.6-4.4%) and CO1 (9.0-16.3%). Again, these distances were highest between the dry and wet forest clades (16S: 4.4%, CO1: 16.3%) and between the dry forest and Santa Rosa clades (16S: 4.1%, CO1: 15.5%). The species delimitation plugin indicated these cladistic structures to be unlikely due to stochastic gene coalescence, indicating *S. baudinii* to represent a complex containing candidate species.

Caecilians

Gymnopis multiplicata (Figure 2.4)

Comparison of the 16S region of all *G. muliplicata* amplified from this study (Figure 2.4), plus an additional three *G. multiplicata*, one *Dermophis parviceps* and seven *D. mexicanus* downloaded from GenBank indicated one sample (MVZ203936) to have been misidentified. The apparently novel *G. multiplicata* lineage was most similar to an individual labelled as the Mexican caecilian *D. mexicanus* (GenBank accession: EU753990) and both were closely related to an individual identified as *D. parviceps* (GenBank accession: EU753994). This focal "*D. mexicanus"* (EU753990) was collected from Costa Rica and shared low similarity to the most similar of the remaining *D. mexicanus* sequences. Interspecific distances indicate the lowest diversity was between the three true clades of *D. mexicanus* (0.0%) and between the *D. parviceps* and the unknown *Dermophis* clade formed by samples MVZ203936 and EU753990 (3.7%). Interspecific distances for all

other clades were high (8.7-11.7%). Where intraspecific distances could be calculated, they were low (0.0-0.2%) at 0% for all clades but the true *G. mulitplicata* one. NBGM distances were lowest between the two clades of *D. mexicanus* (0.0%) and between the *D. parviceps* and unknown *Dermophis* clade (3.7%), but otherwise high (8.7-11.6%).

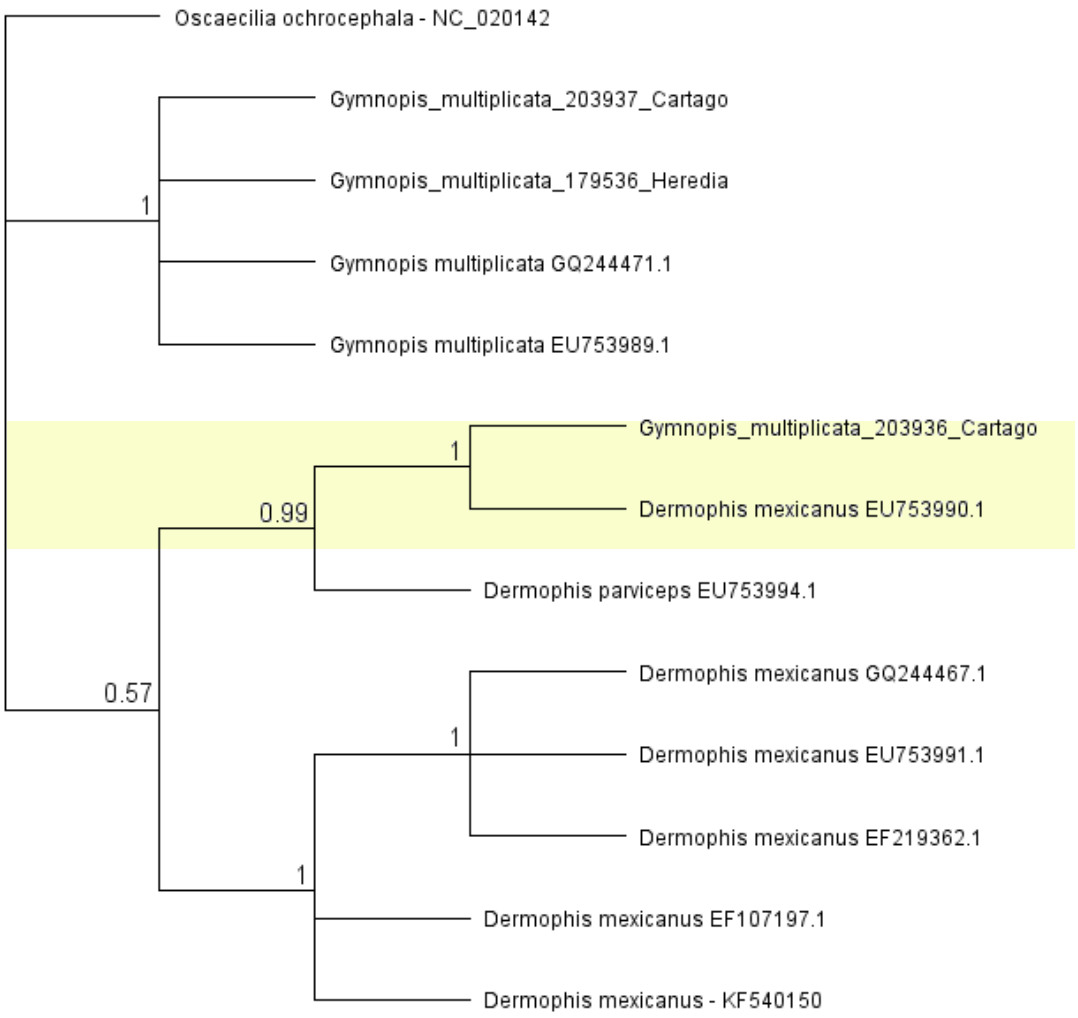


Figure 2.4. Relationships among Costa Rican *G. multiplicata* individuals investigated in this study from museum specimens and GenBank, constructed using 16S gene. The three individuals from this study are indicated by the presence of collection locations Cartago and Heredia. Yellow shading indicates potentially misidentified species.

Discussion

Here, I have detected six candidate species from within four widespread species. These candidate species are defined by combinations of high (<0.95) posterior probability, high interspecific distances (>2% for 16S and >8% for CO1) and low (<0.05) *P(Randomly Distinct)* values. Candidate species were found within the putative species complexes of: *Lithobates warszewitschii* (2 new candidates), *Smilisca baudinii*, *Dendropsophus ebraccatus* (2 new candidates) and *Rana pipiens*. Several species used in my analyses have recently been investigated or reclassified, so have not been discussed here. These include *Craugastor ranoides* (Puschendorf et al. unpublished), *Rhinella marina* (Frost 2018), *Pristimantis caryophyllaceus* (Batista et al. 2014), *P. ridens* (Wang et al. 2008) and *Diasporus diastema* (Hertz et al. 2012, Batista et al. 2016). The cladistic structures for these species found here support taxonomic patterns found in recent studies (Wang et al. 2008, Hertz et al. 2012, Batista et al. 2014, 2016). Other species such as *Leptodactylus fragilis* have not been discussed through their low level of diversity at the 16S gene. However, this species also displays relatively high diversity at the CO1 region, which may be a result of nuclear copies of mitochondrial DNA (numts) (Hazkani-Covo et al. 2010).

Genetic diversity indicative of cryptic speciation has previously been found from extirpated sympatric populations of *Lithobates warszewitschii* from El Cope in Panama (Crawford et al. 2010). Thus, candidate species within the proposed *Lithobates warszewitschii* complex have already been lost (Crawford et al. 2010). My findings support the hypothesis that *L. warszewitschii* is a complex of multiple species (Crawford et al. 2010). In this study, one distinct Costa Rican and two Panamanian, apparently allopatric, clades of *L. warszewitschii* were observed. The *L. warszewitschii* holotype was collected from Chiriqui, western Panama (Savage 1970), so it is likely the Brewster candidate, if a true species, would not retain this name. The separation of the Costa Rican and Panamanian candidate species may be expected through isolation by distance (Wright 1943). However, Brewster and El Cope are separated by a distance of only ~150km, which is about 3.5 times lower than the distance separating the ACG and El Cope. Yet, the Brewster clade appeared most distinct, with the Costa Rican and El Cope clades most similar. Therefore, a more isolating reproductive barrier may be present between Brewster and the other two populations than between El Cope and the ACG. The barriers currently limiting gene flow between Panamanian populations may include the presence of the Panama Canal and artificially constructed lakes, as *L. warszewitschii* is one of the least aquatic species of Central American ranids (Leenders 2016). However, such marked genetic diversity cannot be explained by 100 years of reduction in gene flow. Historic drivers of speciation such as the cyclical expansion of arid environments coupled with contraction of rainforest could have contributed to the diversities between populations (Smith et al. 2012). These climate-driven

habitat changes would have periodically increased areas of less suitable habitat for *L. warszewitschii* (Leenders 2016), hence increased population fragmentation and driven allopatric speciation (Smith et al. 2012). These habitat changes have likely driven some extent of the diversity found among most species studied here.

The *Rana pipiens* complex has undergone multiple changes in the number of species it contains within the last two centuries (Hillis 1988, Hillis & Wilcox 2005). *Lithobates forreri* and *L. taylori* are the only known Costa Rican forms subsumed under the name *R. pipiens*. Yet, my analysis indicates the complex to be comprised of additional undescribed *Lithobates* species, which separated prior to both the formerly mentioned species. Both '*Rana pipiens'* individuals originate from the same area, Cartago, and no individuals from this area are represented in the clades containing *L. taylori* and *L. forreri*. The *R. pipiens* complex in Costa Rica is split by the country's Cordilleras, with *L. forreri* occurring on the Pacific versants and *L. taylori* found on the Caribbean versants. Thus, the candidate species found here likely coexist with *L. taylori*, or are mistakenly identified as *L. taylori*. Both '*R. pipiens*' individuals assessed in this study were collected near to Refugio Nacional Tapanti, Costa Rica, which experienced amphibian population declines from the outbreak of the amphibian chytrid fungus (Bolaños 2002, Puschendorf et al. 2006). Therefore, if this candidate species represents a true species, it should be further assessed to determine its range size and whether it still persists around Refugio Nacional Tapanti.

Dendropsophus ebraccatus has an extensive range on the Atlantic slope, spanning from isolated populations in southern Mexico, Guatemala, Belize,

Honduras and northern Nicaragua to continuous populations from southern Nicaragua to northern Ecuador (Leenders 2016). On the Pacific versants, this species occurs in Costa Rica and Colombia (Leenders 2016). *D. ebraccatus* has previously been found to consist of five deeply-diverged lineages from Costa Rica and Panama (Robertson et al. 2009), with high variability in the phenotype (Ohmer et al. 2009). Therefore, it is unsurprising that the individuals sampled in this study from Costa Rica, Colombia and Ecuador therefore have been indicated as candidate species. These candidate species likely represent three deeply diverged lineages, given the diversity found within populations separated by smaller distances (Robertson et al. 2009). The diversities among Costa Rican and Colombian and Costa Rican and Ecuadorian *D. ebraccatus* are likely explained in some part by the large distances of over 1500km between these sampled populations (Wright 1943). The specimen used for diagnosing *D. ebraccatus* into the genus *Dendropsophus* was collected in Belize (Faivovich et al. 2009). Therefore, the two new species inferred from my analyses are most likely represented by those individual forming the Colombian and Ecuadorian clades.

The range of *Smilisca baudinii* is unusually large for an American tree frog (McCranie 2017), spanning from Texas to southern Costa Rica, with an altitudinal range from the lowlands to 2000 meters (Duellman 2001). Adult *S. baudinii* differ in size, proportion, colouration and markings between Caribbean and Pacific slopes, and across their large range (Duellman 2001). Similarly, in tadpoles tail width and length appear to vary among populations (Duellman 2001). The taxonomic status of *S. baudinii* has recently been questioned, with morphological evidence indicating differences between

Caribbean lowland and Pacific versant populations from Mexico to Costa Rica (McCranie 2017). *Hyla manisorum* is currently considered a synonym of *Smilisca baudinii*, but has been proposed to represent a distinct species, *Smilisca manisorum* (McCranie 2017). Populations of the Caribbean lowlands have been indicated to be of the species *S. manisorum* (McCranie 2017). Additionally, McCranie (2017) has suggested that populations of individuals sampled from Honduras and Guatemala possibly represent an additional species that can be morphologically distinguished from both the Pacific versant and Caribbean lowland populations. The results of my molecular investigation support the findings of McCranie (2017), and indicate *Smilisca baudinii* to be a complex of species. Given the geographical locations of the clades I have found, one of the syntopic clades from the Pacific versant is likely a member of the species *Smilisca baudinii*. Individuals collected from the Caribbean versant rainforest likely represent an additional species. However, I am unable to distinguish whether this population is part of the proposed species *Smilisca manisorum* or represents another member of the *Smilisca baudinii* complex. The presence of three clades further supports evidence that a third potential species is within the *S. baudinii* complex (McCranie 2017). However the third clade found here may represent a different species to that inferred by McCranie (2017). Further sampling of the proposed *Smilisca baudinii* complex should be undertaken across both latitudinal and longitudinal gradients. These samples should then be compared morphologically to those used in McCranie (2017), and genetic analyses of these individuals should be undertaken to determine the number of cryptic species within the *Smilisca baudinii* complex. The holotype of *S.*

baudinii was collected from a non-specific location in Mexico (Smith & Taylor 1950), thus the species found here should be compared to this holotype to determine whether the presumed *S. baudinii* clade from the dry forest should retain this name.

The consistent groupings of morphologically similar species used in this analysis highlights disadvantages associated with only using morphology in species identification. A combined approach inclusive of molecular methods for host identification reduces issues associated with using morphology alone (Bickford et al. 2007). In this study, species such as *Atelopus varius* and *A. senex* (now thought to be extinct) were grouped into the same clade by my analyses, indicating misidentification of these individuals. Additional problems were found in the identification of the caecilian *Gymnopis multiplicata*. Seven caecilian species are known from Costa Rica (Leenders 2016). Although all occur within the range of *G. multiplicata*, all members of the genus *Dermophis* differ in having eyes not covered by bone and in the positioning of the tentacle, theoretically making *G. multiplicata* easily recognisable (Leenders 2016). Yet this apparently novel clade of *G. multiplicata* clusters with two *Dermophis* individuals, indicative of its status within the genus *Dermophis*. Using its collection location and elevation, one possible identification could be *D. glandulosus* (Savage & Wake 2001, Leenders 2016). However, firm identification of this individual under a known or otherwise species is not possible using the data currently available on GenBank. The individual most closely related to the focal '*G. multiplicata*' individual was identified as *D. mexicanus*, collected from Costa Rica. However the taxonomy of Costa Rican caecilians was revised post-collection

of this individual, to exclude *D. mexicanus* from Costa Rica (Savage & Wake 2001). Thus, this individual is likely another member of the genus *Dermophis*, but with no accompanying collection specifics, it cannot be identified any further. These findings highlight both identification problems with caecilian amphibians and the limitations with using sequences from GenBank, which has no capacity for image upload. Given that each of these sequences on GenBank has been used for some taxonomical analyses, this confusion could have implications for current taxonomic findings within Mesoamerican caecilians. This should be resolved through re-examination of the preserved samples and further *in situ* sampling.

One individual identified as the glass frog *Hyalinobatrachium colymbiphyllum* formed its own clade outside of the *Hyalinobatrachium* group containing other *H. colymbiphyllum* and *H. fleischmanni*. However, distances at 16S were extremely low between the focal *H. colymbiphyllum* and the *H. fleischmanni* clade, indicating the individual to have been misidentified. However, CO1 distances were not calculated, so further investigation of this individual may be merited.

Although molecular datasets are generally reliable in diagnosing candidate species (Vieites et al. 2009) this study is limited by the use of mtDNA only. Therefore, the inferred candidate species, at minimum, indicate limited panmixis among females only. Further studies should include nuclear markers to support these findings. Additional morphological and bioacoustics data are required to confirm these clades as new species (Vences & Wake 2007, Padial et al. 2010). Although the methods used here are useful for indicating diversity, another limitation has been the reliance upon sequences

downloaded from GenBank – which does not include image uploads. Therefore an assumption of this study is that those depositing sequences to GenBank have correctly identified the organism to species level, which is likely not always the case (Valkiūnas et al. 2008). This inconsistency of correct identification is even exemplified during this study, especially for the genus *Atelopus* and Gymnophinan amphibians. Another issue was the availability of complete taxonomic groups. For example, five *Dermophis* species exist in Costa Rica, yet I was unable to obtain 16S sequences for four of these.

My findings of multiple cases of cryptic evolutionarily significant units across the geographic ranges of several Costa Rican species indicate amphibian diversity to remain underestimated across Central America. All four proposed complexes are widespread, thus have not been previously recognised as conservation priorities. The potentially smaller populations of candidate species within these complexes, however, should be investigated further for description and conservation assessment. These preliminary analyses are not exhaustive, but provide an indication of where further studies should be undertaken. Taxonomy of populations currently under the focal species discussed here may be in urgent need of addressing. It is apparent that defining units of biodiversity using morphology alone can be misleading. At the same time, researchers should be cautious in using sequences collected from individuals with no accompanying images or morphometrics. An understanding of the partitioning of diversity is essential for its effective conservation, as unidentified units of diversity cannot be purposely protected

(Mace 2004). This study along with numerous previous works has shown that Costa Rican amphibian biodiversity remains underestimated.

Chapter 3 Uneven distributions of two amphibian pathogens among habitats and hosts in northern Costa Rica

Abstract

Two important pathogens for amphibian conservation are chytrid and ranavirus, as both are able to drive population declines and have been linked to species extinctions. Additionally, ranavirus can cause mortality in reptiles. In Costa Rica the emergence of chytrid resulted in numerous extirpations and extinctions among high elevation native amphibian fauna. Due to their enigmatic nature and large-scale, many declines were never fully investigated. Although chytrid is the assumed driver of enigmatic declines, it has often not been confirmed from affected populations. Similarly, little research has been conducted to investigate the distribution patterns of ranaviruses amongst tropical habitats and hosts, with most research focusing on ranavirus in temporal regions. Here, I collected amphibian and reptile samples from seven wet and dry forest sites of the Área de Conservación Guanacaste, northern Costa Rica, and tested these for both pathogens. I found infections to be uneven among taxa for both pathogens. Ninety-seven per cent of chytrid infections were documented from wet forest habitats as opposed to dry forests. A positive association was found between temperature and ranavirus prevalence, which was higher among dry forests than wet forests. Regardless of habitat type amphibian populations of Costa Rica are likely exposed to either chytrid or ranavirus, or both pathogens simultaneously. Costa Rica is set to experience rapid warming and an

increased frequency of short-term warming events, which have already been linked with chytrid outbreaks. Such events could result in further outbreaks of chytrid and previously unsuspected ranavirus outbreaks.

Introduction

Chytrid and ranavirus are important amphibian pathogens due to their capacity to drive population declines and extinctions (Berger et al. 1998, Teacher et al. 2010, Price et al. 2014, Earl & Gray 2014, Rosa et al. 2017). In comparison to ranavirus, the important drivers of chytrid outbreaks are generally well understood. Optimal growth for chytrid in culture is between 17°C and 25°C, and death occurs above 30°C (Piotrowski et al. 2004), although local variations in thermal optima have been documented from Australia, indicating local adaption (Stevenson et al. 2013). This apparent thermal preference is reflected in nature as wild Australian frogs were less likely to be infected when a higher proportion of their bodies were above the upper optimal thermal range of chytrid (Rowley & Alford 2013). Additionally, Australian species, which experienced catastrophic population declines in wet habitats, persist in xeric environments (Puschendorf et al. 2011). Despite exhibiting high chytrid prevalences (Puschendorf et al. 2011), these persisting populations appear to have avoided collapse due to the availability of warm (>30°C) microclimates (Puschendorf et al. 2011, Daskin et al. 2011). In Costa Rica, chytrid prevalence is significantly lower in drier, warmer areas (Puschendorf et al. 2009, Zumbado-Ulate et al. 2014) and elsewhere it has also been shown to increase with elevation and during cooler months (McDonald et al. 2005, Woodhams & Alford 2005, Sapsford et al. 2013).

Whilst amphibian populations occurring at relatively high elevations (Young et al. 2001, Stuart et al. 2004), in wetter, cooler environments were generally more susceptible to enigmatic declines (Bolaños 2002). However, warming climates have been linked to chytrid epizootics at higher elevations due to changes brought about in cloud cover, which constrict ambient temperature ranges towards the optimum for chytrid growth and reduce the availability of warm microclimates (Pounds et al. 2006).

The published data exploring associations between ranaviruses and environmental conditions are almost exclusively of laboratory origin (e.g. Chinchar 2002, Rojas et al. 2005, Ariel et al. 2009, Echaubard et al. 2014). Ranavirus replication is generally greatest at relatively high temperatures (Chinchar 2002, Ariel et al. 2009, Echaubard et al. 2014), but replication optima appear strain specific and can be highly variable (Echaubard et al. 2014). For United Kingdom isolated FV3 ranaviruses, replication occurs fastest at 30°C (Cunningham 2001, Chinchar 2002, Echaubard et al. 2014). For the closely related ATV, of United States origin, the replication rate is highest at 26°C (Rojas et al. 2005). Yet two FV3-like ranaviruses from the United States, ‘azacR’ and spotted salamander Maine virus (SSME), replicate best at 22°C and 14°C, respectively (Echaubard et al. 2014). Field studies have indicated a possible thermal dependence of ranavirus, as amphibian die-offs predominantly occur during warmer months (Chinchar 2002, Green et al. 2002), but data demonstrating this association are generally lacking. Additionally, these associations could reflect the timing of temperature associated events, such as high-density breeding events and metamorphosis.

Differences in the environmental conditions that affect chytrid and ranavirus likely contribute to explaining some variation in species and population susceptibilities to infection (Puschendorf et al. 2005, 2009, Bancroft et al. 2011, Olson et al. 2013). But even when exposed, certain taxa appear more susceptible than others (Woodhams et al. 2007, Hoverman et al. 2011, Hoverman et al. 2012a, Olson et al. 2013). This may be due to immunity factors such as the composition of the skin microbiome and peptides or life history (Woodhams et al. 2007, Harris et al. 2009, Hoverman et al. 2011, Hoverman et al. 2012a, Voyles et al. 2018).

In Costa Rica chytrid has been the assumed driver of enigmatic amphibian declines (Skerratt et al. 2007), which were non-random in host and geographic distribution (Stuart et al. 2004). Systematic monitoring of amphibian populations has been rare (exceptions include Monteverde and Las Tablas, e.g. Lips 1998, Pounds et al. 1999), thus in most areas declines were anecdotally severe, but remain unexamined. Costa Rican individuals from the taxonomic families Bufonidae, Ranidae, Hylidae and Leptodactylidae were especially prone to decline (Bolaños 2002), but individuals of these families have not explicitly been shown to be more likely to be infected with chytrid in Costa Rica. Ranavirus has also recently been confirmed from nine of 21 tested Costa Rican amphibian species (Whitfield et al. 2012, 2013), but other host taxa have yet to be tested. In one study, a significant association between ranavirus and chytrid infection was identified in one species (Whitfield et al. 2013), but in general very little is known or can be predicted about the host and geographic ranges of this pathogen in under-studied systems such as Costa Rica. Although the effects of ranavirus

upon Costa Rican amphibians is unexplored, this pathogen has previously been linked to a die-off event of amphibians in nearby Nicaragua (Stark et al. 2014), highlighting its potential to cause disease in, and potentially threaten, Costa Rican populations. However, without further information on this pathogen's range it is difficult to predict when and where ranavirus outbreaks could occur in these systems.

In the Área de Conservación Guanacaste (ACG), north-west Costa Rica, anecdotal amphibian declines and extirpations have occurred, often at higher, cooler elevations. Despite the assumption that these declines were due to disease, alongside the detection of chytrid (Zumbado-Ulate et al. 2014) and historic identification of a potential ranavirus (Speare et al. 1991), chytrid has not been surveyed for at higher elevation forests and ranavirus has never been surveyed at any location here. The ACG is comprised of a range of highly variable habitats, spanning from Pacific mangroves, across dry, rain and cloud forests, over two volcanic systems and onto the Caribbean slopes of northern Costa Rica. Therefore, this area was identified as an ideal area for investigating the host, geographic and environmental ranges of chytrid and ranavirus. The determination of infection patterns can thus ultimately be used to guide monitoring of infected populations, to advance the research of these pathogens in under-studied systems. Therefore, the aims of this study were to determine whether environmental and taxonomic factors correlate with the occurrence of either pathogen, which can be used to infer the risk of infection of similar taxa under similar environmental conditions. I predict that ranavirus and chytrid are present in the ACG and that their distributions can be determined by environmental

factors. I also predict the host range of Costa Rican ranaviruses to include amphibian and reptile taxa.

Methods

Field sampling

I sampled 844 amphibians from three habitat types (‘cloud forest’, ‘rainforest’ and ‘dry forest’) in the Área de Conservación Guanacaste (ACG), north-west Costa Rica (Figure 3.1). In total three dry forest (n=301), one cloud forest (n=41) and three rainforest (n=502) sites were sampled from. All sampling occurred between June 2014 and August 2017. All three dry forest sites and one rainforest site (Caribe) contain relatively young forest in recovery, as these have been naturally transformed from pasture within the last three decades. The remaining sites contain older forests and some pastures.

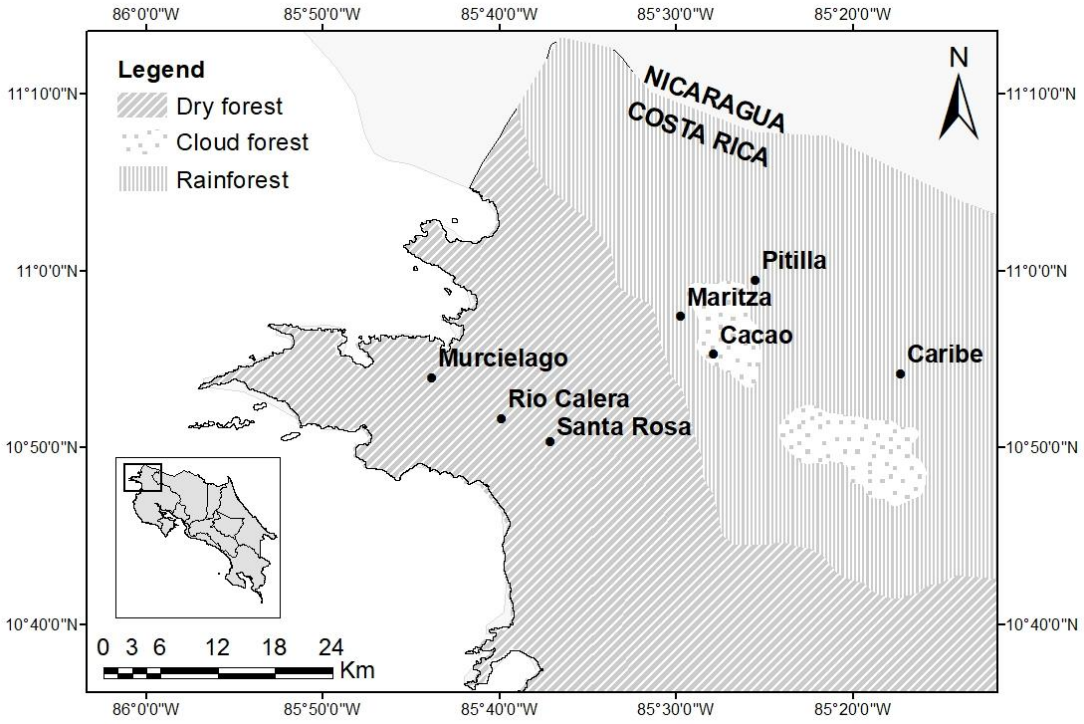


Figure 3.1. Sites used for amphibian and reptile sample collection, within the Área de Conservación Guanacaste, and grouped habitat types in north-west Costa Rica. Map layer downloaded from www.acguanacaste.ac.cr and altered using ArcMap 10.5.1.

Live, post-metamorphic amphibians were opportunistically collected from a variety of forested and pasture habitats, during both diurnal and nocturnal time periods to maximise the diversity of samples. Each animal was visually checked for physical abnormalities and signs of disease (e.g. Bertelsen & Crawshaw, 2003; OIE, 2016), including righting reflex, skin condition and body condition, by a handler wearing vinyl gloves (Cashins et al. 2008). Gloves were changed between individuals. Anurans were sampled for chytrid following a standard protocol, by firmly swabbing the ventral parts of the body using fine tipped sterile tubed dry swabs, using five strokes over the venter, drink patch, each thigh, and the rear feet, or between each toe if the

animal was large enough to do so (Brem et al. 2007, North & Alford 2008). Toe-clips were taken for ranavirus screening by cutting the first interphalangeal joint of the second digit of the right hind foot with surgical scissors and samples were stored in 95% ethanol solution (St-Amour & Lesbarrères 2007). Scissors were flame disinfected between individuals. Toe-clips also served to identify individuals that had been sampled, and therefore prevented repeated sampling of the same individual.

Amphibian and reptile cadavers were also opportunistically collected when encountered (n=86). Most of these were roadkill, collected within the ACG (usually from Santa Rosa). Cadavers were processed a maximum of 24 hours after collection, except in a few cases, where carcasses had been collected by other researchers and stored in a freezer. Cadavers were examined for any visible signs of disease before being dissected, however this examination was limited when carcasses were very damaged. During dissection, where possible, cadavers were inspected for internal signs of disease and parasites (e.g. signs listed in Densmore & Green, 2007). A sample of liver was removed, or unidentified tissue was collected if the liver was not visibly demarcated and could not be recognised (in very damaged cadavers). All tissue samples were stored in 95% ethanol and transported to the University of Plymouth in the UK for further processing.

Laboratory analyses

DNA was extracted from tissues for ranavirus detection using a standard ammonium acetate protocol (Nicholls et al. 2000). Molecular grade water was used as a negative extraction control and FV3 infected tissues were

used as extraction positive controls, all samples and controls were run in duplicate. A nested-PCR, using primers from Mao et al. (1997) and Meng et al. (2013), was used to amplify ranavirus DNA. Eight-microlitre reactions consisted of 0.05µM of primers for initial reactions (Mao primers) and 0.4µM of primers for nested reactions (Meng primers). Reaction mixes also included 4µl DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Massachusetts, USA), 1.2µl water and 2µl of template. Thermocycler settings for both the initial and the nested PCR steps were as follows: an initial 23-cycle stage consisting of a 95°C strand disassociation step, 62°C touchdown (decreasing by 0.5°C per cycle) annealing step, and a 72°C elongation step, then a 25-cycle stage of a 95°C strand disassociation step, 50°C annealing step and a 72°C elongation step. Each step was 30 seconds in duration. PCR products were visualised on 2% agarose gels. Samples were considered positive when amplification occurred in both replicates or if sequences were obtained from samples where amplification occurred in only one replicate. Amplicons of any samples with a band of the correct size (320 base pairs [bp]) were sent to Macrogen for Sanger sequencing of the forward strand to confirm specific amplification and to obtain genotype information. Trace files were uploaded to MEGA7 (Kumar et al. 2016) and sequences were checked against chromatograms and trimmed at the 3' and 5' ends when low quality base calls were present. Sequences were identified as being from ranavirus using the standard nucleotide Basic Local Alignment Search Tool (BLAST +2.8.0; Altschul et al. 1990; https://www.ncbi.nlm.nih.gov/BLAST) with default settings.

For chytrid detection, DNA extractions and quantitative real time TaqMan PCR assays were performed using methods adapted from Boyle et al. (2004), where swabs were used instead of tissues. Four standards of 100, 10, 1 and 0.1 chytrid zoospore genomic equivalents and water, as negative controls, were used in duplicate for each qPCR run. Each sample was run in duplicate and single replicate positives were re-run. If, after 3 repeated runs of duplicates, single positives were still obtained the sample was considered negative (as in, e.g. Wombwell et al. 2016). Genomic equivalent values generated by the assay were used as a proxy for chytrid infection load. Samples were considered positive when sigmoidal amplification occurred for both duplicates and mean genomic equivalent values were ≥0.1 (Bai et al. 2010, Bletz et al. 2015).

Statistical Analyses

For all analyses, only data from amphibians were used as reptile sample sizes were low. Binary logistic regressions were performed in 'R', using package 'lmer4', as Generalized Linear Models with binomial errors and a logit link (R Core Team 2016). One model was run per pathogen, and each included the dependent variable 'infection status' (for ranavirus or chytrid) and covariates 'temperature' and 'taxonomic family'. Weather data is routinely recorded by ACG staff (maximum and minimum daily temperature and total rainfall) but there are missing data points in these measurements due to sampling irregularities. I also recorded temperature at the time of sampling for a subset of samples. Temperature was used as a proxy for weather in models, as these data were available from all sites, as opposed to rainfall data, which were completely unavailable for some sites. The variable

T_{mean} was calculated by taking the mean of the daily minimum and maximum temperatures recorded by ACG staff. To minimise the amount of missing data, T_{mean} was used as a predictor of the temperature measurements at time of sampling in a linear model, and any missing values of T_{mean} were replaced with predicted values from the linear model when sample temperature measurements were available.

Fisher's exact tests were performed in 'R', using package 'stats', to investigate differences in infection by each pathogen between the sexes. These tests were limited to taxonomic families that had sufficiently high sample sizes of individuals for which sex could be determined.

Chytrid loads showed a right-skewed distribution, so were logarithmically transformed for analyses. Linear models with transformed chytrid loads were performed in 'R', using package 'stats', to investigate the effect of temperature on chytrid load. For these analyses, only individuals identified as infected were included. Additional linear model analyses were conducted at the family level for those widely represented across the focal temperature gradient. This allowed further investigation of the effect of temperature on chytrid load.

Results

Environmental and taxonomic distribution of pathogens

I found eighty-two individuals of 21 species and 10 families to be positive for ranavirus (Table S 3.1), resulting in an overall prevalence of 8.9±1.9% (95% confidence intervals [CI]). Of 16 identified reptile species, two, one snake

(*Porthidium ophryomegas*) and one turtle species (*Rhinoclemmys pulcherrima*), tested positive for ranavirus. This pathogen was detected from all seven sampled sites (Figure 3.2). I identified the sequence of the MCP gene of this virus to be FV3-like.

During this study unexplained amphibian die-offs in the dry forest were reported by ACG staff. Dead frogs were encountered by ACG staff on 10th May 2017 at Sendero Los Patos in Santa Rosa (Figure S 3.1), but not collected, examined or tested for any abnormality. The species involved were *Rhinophrynus dorsalis* and *Incilius luetkenii*, which I found to be infected with ranavirus in the dry forest sites at a prevalence of 50% (1.3-98.7% CI) and 16.9% (10.8-24.7% CI), respectively.

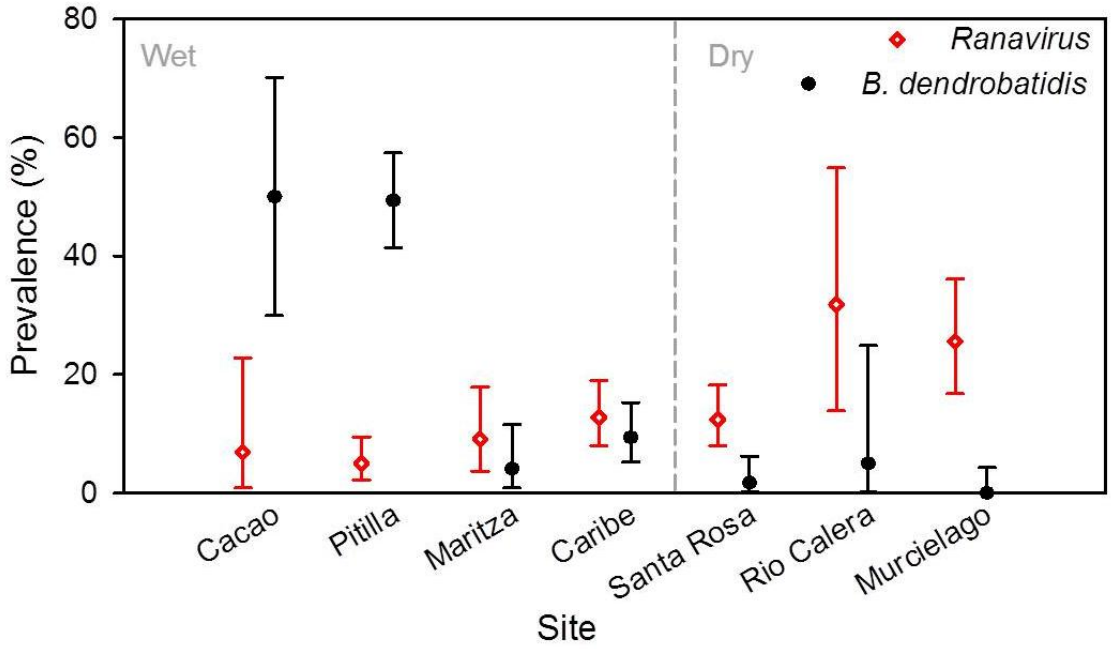


Figure 3.2. Mean ranavirus and chytrid prevalences (with 95% confidence intervals) among all sampled sites in the Área de Conservación Guanacaste, Costa Rica. Dashed line represents separation of wet and dry forest types.

Chytrid was found at an overall prevalence of 16.4±2.8% from 13 amphibian species of 5 families. Chytrid was detected from all four wet forest sites, but only three out of 342 individuals were infected from two of the dry forest sites (Figure 3.2).

No samples were collected from the Rio Calera between June 2015 and November 2016. As a likely result of the 2015-2016 El Niño, the river here dried up, with only a small single pool remaining on the riverbed. Subsequent visits were undertaken twice a month between June and August 2015 and in July 2016, but no amphibians were observed. In November 2016, two tungara frogs (*Engystomops pustulosus*) were sampled (no pathogens detected) from a puddle near to the Rio Calera, but no amphibians were found whilst walking the riverbed. No amphibians were found here during a final visit in August 2017.

Statistical findings

The effects of temperature and taxonomic family upon infection likelihood were examined using logistic regression. Temperature (Figure 3.3 A) showed significant positive effects upon ranavirus infection likelihood (Table 3.1). For every additional 1°C increase in temperature ranavirus was 1.4 times ($P < 0.001$) more likely to be detected from individuals. Individuals of the families Centrolenidae ($P < 0.025$), Hylidae ($P < 0.037$), Leptodactylidae ($P < 0.015$), Microhylidae ($P < 0.001$) and Ranidae ($P < 0.005$) were 7.2, 2.5, 3.6, 9.6 and 4.1 times more likely to contain infected individuals than individuals of the family Bufonidae, respectively (Table 3.1, Figure 3.4).

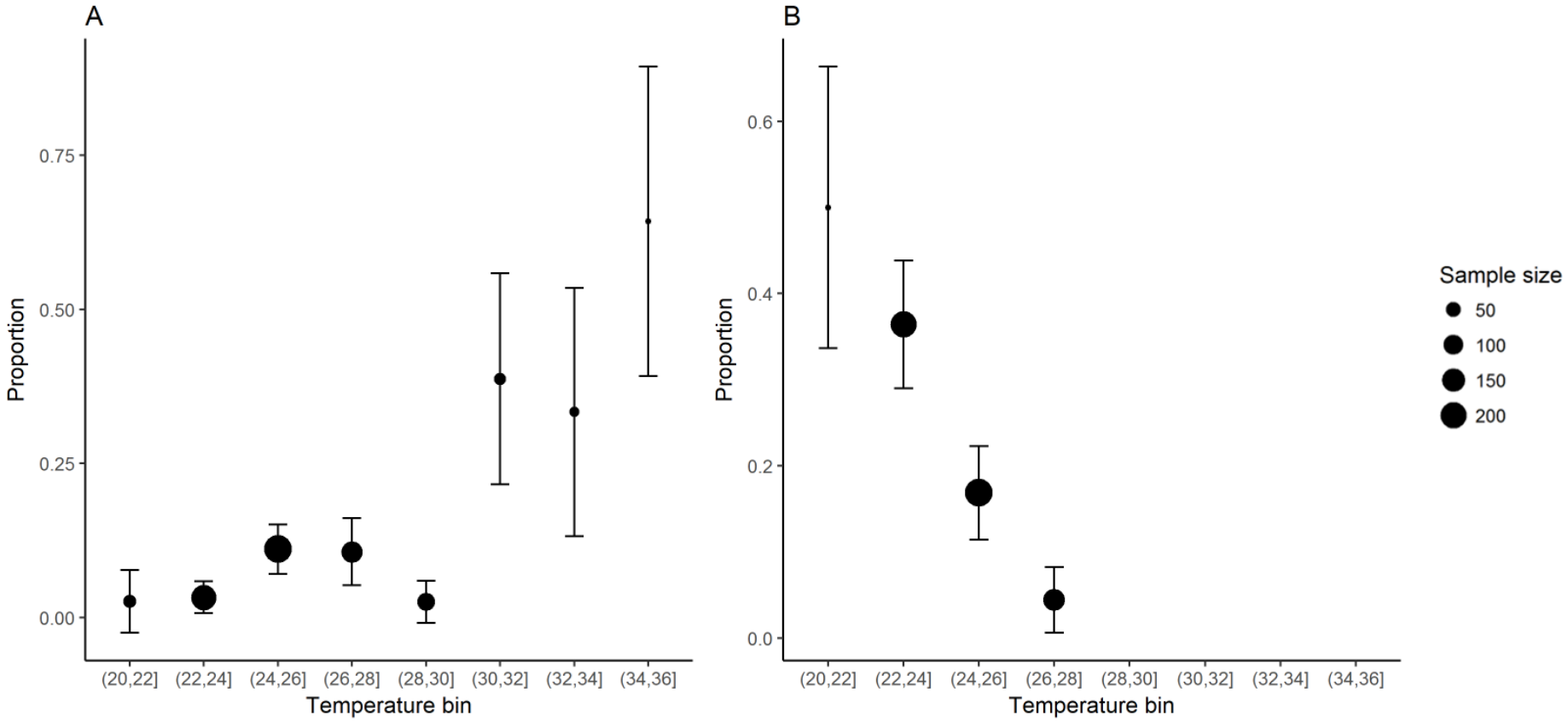


Figure 3.3. Prevalence of A) ranavirus and B) chytrid by 2°C temperature bins from individuals sampled from the Área de Conservación Guanacaste, Costa Rica. Exclusive '(' and inclusive ']' notations are used to indicate bin extents.

Table 3.1. Factors with a significant effect upon ranavirus infection likelihood in the Área de Conservación Guanacaste, Costa Rica.

Variable	Coefficient (β)	SE	Wald χ^2	P value
Temperature	0.332	0.055	6.021	0.001
Centrolenidae	1.975	0.877	2.251	0.024
Hylidae	0.908	0.434	2.091	0.037
Leptodactylidae	1.273	0.520	2.446	0.014
Microhylidae	2.259	0.500	4.516	0.001
Ranidae	1.400	0.492	2.848	0.004

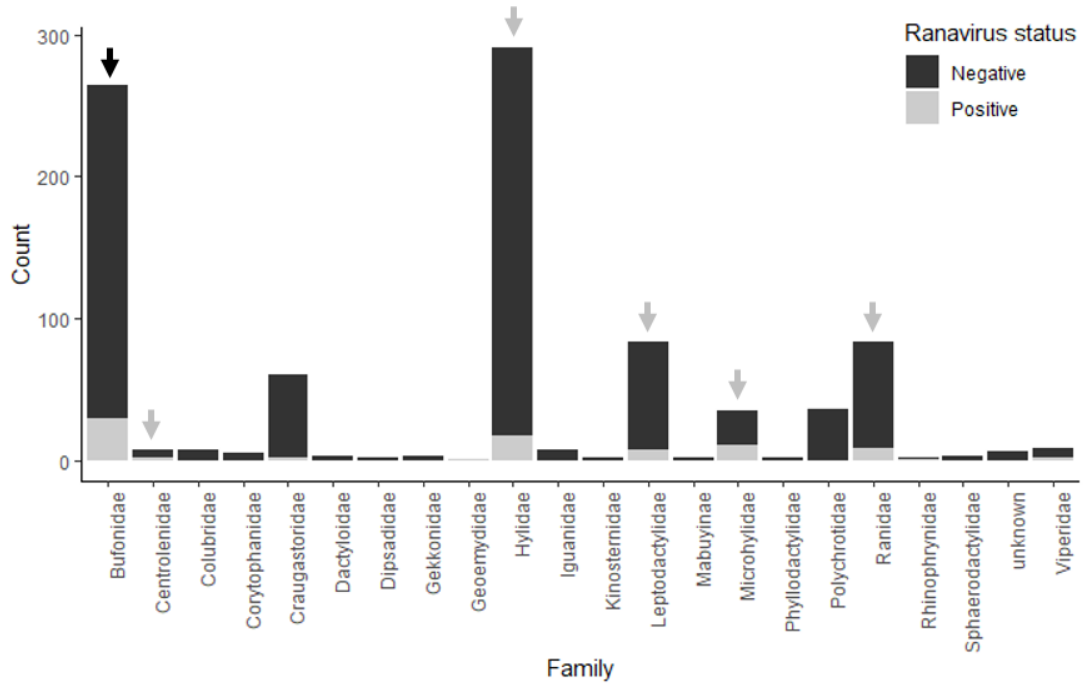


Figure 3.4. Ranavirus infection status by taxonomic family from individuals sampled from the Área de Conservación Guanacaste, Costa Rica. Grey arrows indicate taxa more likely to contain infected individuals than the family Bufonidae, indicated by a black arrow.

For chytrid, temperature showed significant negative effects upon infection likelihood (Figure 3.3 B**;** Table 3.12). For every 1°C decrease in temperature chytrid was 1.4 times ($P < 0.001$) more likely to be detected from individuals. Individuals of the taxonomic families Centrolenidae ($P < 0.040$), Hylidae ($P < 0.001$), Leptodactylidae ($P < 0.034$) and Ranidae ($P < 0.001$) were 12.5,

27.0, 5.0 and 12.2 times, respectively, more likely to be infected with chytrid than individuals from the family Bufonidae (Table 3.2 and Figure 3.5).

Table 3.2. Factors with a significant effect upon chytrid infection likelihood in the Área de Conservación Guanacaste, Costa Rica.

Variable	Coefficient (β)	SE	Wald χ^2	p value
Temperature	-0.314	0.067	-4.674	0.001
Centrolenidae	2.529	1.234	2/050	0.040
Hylidae	3.297	0.602	5.474	0.001
Leptodactylidae	1.612	0.757	2.128	0.033
Ranidae	2.504	0.682	3.670	0.001

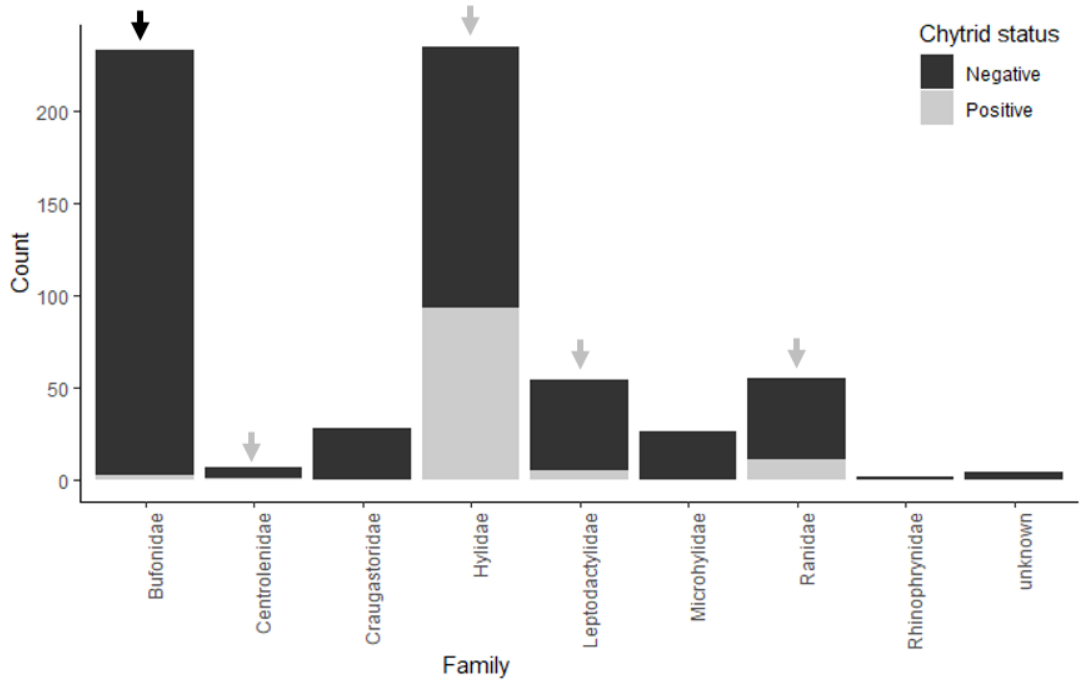


Figure 3.5. Chytrid infection status by taxonomic family from individuals sampled from the Área de Conservación Guanacaste, Costa Rica. Grey arrows indicate taxa more likely to contain infected individuals than the family Bufonidae, indicated by a black arrow.

Differences in infection prevalence between sexes of the family Hylidae, which had the highest sample sizes per pathogen and high prevalence, were examined using Fisher's exact tests. For both ranavirus (est. odds ratio < $1x10^{-30}$, $P < 0.049$) and chytrid (est. odds ratio = 0.2, $P < 0.001$) infections, these differences were significant, with infection prevalence greater in males than in females (Figure 3.6). Additionally, male hylids accounted for seven of the nine coinfections detected.

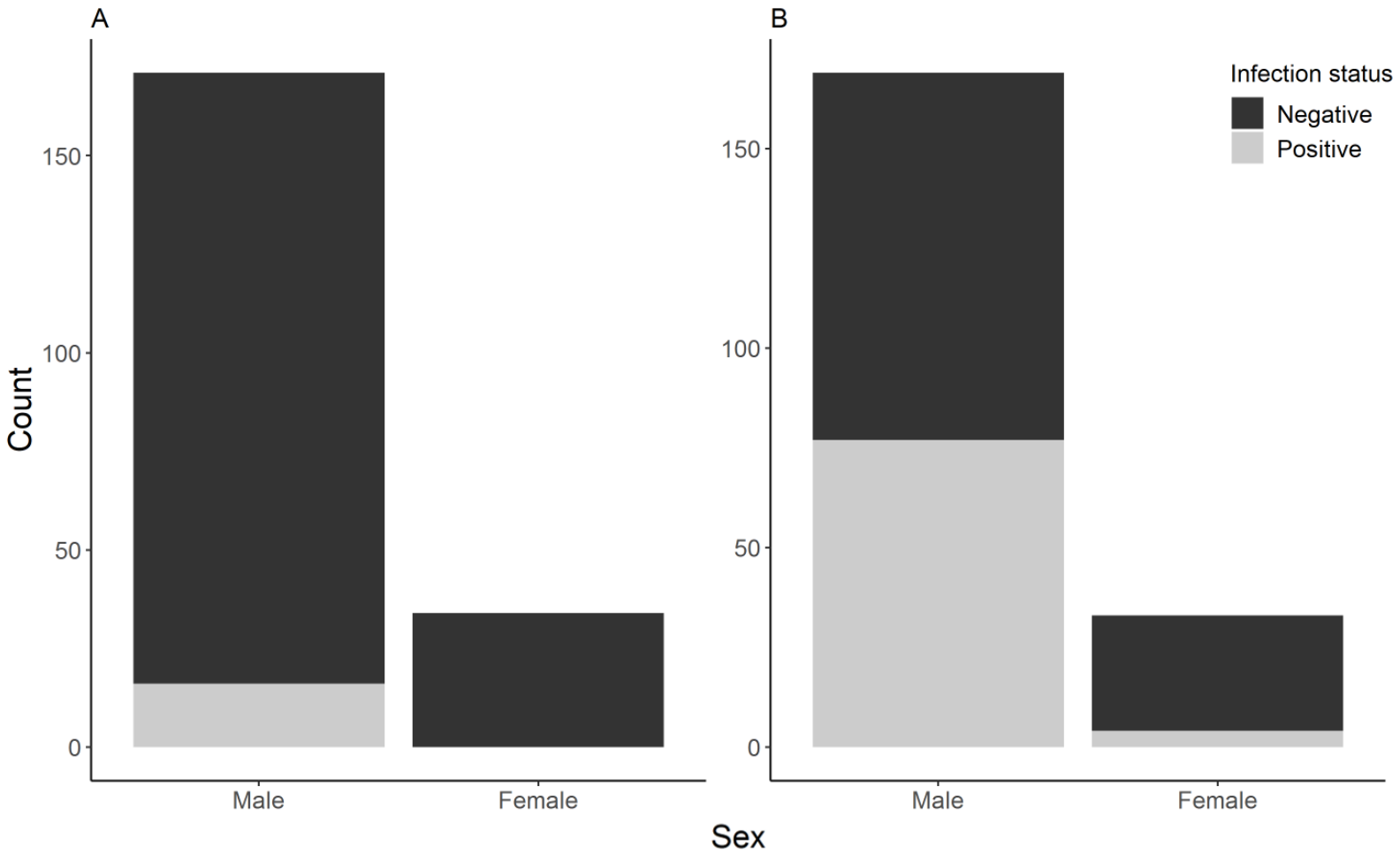


Figure 3.6. Infection status by sex in Hylidae for A) ranavirus and B) chytrid from individuals sampled from the Área de Conservación Guanacaste, Costa Rica.

Models containing only infected individuals were used to assess taxonomic, site and temperature effects on chytrid load. Chytrid loads were higher among individuals of the family Hylidae ($F(4, 109)= 3.929$, $P < 0.004$) and at Pitilla ($F(5,108)=5.688$, $P < 0.008$) (Figure 3.7 and Figure 3.8). The trend between temperature and chytrid load was explored using two taxa that were sampled across a temperature gradient from 20 to 28°C. The linear model indicated an effect of temperature on chytrid load for individuals of the family Hylidae ($F(1, 91)=17.220$, $P < 0.001$), but no significant trend was found between temperature and chytrid load of Ranids.

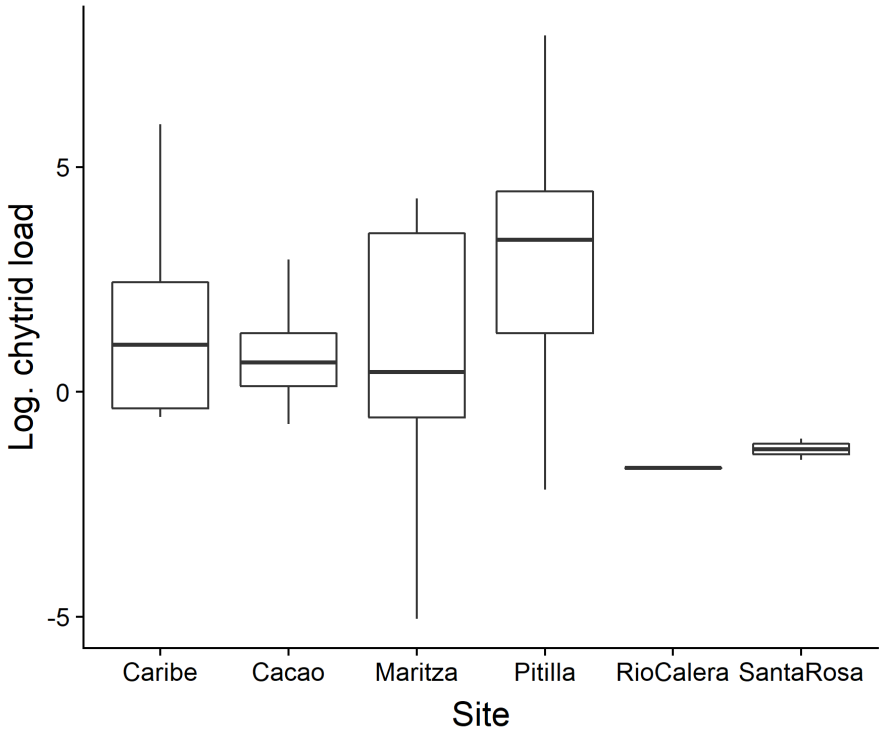


Figure 3.7. Median logarithmically transformed chytrid loads at each site within the Área de Conservación Guanacaste, Costa Rica, with one or more positive individuals sampled. Whiskers represent the highest and lowest data values, boxes represent the upper and lower quartiles of data values, with the median indicated as a horitzonal line contained in the box.

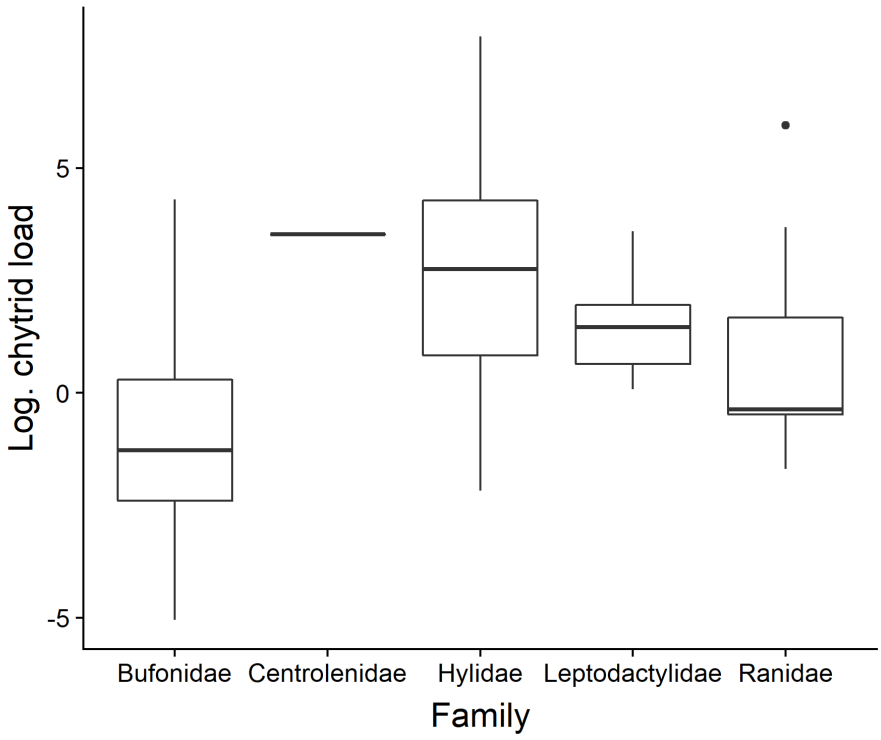


Figure 3.8. Median logarithmically transformed chytrid loads across each taxonomic family sampled within the Área de Conservación Guanacaste, Costa Rica, with one or more positive individuals sampled. Whiskers represent the highest and lowest data values, boxes represent the upper and lower quartiles of data values, with the median indicated as a horitzonal line contained in the box. Point represents outlier in data.

Discussion

Here, I have demonstrated widespread infection by ranavirus from a strikingly broad geographic and host range, which included both amphibian and reptilian representatives. Whilst ranavirus was consistently detected from both wet and dry forests in all sampled sites within the ACG, chytrid was rarely detected from the dry forests. These results combined with the detection of ranavirus from La Selva Biological Station, Costa Rica (Whitfield et al. 2012) and Ometepe, Nicaragua (Stark et al. 2014) suggest an

extensive distribution of this pathogen in southern Central America and across altitudinal gradients. As ranavirus infection has only previously been reported from Neotropical amphibians (Whitfield et al. 2012, 2013, Stark et al. 2014), the reptile samples from this study highlight an infrequently-examined distribution of ranavirus among these taxa. Duffus et al. (2015) reported ranavirus to have been detected from at least 175 species of 52 families. Here, ranavirus was detected from thirteen amphibian and two reptile species and from one amphibian and two reptile families, for which it is not known to have been reported from (Duffus et al. 2015). Despite a likely increase from the figure reported by Duffus et al. (2015), the results of this study have considerably widened the range of known ranavirus hosts, including, as far as I am aware, the first known wild ranavirus infected reptile species of the Neotropics.

I have shown both predator and prey species to be infected, alongside numerous species that congregate en masse at the same breeding sites. Ranavirus transmission is thought to be related to host density (Greer et al. 2008), although vectors (Kimble et al. 2015) and reservoirs (Brenes et al. 2014) likely play a role. High rates of ranavirus transmission may be driven by high densities of individuals, which in turn may drive disease outbreaks or even extinction (De Castro & Bolker 2005). But transmission through density independent means, such as consumption of infected carcasses could also drive transmission (De Castro & Bolker 2005). For instance, several times I observed reptilian predators consuming road-killed amphibian or reptile carcasses whilst sampling in the dry forests. In one such case, a *Leptodeira annulata* was found preferentially consuming a desiccated, road-killed

anoline lizard carcass, despite being surrounded by hundreds of small live amphibian prey (Figure S 3.2). Additionally, mosquitoes were often observed feeding on amphibians. These insects have previously been suspected as vectors of ranavirus transmission (Kimble et al. 2015), but have not been explicitly demonstrated to fulfil this role. Such interactions may sustain pathogen transmission, even when densities of conspecifics are low. Tracking the dynamics of pathogen transmission in this environment, where so many individuals can be infected, would likely prove a difficult task. However, the contributing factors, such as vectors, predation and direct and indirect contact could be investigated experimentally.

Infection proportions for ranavirus and chytrid differed among taxonomic families. This study is the first to have detected ranavirus from wild individuals of one of the families found more likely to be infected, Microhylidae – which is also known to have experienced population declines (Lips et al. 2003a). The mechanisms driving taxonomic differences in susceptibility are unclear. But given the life history of this taxon, a lack of exposure, resulting in immunological naivety, may be partly responsible (Hoverman et al. 2010). Microhylids have unusual life histories, they remain underground for the majority of time and emerge simultaneously during periods of heavy rain for breeding (Savage 2002). This mass emergence could result in spikes of ranavirus infection through increased exposure or physiological changes (e.g. Langwig et al. 2015). The synchronised emergence of these frogs likely brings them into contact with vectors, reservoirs and host densities at levels not experienced underground. Additionally, emergence from underground burrows could result in an

increase in body temperature of the frogs, towards the optimum replication temperature for ranavirus. Similar body temperature changes driven by life history events have previously been shown to initiate disease prevalence spikes in bats (Langwig et al. 2015). However, the families Microhylidae and Centrolenidae, which were found to be at higher infection risk than Bufonidae, were comprised of a single sampled species, thus effects may also be species specific and diluted through my combining of taxonomic units into family groups. One Microhylid species (*Gastrophryne carolinensis*) from the USA has previously been examined for ranavirus in the wild with none of the 26 tested individuals found to be infected (Hoverman et al. 2012a). This lack of detection may in part be due to the low sensitivity of the methods used in Hoverman et al.'s study (see Chapter 4), which may have missed low intensity infections. However, *G. carolinensis* has been experimentally exposed to ranavirus, with a low number of resultant detectable infections and low mortality rates (Hoverman et al. 2010, 2011). Although this species is phylogenetically close to the Microhylid *Hypopachus variolosus* tested here, host community structure, environmental conditions and interspecific variation in immunity and life history could all account for these differences. Notably, three of the families found more likely to be infected with ranavirus, Hylidae, Ranidae and Leptodactylidae, have previously been found to contain Costa Rican species especially prone to enigmatic declines (Bolaños 2002). Additionally, my findings are congruent with previous studies, which have found the family Ranidae to be more likely to contain infected individuals than other families that have been previously tested (Hoverman et al. 2011, 2012a).

Individuals of the families Centrolenidae, Hylidae, Ranidae and Leptodactylidae were more likely to be infected with both ranavirus and chytrid than individuals of the family Bufonidae. The family Centrolenidae has previously been found to contain individuals infected at a relatively high prevalence for both pathogens (Whitfield et al. 2013) and this taxon has experienced declines (Lips et al. 2003a), although some populations appeared to recover relatively rapidly (Voyles et al. 2018). Severe declines were also recorded from the families Hylidae, Ranidae and Leptodactylidae in Costa Rica, which affected nearly one fifth to half of their constituent species (Bolaños 2002). Globally the family Hylidae is reported to be more susceptible to chytrid infection than all other tested families (Olson et al. 2013), so this pattern appears to remain true at a more localised scale in Costa Rica. For both pathogens, taxonomic differences in infection may be due to differences in host genetic diversity (Gantress et al. 2003, Pearman & Garner 2005, Ellison et al. 2017), host microbial community structure (Lam et al. 2010), life history (Bancroft et al. 2011, Hoverman et al. 2011), habitat and community differences (Stuart et al. 2004, North et al. 2015), or a combination of these.

Life history appears to explain some degree of this variation. When hylids were examined, it was apparent males were at higher infection risk for both pathogens than females. Additionally, whilst coinfections were found to be rare, male hylids from wet forests accounted for seven of the nine recorded cases. As the same was not true for females, it is unlikely this effect is due to intraspecific factors. Instead, temporal reproductive events could result in increased transmission for each pathogen. The males of many of these

species congregate in high densities around breeding pools throughout the breeding season, are territorial and engage in both intra- and inter-specific combat (Savage 2002). Conversely, females generally spend less time aggregated around ponds containing high densities of potentially pathogen-shedding individuals.

The positive correlation between ranavirus prevalence and temperature found in this study aligns with what is already known of the virus's biology from laboratory experiments and field sampling (Chinchar 2002, Echaubard et al. 2014, Price et al. 2018). Here, I have been able to more explicitly demonstrate this trend *in situ*. The effects of ranavirus upon amphibian mortality rates and populations have not yet been explored in Costa Rica. If ranavirus is driving mortality among Costa Rican amphibians it would be rational to expect these events to be most common in the areas with highest prevalence, especially given the reported amphibian die-offs in Santa Rosa. However, individuals of the dry forests could be able to rapidly clear infection at temperatures that are optimal for the host's immune system, as has previously been found with chytrid (Ribas et al. 2009). Differences in host community structure (Keesing et al. 2006), genetic diversity (Spielman et al. 2004) or more frequent exposure driving acquired resistance (McMahon et al. 2014) could also limit the effects of disease among dry forest amphibians. Thus, although prevalence is high in these areas disease may be uncommon. Similarly, outbreaks may be expected to be less common in areas of lower prevalence. However, factors such as the reduction of host populations at higher elevations through chytrid outbreaks could have led to populations with lower genetic diversity (Sutton et al. 2011). In turn,

individuals from these areas may be susceptible to disease, but less able to respond and limit ranavirus proliferation when infected. Thus, these hosts could be rapidly undergoing morbidity and/or mortality. These individuals may be harder to detect than non-moribund individuals that have very recently become infected or are less susceptible (Wobeser & Wobeser 1992). Population monitoring and disease progression studies are essential for gaining clarity on disease dynamics and effects among these Costa Rican populations.

The results of my study align with previous research which has indicated wetter, cooler environments in Costa Rica to be more suitable for chytrid (Puschendorf et al. 2009). Very few individuals with chytrid were found from the dry forest and chytrid prevalence was negatively associated with temperature, supporting the hypothesis that environmental conditions are important for this pathogen (Woodhams & Alford 2005). These results align with previous research indicating these habitats as chytrid refuges for amphibians (Puschendorf et al. 2009, Zumbado-Ulate et al. 2014). Chytrid infection loads appeared to be associated with temperature for one taxonomic family. However further testing of a higher number of samples will be required to confirm these results, as the drivers of chytrid infection likely include an array of factors, including host susceptibility, pathogen virulence, host communities and local environmental conditions.

The capacity of ranavirus and chytrid to drive declines and their wide-ranging occurrence alongside continuing climatic warming is of conservation concern and may present additional challenges for threatened and recovering amphibian populations. Costa Rican amphibians have already undergone

losses from the effects of climate change and chytridiomycosis (Pounds et al. 2006). Future warming will likely lead to altered disease dynamics due to the association between each pathogen and environmental conditions (Harvell et al. 2002), and such climatic changes are linked to infected amphibian mortality rates (Woodhams et al. 2003, Rojas et al. 2005, Pounds et al. 2006, Laurance 2008).

At the ACG amphibian losses have occurred at higher altitudes, where I found chytrid prevalences to be highest (up to 50%), yet I found ranavirus prevalence to increase at higher temperatures, which are typical of lower elevation dry forest sites. Lower elevation dry forests are thought to be unsuitable for chytrid and thus considered a refuge for amphibians (Puschendorf et al. 2009, Zumbado-Ulate et al. 2014). Critically endangered species have survived in these forests after their extirpation from higher altitudes (Puschendorf et al. 2005). This study has shown individuals of one such species (*Craugastor ranoides*), within one of its last remaining populations, to be infected with ranavirus. If Costa Rican ranaviruses have the capacity to drive amphibian mortality events, this presents a further threat to such species, with amphibian populations at both lower and higher elevations in the ACG possibly being reduced by different pathogens. Although no studies have investigated the association between ranavirus and Costa Rican amphibian mortalities or declines, this pathogen has been linked to one previous die-off in Nicaragua (Stark et al. 2014). This demonstrates Neotropical amphibians to be susceptible to ranavirus driven mortality events (Stark et al. 2014). Additionally, the mortality event in Santa

Rosa merits further investigation of the effects of ranavirus on native populations.

Little is known about ranaviruses in Central America, yet the associated amphibian hosts here have undergone extremely severe population declines and appear susceptible to ranavirus-driven die-offs (Stark et al. 2014). Therefore, further research should be undertaken to determine the range extent of Central American ranaviruses. Given the results of this study, detection appears most likely at higher temperature habitats and include some hosts which have rarely previously been tested. Such studies are essential for the development of ranavirus research in under-studied tropical regions and the monitoring and identification of further disease threats to Neotropical species.

Chapter 4 Choice of molecular assay determines ranavirus detection probability and inferences about incidence and impact

Abstract

Infectious diseases are emerging at an increasing rate and reliable diagnostic strategies are essential for effective surveillance and research. Ranavirus is an emerging pathogen that can cause morbidity, mortality and population declines in ectothermic hosts. Despite the economic and conservation importance of these viruses, there is no standardized approach to diagnostics, with many molecular assays currently in use. Here, I compared the inter-assay variation and intra-assay precision among two quantitative and two conventional polymerase chain reaction assays, using laboratory propagated virus and field-collected amphibian and reptile samples. The assays varied in their sensitivity and specificity as well as the sequence similarity of oligonucleotides to a set of published ranavirus isolates. Some assays exhibited poor sensitivity resulting in a high proportion of false negative results whilst false positive results were also generated. Amplicon sequences from the two conventional PCRs were used to confirm results but were also shown to be useful for inferring viral lineage. Inaccurate results bias estimates of the distribution and impacts of ranaviruses, and together these findings emphasize that molecular assays should be chosen carefully in the context of study aims.

Introduction

Disease emergence is occurring at an unprecedented rate (Jones et al. 2008) requiring surveillance systems which incorporate sensitive and reliable diagnostic assays that limit bias (Kuiken et al. 2005, Hyatt et al. 2007, Lachish et al. 2012, DiRenzo et al. 2017). Molecular methods – predominantly nucleic acid-based techniques such as conventional polymerase chain reaction (PCR), quantitative (q)PCR and sequencing – do not usually provide explicit information about pathology but are increasingly used for pathogen detection due to their perceived ease of use, reducing costs and rapid turnaround times (Boyle et al. 2004, Hyatt et al. 2007, Black et al. 2017). They also often have broader applications, greater sensitivity and specificity and require less training and skill than traditional techniques, such as histology (Hyatt et al. 2007, Lorch et al. 2010, Skerratt et al. 2011).

Ranaviruses (family *Iridoviridae*) have caused incidents of mortality and morbidity in amphibians, reptiles and bony fish around the world (Gray et al. 2009, Price et al. 2017). The distribution of ranavirus and the drivers of outbreaks are often unclear. It has been suggested that ranaviruses are ubiquitous (Warne et al. 2016), but severe disease outbreaks are patchy in distribution and many studies have linked disease emergence to human actions including translocations of infectious materials (Price et al. 2014, 2016, Rijks et al. 2016). Ranaviruses have been associated with population declines of a frog species in the United Kingdom and entire amphibian assemblages in Iberia (Teacher et al. 2010, Price et al. 2014, Rosa et al. 2017), underlining their importance as pathogens of potentially significant

conservation concern. Reliable, sensitive and specific diagnostic methods are essential for detecting low intensity infections, which will aid a more complete understanding of ranavirus presence, disease ecology and epidemiology.

The majority of laboratories use a single molecular assay when screening samples for ranavirus infection, but approaches are not standardized despite the availability of published guidelines from the World Organisation for Animal Health (OIE 2017, Black et al. 2017). Eleven different PCR methods for ranavirus detection were used in the published literature between 2009-2014, but information about the sensitivity or specificity of these assays was usually lacking (Black et al. 2017). Therefore, results may not be reproducible, low intensity infections may be missed, prevalence estimations may be biased, and pathogen species may be misidentified. Sequencing of PCR amplicons can be used to confirm the specificity of an assay and the sequences can also be used to investigate a pathogen's taxonomy and genetics (OIE 2017). Nevertheless, even when the primary goal is presence-absence detection, laboratories may favor qPCR and have frequently utilized unpublished methods (Black et al. 2017). Additionally, there appears to be both uncertainty and inconsistency about how to define a positive result from qPCR assays (e.g. Miller et al. 2015, Black et al. 2017). Here, I compare the inter-assay variation and intra-assay precision of four molecular methods used in ranavirus detection. I hypothesise that ranavirus detection is affected by molecular method choice.

Methods

Two conventional PCRs (from Mao et al. 1997 and Meng et al. 2013; Table 4.1) and two probe-based qPCRs (Brunner et al. 2004– cited in Picco et al. 2007 and Leung et al. 2017; Table 4.1), respectively referred to as “Mao PCR”, “Meng PCR”, “Brunner qPCR” and “Leung qPCR”, were compared for their sensitivity and specificity. All assays targeted the viral major capsid protein (MCP) gene, the main viral coat protein, conserved among all members of the *Iridoviridae* (Eaton et al. 2007). Oligonucleotide sequences (primers and probes) were compared to an alignment of 21 ranaviruses with published whole genomes (Table S 4.1) to obtain orientations and annealing positions and to check for polymorphisms in binding regions. Assay performance was compared using both cultured ranavirus isolates and field-collected samples.

DNA was extracted alongside extraction controls (nuclease free water as a negative control and cell-cultured ranavirus as a positive control) using an ammonium acetate protocol and purified using ethanol washes (Nicholls et al. 2000). Samples were stored at -20°C until use. Conventional PCRs were run in 8µl reactions and qPCRs in 20µl reactions. Two microliters of template DNA were used in every reaction, regardless of assay used. A PCR positive control consisting of DNA extracted from a cultured ranavirus isolate (RUK13; GenBank accession KJ538546; Price, 2014) and a no-template negative control consisting of nuclease-free water were run in duplicate on each PCR plate.

The Mao PCR reaction mixtures comprised forward and reverse primers MCP4 and MCP5 (Table 4.1; Mao et al. 1997) at a final concentration of 0.5μM, 4μl of 2X DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Massachusetts, USA), and nuclease-free water to take the total volume excluding template to 6μL. The nested PCR used the Mao PCR as the first step but both primers were diluted to a final concentration of 0.05μM. The second step of the nested PCR used primers MCP-IF and MCP-IR from Meng et al. (2013) at a concentration of 0.4μM, but all other reagents and concentrations were the same as used for the Mao PCR. The Brunner qPCR used the rtMCP-for and rtMCP-rev primers (Picco et al. 2007) at a final concentration of 0.9μM with 10μl TaqMan Universal 2x PCR Master Mix (Thermo Fisher Scientific, Massachusetts, USA), rtMCP-probe at a final concentration of 0.25μM, and nuclease-free water to take the volume to 18μL (Brunner et al. 2004 - cited in Picco et al. 2007; Whitfield et al. 2012, 2013). Reaction mixtures for the Leung qPCR comprised forward and reverse primers (MCP_F and MCP_R) at a final concentration of 0.5μM, 10μL TaqMan Universal 2x PCR Master Mix (Thermo Fisher Scientific, Massachusetts, USA), MCP_probe at a final concentration of 0.25μM, and nuclease-free water to take the volume to 18μL (Leung et al. 2017).

The Mao and Meng PCRs were run on a Techne PCRmax Alpha Cycler 1 (Fisher Scientific, Loughborough, UK) and thermocycler settings comprised a 10-minute strand dissociation step at 95°C, 23 cycles of 95°C for 30 seconds, 62°C (decreasing by 0.5°C per cycle) for 30s, and 72°C for 30s, 25 cycles of 95°C, 50°C and 72°C each for 30s, and a final elongation step of seven minutes at 72°C. The qPCRs were run on a StepOne Real-Time PCR

system (Applied Biosystems, California, USA) and began with a two-minute step at 50°C to prevent carry-over contamination with Uracil-N glycosylase (UNG) followed by a 10-minute strand dissociation step at 95°C. The Brunner qPCR was run for 50 cycles comprising steps of 95°C for 20 seconds, 54°C for 20 seconds and 72°C for 30 seconds. The Leung PCR was run for 50 cycles comprising steps of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds. Conventional PCR products were visualized on 100ml 2% agarose gels containing 5µl SYBR Safe DNA Gel Stain (Invitrogen, California, USA) alongside a 100bp ladder (Thermo Fisher Scientific, Massachusetts, USA). Positive results were defined in each of two ways: 1) using a “majority-rules” approach where a consensus was taken by considering the overall majority returned from replicates (Miller et al. 2015), 2) by taking amplification in any replicate as evidence of a positive sample.

Inter-assay variation

To investigate inter-assay sensitivity, first, the limit of detection of each of the four assays was compared using laboratory isolates grown in culture. Isolates were used from two of the lineages of the amphibian-associated ranaviruses (FV3-like and CMTV-like) (Price et al. 2017). A ten-fold dilution series of DNA extractions of each isolate (1 to 1^{-10}) was used as template DNA.

To investigate the relative sensitivities and specificities of the assays, the Meng PCR was considered a ‘gold standard’ on account of the high sensitivity of nested protocols (Hafez et al. 2005, Miller & Sterling 2007) and the capacity to confirm the specificity of amplification by sequencing

amplicons, as is recommended by the OIE (OIE 2017). DNA extractions of samples from an archive comprising toe-clips and liver tissue from a diverse assemblage of Costa Rican herpetofauna (Table S 4.2) were screened using the Meng PCR and positive results confirmed by sequencing (Chapter 3). Forty “known positive” and forty “known negative” samples were selected at random from this larger set. Sensitivities of the Mao PCR, Brunner qPCR and Leung qPCR were calculated relative to the Meng PCR (gold standard) as the proportion of the total known positive samples that amplified. Specificities were also calculated relative to the gold standard as the proportion of known negatives that did not amplify.

Fisher’s exact tests were run to determine whether assays performed equally to one another.

Intra-assay precision

Intra-assay precision - defined as an assay’s capacity to generate consistent results among replicates - was investigated by using each assay to run six replicate reactions per sample. Fifteen known positive and fifteen known negative samples were selected at random from the Costa Rican sets used above. The intra-assay precision was calculated as the proportion of replicates that returned the expected result.

Table 4.1. Primer and probe sequences of ranavirus diagnostic PCRs compared for sensitivity and specificity.

Assay	Amplicon size (bp)	Oligo ID	Sequence (5'->3')	Strand	Position in coding sequence
Mao PCR	531	MCP 4	GACTTGGCCACTTATGAC	Sense	43-60
		MCP 5	GTCTCTGGAGAAGAAGAA	Anti-sense	556-573
Meng PCR	320	MCP-IF	TCGCTGGTGTTGCCTATCAT	Anti-sense	457-476
		MCP-IR	CTGCCAAGATGTCGGGTAAC	Sense	158-177
Brunner qPCR	70	rt-MCP-for	ACACCACCGCCCAAAAGTAC	Sense	1298-1317
		rtMCP-rev	CCGTTCATGATGCGGATAATG	Anti-sense	1347-1367
		rtMCP-probe	CCTCATCGTTCTGGCCATCAACCAC	Sense	1320-1344
Leung qPCR	97	MCP_F	GTCCTTTAACACGGCATACCT	Sense	381-401
		MCP_R	ATCGCTGGTGTTGCCTATC	Anti-sense	459-477
		MCP_probe	TTATAGTAGCCTR*TGCGCTTGGCC	Anti-sense	432-455

**R* is the IUPAC nucleotide ambiguity code for the bases Adenine or Guanine

Comparison of phylogenetic signal contained in Mao and Meng amplicon sequences

The phylogenetic signal contained in the respective MCP regions amplified by the Meng and Mao PCRs was compared using phylogenetic trees. A multiple-sequence alignment of the complete coding sequence of the MCP gene of 21 ranavirus isolates with published whole genomes (Table S 4.1) was trimmed to the maximum possible length of both the Meng and Mao amplicon sequences by mapping the respective primer pairs of each assay to the alignment. Phylogenetic trees were then constructed with Mr Bayes v3.2.2 (Huelsenbeck & Ronquist 2001) using a generalized time-reversible model of nucleotide substitution with rate variation modelled as a gamma distribution with four rate categories. Two runs of four MCMC chains were run for 500,000 generations and the default program settings were used for other parameters.

Results

Polymorphisms within binding regions of primers and probes

The Mao PCR primers were 100% sequence identical to all 21 ranavirus isolates but there were mismatches (single nucleotide polymorphisms) between the oligonucleotide sets of the other assays and some of the 21 isolates (Table 4.2). The Meng_IR priSmer had between one and four mismatches with eight of the isolates and appeared to be poorly suited for use with the whole group of ATV-like ranaviruses (as previusly found in Price 2016, Price et al. 2017).

Table 4.2. Single nucleotide polymorphisms (SNPs) within regions of the major capsid protein (MCP) gene complementary to primer and probe sequences. Full names and accession numbers for virus isolates are provided in Table S 4.1.

Assay	Oligo ID	Isolate	SNP count
Mao PCR	MCP 4	Not applicable	100% identical to all isolates
	MCP 5	Not applicable	100% identical to all isolates
Meng PCR	MCP-IF	SERV	1
	MCP-IR	SERV	2
		Rmax	2
		CodIV	2
		TFV	1
		EHNV	2
		ESV	2
		ToRV1	4
		ATV	2
Brunner qPCR	rt-MCP-for	SERV	1
		PPIV	1
	rtMCP-rev	Not applicable	100% identical to all isolates
	rtMCP-probe	SERV	1
		GGRV	1
		PPIV	1
Leung qPCR	MCP_F	SERV	3
		ESV	1
	MCP_R	SERV	1
	MCP_probe	SERV	1
		GGRV	1
		ATV	1
		BIV	1

Sensitivity of assays in detection of cultured ranavirus isolates

The Leung qPCR was two orders of magnitude more sensitive than other assays regardless of the viral lineage of the cultured isolate (FV3 or CMTV; Table 4.3). The Meng PCR and Brunner qPCR performed similarly, exhibiting slightly greater sensitivity than the Mao PCR (Table 4.3).

Table 4.3. Limit of detection of four PCR methods for detecting ranavirus isolates from two major groups of amphibian-associated ranaviruses (frog virus 3-like [FV3] and common midwife toad virus-like [CMTV]). Isolates were diluted from 1 to 1e-10.

Isolate	**Limit of Detection**			
	Mao	Meng	Brunner	Leung
FV3	$1e^{-6}$*	$1e^{-6}$*	$1e^{-6}$	$1e^{-8}$
CMTV	$1e^{-6}$*	$1e^{-6}$	$1e^{-6}$	$1e^{-8}$

* Amplification in one of two replicates at the dilution given

Assay performance against field-collected samples

The assays did not perform equally against the field-collected samples (Fisher's exact test, $P < 2x10^{-19}$). The Leung qPCR was the most sensitive assay, detecting all of the known positives and therefore demonstrating 100% comparative sensitivity with the results from the Meng PCR confirmed by sequencing (the proposed gold standard; Figure 4.1 A). The Brunner qPCR was much less sensitive, only correctly identifying 11 of 40 (27.5%) positive samples if a majority-rules approach was applied. If amplification in any replicate was used to define a positive result, then an additional nine known positives were correctly identified taking the total sensitivity to 20 of

40 (50%; Figure 4.1 A). The Mao PCR performed extremely poorly, correctly identifying only one known positive (2.5%) using a majority-rules approach with just two additional samples giving an ambiguous result, and the remaining 37 samples showing no amplification in either replicate (Figure 4.1 A).

There was no significant difference between the assays in terms of specificity against known negative samples (Fisher's exact test, $P > 0.10$). Using a majority-rules approach, all assays showed 100% specificity, correctly identifying all of the known negatives. However, taking any amplification (either replicate) as a positive result reduced the Brunner qPCR's comparative specificity to 92.5% (three of the known negative samples showed amplification in one replicate; Figure 4.1 B). Since the Brunner qPCR was significantly less sensitive than the Meng PCR or Leung qPCR in screens of known positive samples it is very unlikely that this amplification was explained by a greater capacity to detect ranavirus than exhibited by those assays. Instead, it is most likely that this amplification was non-specific and that the Brunner qPCR generated false positive results for these three (out of 40) samples.

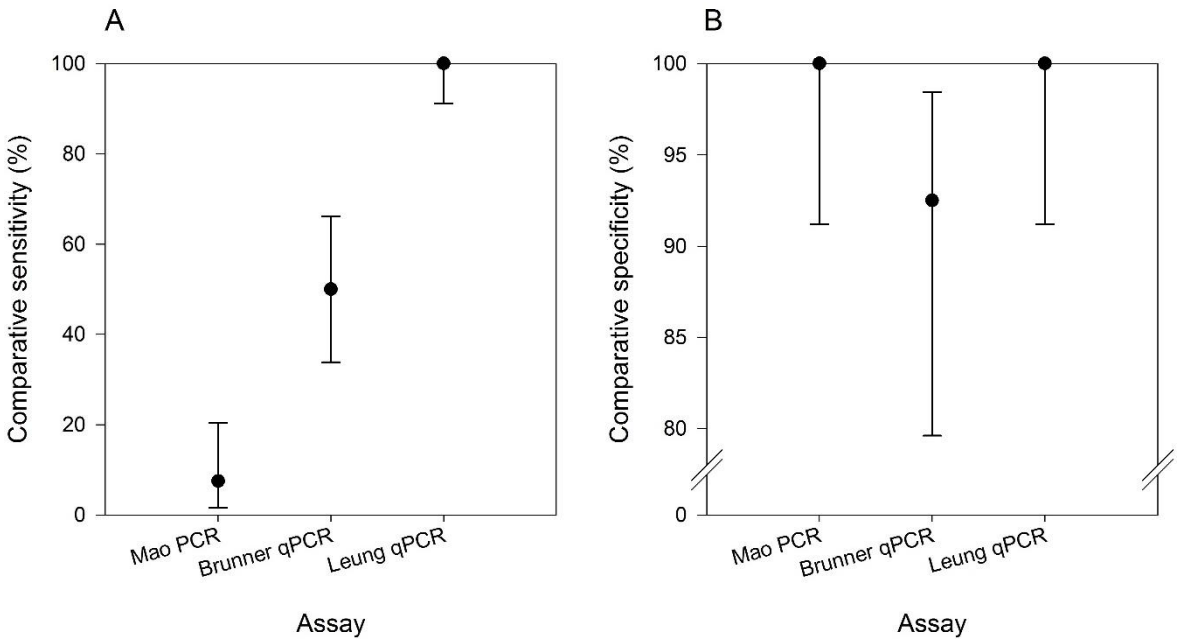


Figure 4.1. Relative performance of four molecular assays in detecting ranavirus from non-lethal samples of Costa Rican amphibians. Assay performance is relative to a nested PCR assay (Meng et al. 2013) used as a 'gold standard'. A) Relative sensitivity tested against known positives, B) Relative specificity tested against known negatives. 95% confidence intervals shown.

Intra-assay precision

The Leung qPCR performed most consistently of the assays tested in terms of the proportions of replicate reactions showing amplification with known positive samples: all samples (n = 15) were identified as positive when applying a majority-rules approach with only a single replicate in a total of 90 (15 samples, 6 replicates each) showing no amplification (Figure 4.2 A). The Brunner qPCR performed relatively poorly: none of the 15 positives would have been called positive using a majority-rules approach and just 38.9% of all replicates showed amplification (Figure 4.2 A). The Mao PCR performed

very poorly with just a single replicate out of 90 yielding a band on gels (Figure 4.2 A). The Brunner qPCR had the lowest intra-assay precision for negative samples, however both qPCRs showed high consistency: 93 of 96 replicates (96.9%) of the Brunner qPCR and 94 of 96 replicates (97.9%) of the Leung qPCR showed no amplification (Figure 4.2 B). The Mao PCR showed 100% consistency among negative samples; no replicate produced bands on gels (Figure 4.2 B). When a majority-rules approach was applied to these results, no false positive results were generated by any assay.

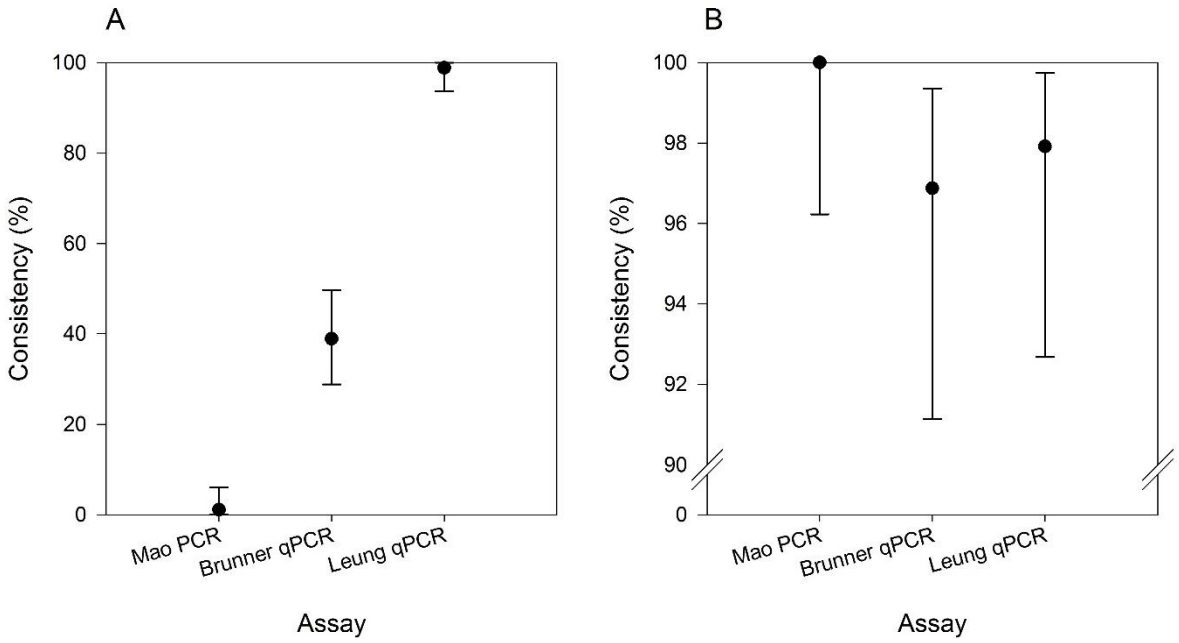


Figure 4.2. Comparison of the capacity of ranavirus molecular assays to generate consistent results among replicate reactions (intra-assay precision). A) Proportion of replicates that returned the expected result with known positive samples. B) Proportion of replicates that returned the expected result with known negative samples. 95% confidence intervals shown.

Phylogenetic signal of PCR amplicons

The phylogenetic signal of the Meng PCR amplicon (320bp) was compared to that of the longer Mao PCR amplicon (531 bp). The two amplicons returned well-supported trees with very similar topologies (Figure 4.3). Neither amplicon contained sufficient signal to return the CMTV-like ranaviruses as the monophyletic group that is obtained when using much larger datasets (Price 2016). The amplicon sequences of both assays incorrectly placed ToRV1 in the FV3-like clade but this isolate is a mosaic of two highly divergent ranavirus types which requires whole genome data to resolve its true position (Price 2016). The shorter Meng PCR amplicon exhibited some loss of resolution relative to the Mao PCR amplicon, especially in distinguishing CMTV-like viruses, but otherwise the two trees were highly concordant and both amplicons yielded accurate information that could be used to infer the ranaviral group (ATV-like, CMTV-like, FV3-like).

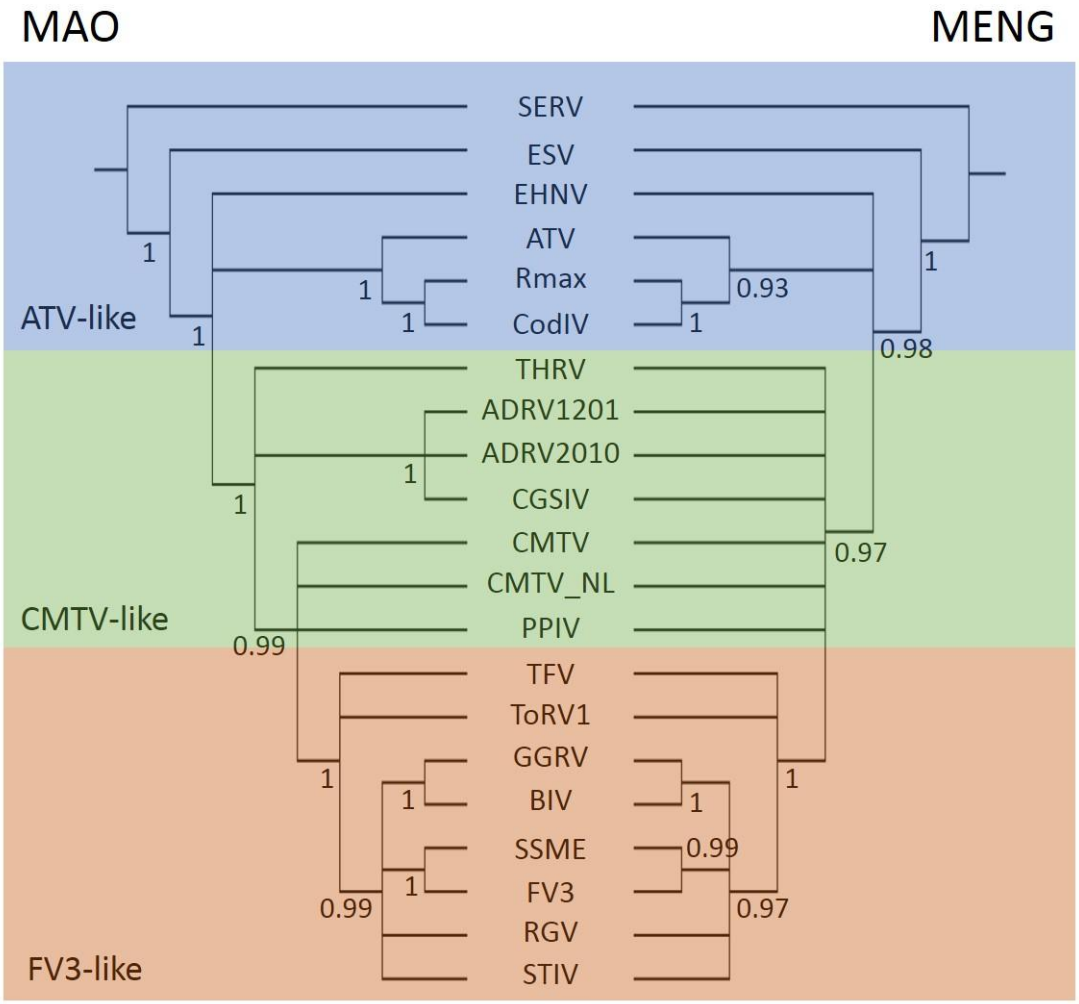


Figure 4.3. Comparison of the phylogenetic signal contained in sequences from PCR amplicons generated by two PCR methods (Mao PCR and Meng PCR). Both assays targeted the viral major capsid protein (MCP) gene, and an alignment of the full locus for amphibian-associated ranaviruses, with published complete genomes, was trimmed to the lengths of the respective PCR amplicons. Trees were constructed using default settings in Mr Bayes and drawn as cladograms (see main text). Support values at nodes are posterior probabilities. Full names of viruses, GenBank accession numbers and citations are provided in Table S 4.1.

Discussion

Ranaviruses are important pathogens which cause an emerging disease, spread by humans in some cases, that has had community-level impacts on amphibians (Picco & Collins 2008, Schloegel et al. 2009, Price et al. 2014, 2016, 2017). Sensitive and reliable diagnostics are required to yield accurate estimates of incidence and prevalence. Here, I found considerable variation in the performance of four molecular assays that are routinely used for ranavirus diagnostics in both surveillance of wild populations and experimental laboratory studies.

Assays varied in their suitability for ranavirus detection based on the viral genotype: in particular, one of the Meng PCR primers contained multiple mismatches to a whole group of amphibian-associated ranaviruses, the ATV-like viruses, affecting the optimal temperature for primer annealing and increasing the probability of false negative results. Assays also varied in their sensitivity and specificity as well as precision of results among replicates. The Leung qPCR showed 100% comparative sensitivity and specificity, but the Mao PCR showed poor sensitivity against my set of field-collected samples and the Brunner qPCR also resulted in false negatives as well as potential false positives. Although the Leung qPCR performed well in all experiments, neither qPCR assay allowed sequencing of amplicons as confirmation of results (OIE 2016) or as a basis for inferences about viral genotype (Black et al. 2017), meaning that alternative assays (or the use of a second assay) may be required when working with samples from regions or species where ranavirus has not been previously detected. In this respect,

the Meng nested PCR is sensitive and yielded reasonable phylogenetic signal compared to the Mao PCR, enabling viral isolates to be roughly placed within the major groups of amphibian-associated ranaviruses (Black et al. 2017), although it appeared to be poorly suited for amplification of a whole group of ranaviruses due to mismatches in primer regions.

The use of an inappropriate assay that yields false negative results is a significant problem for estimates of the current ranavirus distribution. Low intensity infections of individuals as well as whole populations/communities where ranavirus occurs at low prevalence may be falsely reported as negative, biasing estimates of ranavirus distribution. This is important for three main reasons: 1) patchy distributions can be taken as evidence of long-distance dispersal and can favor the inclusion of agents outside of the straightforward host-pathogen interaction in conceptual models explaining pathogen distributions. 2) If, for example, assay sensitivity is only sufficient for detecting infections from visceral organs of individuals experiencing active disease, perceptions of the impact, host range and virulence of these viruses could be biased, which may influence the focus of both management approaches and research effort. 3) Assays with poor sensitivity could also bias understanding of the genetic diversity among ranaviruses (in the context of virulence and other key viral life history traits) if some genotypes have low transmission rates, increased latency, and so on. As with false negatives, false positives bias estimates of distribution and impact but might also have important consequences for conservation and commercial activities. Considering amplification of any replicate indicative of infection led to probable false positives during this study, whereas using a majority-rules

approach reduced this, indicating it to likely be a more accurate approach, which should be adopted as standard. Given its OIE status, there are pressures to consider ranavirus presence when undertaking translocations of herpetofauna for both commercial and conservation purposes. False positive results could interfere with important economic activities such as the culture and supply of amphibians for food or conservation actions.

In spite of the notifiable status of ranavirus disease and OIE guidelines for diagnostic tests, there is currently no standardized approach to diagnostics (OIE 2016, Black et al. 2017). Many molecular assays have been used for ranavirus detection, often without checks on the appropriateness or power of the assays (Jaramillo et al. 2012, Black et al. 2017). My study highlights the importance of selecting and optimising methods that are compatible with study aims.

Chapter 5 Detection of lethal virus from extinct and extirpated amphibian populations

Abstract

Over the last forty years, Central American amphibians have experienced population declines and extinctions that have been associated with outbreaks of the chytrid fungus (*Batrachochytrium dendrobatidis*). More recently, another pathogen, which has been associated with amphibian die-off events and severe population declines, ranavirus, has been detected from Costa Rican amphibians. Few ranavirus surveys have been undertaken in tropical regions, where past amphibian declines were often enigmatic and especially severe, and few declining populations and diseased individuals were ever tested for this and other pathogens. Therefore, almost nothing is known about the ecology and host-effects of this pathogen in Costa Rica, including the length of time it has been present among local populations. Here, I tested 385 samples taken from amphibian and reptile museum specimens collected between 1975 and 1989 to investigate the historical occurrence of ranavirus in Costa Rica around the time of amphibian declines. Using quantitative and conventional Polymerase Chain Reactions and sequencing I found an FV3-like ranavirus from 22 individuals, including lizards, frogs and one caecilian. The earliest confirmed records of ranavirus infection were from 1976, indicating this pathogen to have been present in Costa Rica prior to and during amphibian declines. Two of the individuals collected in 1976, were of species that suffered rapid declines within the following decade and one is now thought to be extinct. Ranavirus was

detected from five sites where dramatic declines occurred soon after, and at the time of, sample collection. Further surveys are necessary to determine whether this pathogen and the disease it causes can be detected from individuals collected during declines and whether ranavirus outbreaks pose future threats to native herpetofauna.

Introduction

The amphibian chytrid fungus has driven many of the world's enigmatic amphibian declines (Berger et al. 1998, Lips et al. 2006), and so has received much of the research focus in investigations of amphibian declines and diseases. Indeed, chytrid has often been detected following declines from many of the areas where amphibians were declining with no other obvious cause (Lips et al. 2006), and is undoubtedly an extremely important factor in amphibian declines. Because the aetiologic agent of chytridiomycosis was only discovered in the late 1990s (Berger et al. 1998), and many declines occurred before this, most investigations of declining populations tested for this pathogen retrospectively (e.g. Puschendorf 2003). In addition, despite the widespread severity and distribution of these amphibian declines, from many locations, diseased animals were rarely found. Of those that were, few surveys ever tested them for pathogens other than chytrid (Duffus 2009), despite signs of disease uncharacteristic of chytridiomycosis and other aetiologic agents having been reported from some specimens collected at the time of declines (Green & Sherman 2001, Burgin et al. 2005, Merino et al. 2005).

Unusually, in one area in Costa Rica, declines were exceptionally well documented, with 40% of amphibian species thought to have been extirpated (Pounds & Crump 1994, Pounds et al. 1999, 2006, Cheng et al. 2011). In 1984 the first amphibian declines were recorded at Monteverde, from the glass frog species *Hylinobatrachium fleischmanni* (Hayes 1991). Researchers were initially unable to locate any glass frog egg masses, then across the following years encounters with these frogs became rarer events (Hayes 1991). This dramatic reduction in abundance followed a very strong El Niño event (Gill & Rasmusson 1983), but was also during the same year (currently known) that chytrid was first detected from Monteverde (Cheng et al. 2011). Further declines followed during another El Niño year in 1987, notoriously affecting the golden toad (*Incilius periglenes*) and harlequin frog (*Atelopus varius*), but also affecting other taxa, including anoline lizards, which are not known to be susceptible to chytrid (Pounds et al. 1999). These reptile declines appear to exemplify a wider-scale problem amongst Central American reptiles, and though some compelling data exist (Pounds et al. 1999, Whitfield et al. 2007, Sinervo et al. 2010), sampling has been sporadic (Chapple 2016), and most evidence is anecdotal.

Central American amphibian declines were suggested to be solely due to chytrid (Lips et al. 2008). Yet, periods of abnormal weather and declines of multiple taxa occurred simultaneously with amphibian declines (Pounds et al. 1999, 2006, Pounds & Coloma 2008, Parmesan & Singer 2008). For some areas of Central America, lags of nearly a decade between chytrid arrival and amphibian declines were recorded (Phillips & Puschendorf 2013). Similarly, in Italy chytrid was common among frog populations that did not

show any signs of chytridiomycosis until three years post-detection, when a heatwave was associated with the spread of another pathogen in the same host populations (Rosa et al. 2007). Together, these studies indicate factors beyond the single effect of chytrid in driving amphibian declines. Likely, the combination of climate change and chytrid was a significant factor, but the involvement of other under-studied factors is probable.

When investigating amphibian mortalities and declines, it is therefore necessary to include lines of evidence taken from beyond the chytrid global panzootic, including patterns of declines among other taxa and other factors such as the presence of other pathogens. One group of pathogens that undoubtedly merit further investigation among Central American herpetofauna are ranaviruses, lethal pathogens with the capacity to affect both amphibians and reptiles, which have been confirmed from Central America (Chen et al. 1999, Hyatt et al. 2002, Marschang et al. 2005, Whitfield et al. 2012). Ranavirus outbreaks can result in nearly 100% mortality within affected populations (Green et al. 2002), and have been associated with multi-species and persistent amphibian population declines in western Europe (Teacher et al. 2010, Price et al. 2014, Rosa et al. 2017). Although, a lack of systematic population-level studies may explain this apparently restricted spatial pattern of declines, as ranaviruses are responsible for multiple large, multi-species die-off events from North America (Bollinger et al. 1999, Green et al. 2002). Ranavirus outbreaks appear to be linked to warmer times of the year (Cunningham 2001), and similarly to chytrid, weather conditions resulting from climate change are predicted to increase the frequency of outbreaks (Price et al. 2018).

Ranaviruses were initially suspected to infect native Central American herpetofauna in the early 1990s, when an iridovirid, likely a species of ranavirus, was detected from a Costa Rican toad (Speare et al. 1991). But its detection was only confirmed from Central American amphibians two decades later, raising conservation concern for native amphibians (Whitfield et al. 2012). A subsequent study documented a mass die-off of tadpoles in nearby Nicaragua that was associated with a ranavirus outbreak (Stark et al. 2014). More recently I detected this pathogen from both Costa Rican amphibians and reptiles, with ranavirus detected from every site tested (Chapter 3). Together, these studies indicate ranavirus to have a high host and geographic distribution, with its presence in Costa Rica possibly spanning back at least three decades.

If present within Costa Rica during periods of decline and abnormal weather, it is not inconceivable that ranaviruses could, among other factors, have been involved in amphibian mortalities and potentially declines. The suspected case of ranavirus in Costa Rica from the early 1990s is indicative that these potentially lethal pathogens are not novel to present-day herpetofauna, but this has not been definitively shown. Therefore, I chose to test this hypothesis and initiate investigations into the potential role of ranaviruses in historic amphibian declines through examining Costa Rican herpetofauna samples collected during these periods. An extensive collection of Costa Rican specimens sampled from around the time of declines, for mostly taxonomic surveys, provides a large assembly of appropriately preserved historical samples. In addition to testing whether this potentially lethal pathogen was infecting native herpetofauna, I also hope to

determine whether a distinct introductory period for ranavirus during amphibian declines can be observed from testing these specimens, or whether this pathogen may have been present in Costa Rica prior to declines.

Methods

Three-hundred and eighty-five tissues from the Museum of Zoology, Berkeley, USA were obtained for processing. Samples were collected (usually as whole organisms for taxonomic research) from central to northern Costa Rica in 1975-1978 (n = 82), 1984 (n = 1), 1986-1987 (n = 298) and 1989 (n = 4). Tissues were from 155 reptiles (lizards and snakes only), 217 anurans and 3 caecilian individuals, comprising 83 species of 15 taxonomic families.

DNA from tissues and extraction controls (nuclease-free water as a negative control and cell-cultured ranavirus as a positive control) were extracted using a Qiagen DNeasy Blood and Tissue Kit, following the manufacturer's instructions. Samples were extracted in chronological order of initial collection. I used a probe based TaqMan quantitative (q) PCR assay to detect ranavirus (as in Chapter 4, page 75; Leung et al. 2017). Samples and controls were run in duplicate and were considered positive when sigmoidal amplification occurred in both wells and as negative when amplification occurred in neither well. Samples where amplification occurred in one well were conservatively considered negative, unless sequencing from conventional PCR indicated otherwise.

To investigate the strain of ranavirus present, and further confirm ranavirus presence, I ran all positives and samples where amplification occurred in a single replicate, with PCR controls, in duplicate on a nested conventional PCR (Chapter 4, page 74), using primers from Mao et al. (1997) and Meng et al. (2013). PCR products were sent to Macrogen Inc for sequencing of both strands. I considered qPCR single-positive samples as positives when a sequence was returned after PCR amplification that could be identified as ranavirus.

Sequences were trimmed, visually checked against chromatograms, aligned and forward and reverse reads were merged in MEGA7 (Kumar et al. 2016). These merged sequences were then compared to sequences in GenBank (Clark et al. 2016) using the Basic Local Alignment Search Tool (BLAST+2.8.0; https://www.ncbi.nlm.nih.gov/BLAST; Altschul et al. 1990), with default settings. Percentage similarity between the generated sequence and named viruses from GenBank was calculated in MEGA7 using the p-distance method. To investigate viral lineage, I compared the ranavirus sequences from this study to named ranaviruses from GenBank. Sequences were imported to Geneious V8 (Kearse et al. 2012) and named ranavirus sequences were downloaded from GenBank. Only unique sequences were retained and aligned. I used the MrBayes (Huelsenbeck & Ronquist 2001) Geneious Plugin for phylogenetic tree generation. Jukes and Cantor model (JC; Jukes & Cantor 1969) was identified as the best-fit model of molecular evolution using TOPALi v2.5 (Milne et al. 2009). Analyses were run for six million generations with an MCMC subsampling frequency of 10,000 and burn in length of 1.5 million.

Where host species was unknown, samples were amplified using primers targeting the 16S region (Crawford et al. 2010; as in Chapter 2, page 19) and both forward and reverse strands were sent for sequencing. Sequences were treated as above, with BLAST+2.8.0 used to identify individuals to species level.

Results

Overall, 385 samples were tested for ranavirus (see Table S 5.1 for museum identities). Of these, 372 were tested using qPCR and 16 were found to be positive (Table 5.1). In addition to these 16 positives, 62 samples that showed amplification in a single replicate from qPCR (Table S 5.2) and 13 samples, which were not tested on qPCR, were tested using a nested PCR. Six of these samples, including two from samples that showed amplification in only a single replicate on qPCR, were successfully sequenced, therefore bringing the total number of positive samples to 22. The 16 qPCR positives were also run on the nested PCR and six were successfully sequenced in order to confirm their genetic identities.

Table 5.1. Species which tested positive for ranavirus through qPCR and/or *nested PCR. †Indicates that a ranavirus sequence has been generated from this species.

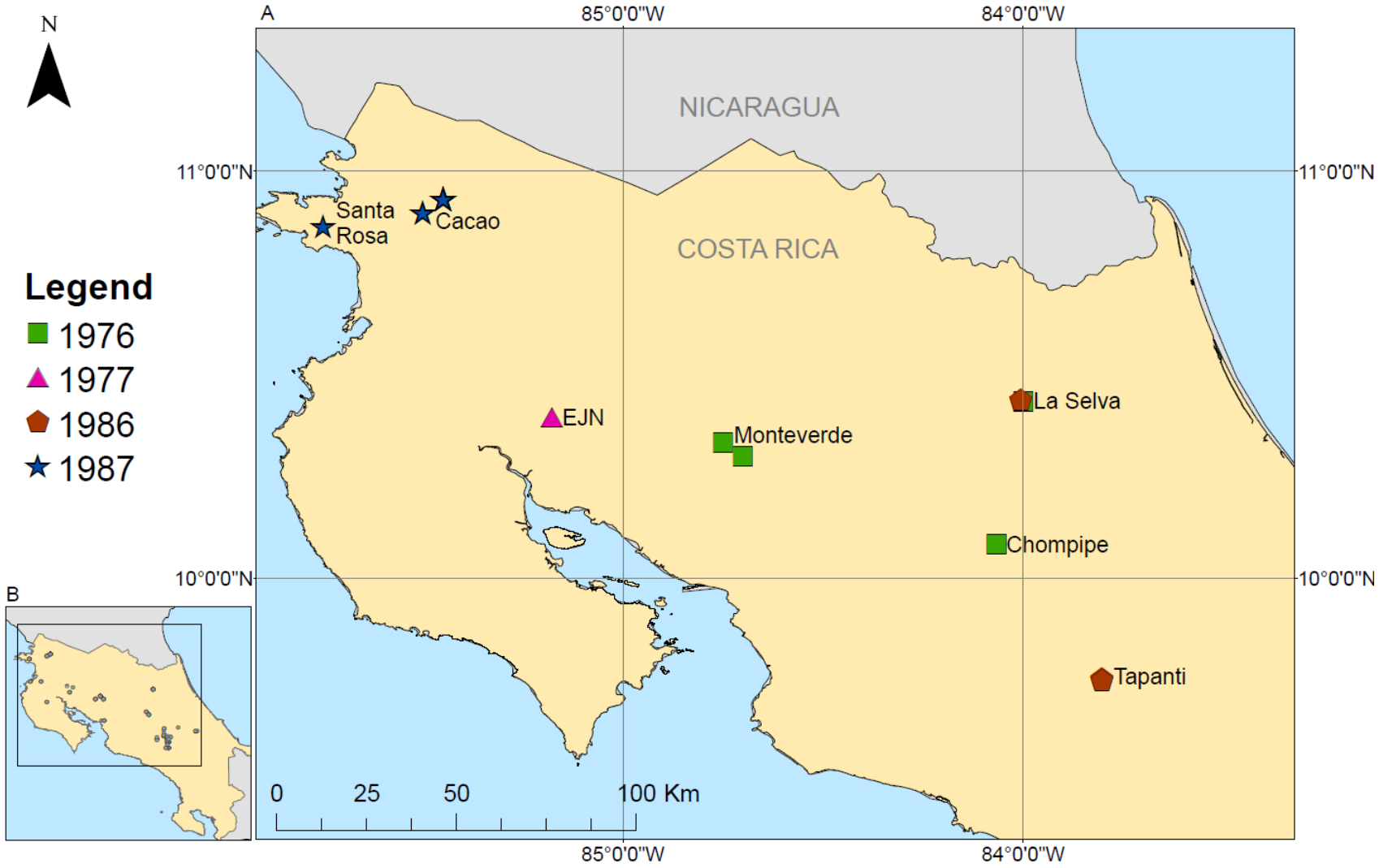
Species	Number	Year	Location
Reptilia	3		
Anolis altae	1	1986	Tapanti
Anolis humilis	1	1986	La Selva
Anolis intermedius	1	1976	Monteverde
Amphibia – anura	18		
Atelopus senex†	2	1976	Chompipe
Atelopus varius†	1	1976	Monteverde
*Craugastor andi**†	1	1987	Cacao
*Craugastor melanostictus**†	1	1987	Cacao
*Craugastor ranoides**†	2	1987	Cacao
Duellmanohyla rufioculis	1	1987	Cacao
Isthmohyla tica	1	1987	Cacao
Leptodactylus melanonotus†	1	1987	Santa Rosa
*Lithobates warszewitschii**†	1	1987	Cacao
Engystomops pustulosus†	1	1987	Cacao
Pristimantis caryophyllaceus†	1	1986	Tapanti
Rana forreri†	5	1977, 1987	Cacao, Enrique Jimenez Nunez
Amphibia – Gymnophiona	1		
Gymnopis multiplicata	1	1976	La Selva
Total	22		

The 12 generated 320bp sequences showed no variation amongst themselves, except for one sequence (“EJN virus”) from Estación Experimental Enrique Jiménez Núñez (EJN), for which one single nucleotide

polymorphism (SNP) was observed. This SNP causes a substitution in the coded amino acid, from aspartate, as in the majority of the examined samples, to glycine. However, this substitution is unlikely to affect the structure of the resulting protein, as glycine has no charge so can fit into both hydrophilic (as with aspartate) or hydrophobic environments. All virus sequences showed highest similarity to FV3 sequences on GenBank, thus all amplicons were identified as FV3-like ranaviruses. Despite the small size of the amplicon sequences, variation between these viruses and other named viruses could be identified, giving confidence to the identification of the amplified virus as FV3-like (as predicted from Chapter 4; Table 5.2). The assay showed high enough resolution to investigate interspecific variation, but information on inter-strain variation was limited when using highly similar viruses: FV3 viruses from multiple locations showed the same percentage similarity as one another to the Costa Rican ranaviruses.

Table 5.2. Percentage similarity between the Costa Rican ranavirus (not shown) and named ranaviruses from GenBank, with accession numbers shown. EJN virus indicates the virus with a SNP detected from Estación Experimental Enrique Jiménez Núñez. Location indicates geographic location sampled from, CR= Costa Rica. Modified accordingly from Waltzek et al. (2014). ►

Ranavirus	% Similarity	Accession #	Citation	Location
EJN virus	99.7	-	-	CR
Frog virus 3	99.4	FJ459783	(Holopainen et al. 2009)	North America
Rana grylio virus	99.1	KY264205	(Kwon et al. 2017)	South Korea
Soft-shelled turtle iridovirus	98.8	DQ335253	(Zhao et al. 2007)	China
Bohle iridovirus	98.4	AY187046	(Marsh et al. 2002)	Australia
Common midwife toad ranavirus	97.8	KJ703123	(Price et al. 2014)	Spain
Rana esculenta virus	97.5	FJ358611	(Holopainen et al. 2009)	Italy
Andrias davidianus ranavirus	97.5	KM609215	(Chen et al. 2013)	China
Tiger frog virus	97.2	AY033630	(He et al. 2002)	China
Pike-perch iridovirus	96.9	FJ358610	(Holopainen et al. 2009)	Finland
Epizootic haemotopoietic necrosis virus	96.6	AY187045	(Marsh et al. 2002)	Australia
European catfish virus	95.3	FJ358608	(Holopainen et al. 2009)	Italy
European sheatfish virus	95.3	FJ358609	(Holopainen et al. 2009)	Germany
Cod iridovirus	95.3	GU391284	(Ariel et al. 2010)	Denmark
Ranavirus maxima	95.3	GU391285	(Ariel et al. 2010)	Denmark
Ambystoma tigrinum virus	95.0	EU512415	(Ridenhour & Storfer 2008)	North America
Short-finned eel ranavirus	93.5	FJ358612	(Holopainen et al. 2009)	Italy
Largemouth bass virus	76.7	FR682503	(Ohlemeyer et al. 2011)	North America
Grouper iridovirus	71.7	KX284838	(Ma et al. 2016)	China


N
A
85°0'0"W
84°0'0"W
NICARAGUA
11°0'0"N
COSTA RICA
Santa Rosa
Cacao
Legend
1976
1977
1986
1987
EJN
Monteverde
La Selva
Chompipe
Tapanti
10°0'0"N
B
0
25
50
100 Km

◄Figure 5.1. A) Ranavirus-positive samples over different sampling years shown by different symbols. EJN = Estación Experimental Enrique Jiménez Núñez. B) Distribution of all samples tested, and indicator of the extent of Figure 5.1. A. For both maps, samples from the same site and year are shown as one point only.

Ranavirus positive species consisted of 13 amphibian species, including frogs and one caecilian, and three lizard species (Table 5.1). Four individuals identified as part of the '*Rana pipiens*' complex were sequenced and found to be most closely related to *R. forreri*, with sequences sharing between 98.7% and 99.1% identity with a sequence of *R. forreri*.

The earliest ranavirus positives were from five individuals collected prior to the current first detection of chytrid in the country and first recorded amphibian declines, in 1976 (Table 5.1). In chronological order of detection, these included one *Gymnopis multiplica* collected from La Selva Biological Station, two *Atelopus senex*, collected from Chompipe near Volcan Barva, one *Atelopus varius* and an *Anolis intermedius*, both collected from Monteverde Cloud Forest Reserve (Table 5.1; Figure 5.1). In addition to the five positive individuals from 1976, one was detected from 1977, three were collected in 1986 and thirteen from 1987.

No distinct year of introduction was observed as ranavirus was detected from all years where sample sizes were relatively high (low sample sizes with negative results have low power [Biau, et al. 2008], thus the pathogen would not be expected to be detected during these years; Figure 5.2). No samples were obtained from 1979-1983, 1985 and 1988.

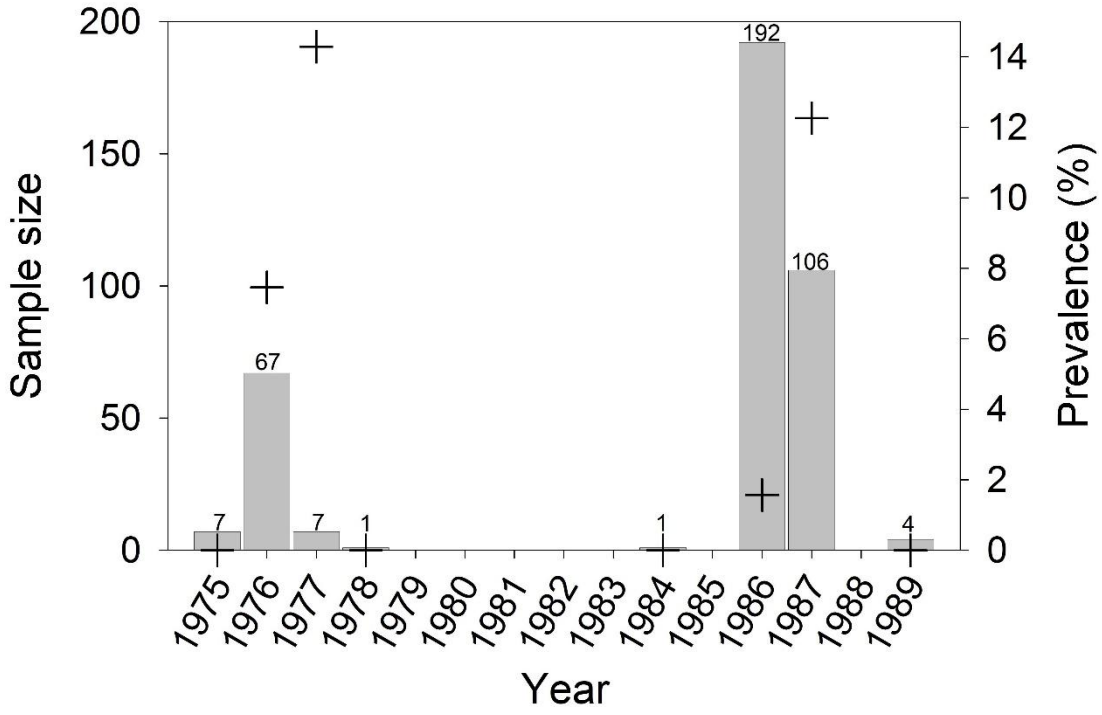


Figure 5.2. Variations of sample sizes across sample collection years. Prevalence estimations for each sampled year shown as '+' and numbers indicate total sample size per corresponding year.

Discussion

Ranaviruses are considered to be an important group of emerging and lethal wildlife pathogens due to their involvement in multiple amphibian population declines (Teacher et al. 2010, Price et al. 2014, Rosa et al. 2017). The herpetofauna of Costa Rica have undergone population declines in association with the emergence of chytrid and climate change (Pounds et al. 2006). But here, I have also found ranavirus to be present within populations that underwent declines, and from species which subsequently became extinct (Bolaños et al. 2008).

Although declines were often best recorded from the areas where higher numbers of samples were collected, hence possibly biasing the sample pool (81% of samples were collected from five protected, decline sites), it is nevertheless clear that ranavirus has historically been present within multiple severely declining herpetofaunal populations. Of the 16 species positive for ranavirus nearly half are known to have undergone local or widespread declines (IUCN 2017). Additionally, amphibian and reptile populations disappeared from five (Cacao, Monteverde, La Selva, Chompipe and Tapanti) of the seven areas in which I detected ranavirus (Pounds et al. 1999, Puschendorf et al. 2006, Whitfield et al. 2007).

In Cacao anecdotal reports indicate severe amphibian declines (Puschendorf et al. 2006), which included *Craugastor ranoides*, *C. andi* and *Isthmohyla tica*. These species are extirpated from this area, (Puschendorf et al. 2005, Solís et al. 2008) considered critically endangered (and possibly extinct for *C. andi*; Pounds et al. 2008; Solís et al., 2008; IUCN SSC Amphibian Specialist Group & NatureServe, 2013), and are among several declined species now known to have been infected with ranavirus from Cacao during periods of decline. In Monteverde, ranavirus was detected from an *Anolis intermedius*. Although this species did not face declines, it highlights that ranavirus was infecting reptiles of an area infamous for its herpetofauna population declines (Pounds et al. 1999). I was also able to show that one lizard species which is known to have been extirpated from Monteverde area (*Anolis altae*) had the capacity to become infected, as I found an individual collected from Tapanti to be positive for ranavirus (Pounds et al. 1999). Additionally, I detected ranavirus from an *Atelopus varius*, pre-decline. This

species is now considered critically endangered and is thought to have been extirpated from most of its range, including Monteverde (Pounds et al. 2010). Well-documented, but slower declines, uncharacteristic of disease outbreaks, have also been reported from La Selva herpetofaunal populations (Whitfield et al. 2007), including from lizard populations. Here, I detected ranavirus from one *Anolis humilis* and surprisingly a caecilian, *Gymnopis multiplicata*. The detection of this pathogen from a caecilian amphibian represents the first case of infection I am aware of from this enigmatic order of amphibians. In Chompipe several amphibian populations disappeared, including a species from which I detected ranavirus, *Atelopus senex*, which is now thought to be globally extinct. Ranavirus was also detected from another species that faced significant declines, *Pristimantis caryophyllaceus*, with infection confirmed from an individual collected in Tapanti (Pounds et al. 2008).

Currently, nothing is known about the potential progression of ranavirus infection to disease among most Costa Rican species, and whether any mortality events could be numerous enough to cause a population-level effect. In North America, ranavirosis tends to be most severe among larval amphibians (Haislip et al. 2011). From the outbreaks of chytridiomycosis, which predominantly affected post-metamorphic individuals, it is known that amphibian populations disappeared rapidly, with diseased individuals being recorded relatively rarely. If ranavirus outbreaks had, or commonly do, occur in Costa Rica, ranavirosis may not have been recognised from diseased individuals. This is especially applicable if, as in the rest of North America, disease is predominantly most severe among larval amphibians (Green et al.

2002, Hoverman et al. 2011, Stark et al. 2014). Amphibian larvae are less-frequently encountered and decompose more rapidly than post-metamorphic individuals, making die-off events less visible than those of adult amphibians (Regester & Whiles 2006). Therefore, further studies in Costa Rica should include larval amphibians in testing, so comparisons of disease prevalence among life stages can be made.

The information generated from sequencing of positive samples is limited due to the small size of the sequenced amplicons. However, enough information was contained within this region for me to identify this ranavirus as being most similar to FV3, a strain that has previously been linked to amphibian die-offs and population declines (Greer et al. 2005, Teacher et al. 2010). Although the quantities of ranavirus detected from these samples was relatively low, my use of internal tissues provide high confidence that positive cases truly represent infection as opposed to environmental contamination. In addition, I have interpreted single positive results with caution, due to the capacity for assays to produce non-specific amplification. However, the qPCR assay I utilised has previously been found to be highly specific and sensitive, more so than the nested PCR also used here for sequencing (Chapter 4). The combination of this high specificity and my results, which showed successful sequencing of several qPCR single positives, are indicative that at least some of these samples are more likely to represent true positives than non-specific PCR amplification. Therefore, ranavirus may have been more widespread than I have found here.

The range of species, geographic locations and years from which ranavirus has now been detected suggest it to have been widespread in Costa Rica for at least four decades (see also Chapter 3). Although sampling efforts were uneven among sites and years, ranavirus was detected from each year where sample sizes were relatively high. However, due to the dilution of sample sizes when divided among areas, I have chosen not to make any inference about time-period or geographic prevalence estimates. Therefore, I have been unable to identify any distinct introductory period for this virus.

I detected ranavirus in herpetofaunal populations at seven sites, six of which are protected and five that underwent known declines after or during the dates infections were detected (Pounds et al. 1999, Puschendorf et al. 2006, Whitfield et al. 2007). It is often thought that the introduction and spread of chytrid drove amphibian declines almost immediately after the pathogen's arrival in Costa Rica (Lips et al., 2006; Cheng et al., 2011; but see Phillips and Puschendorf, 2013), but ranavirus seems to have been present within Costa Rica for around a decade prior to declines. One of two hypotheses may explain my results: 1. Ranavirus is endemic to Costa Rica and amphibians have been co-existing with infections for thousands or millions of years, as has been suggested for a North American ranavirus in ambystomatid amphibians (Storfer et al. 2007). This hypothesis is based on the endemic pathogen hypothesis that has previously been discussed for multiple pathogens (Rachowicz et al., 2005). It also provides support to the hypothesis that ranaviruses are ubiquitous (Warne et al., 2016). 2. Ranavirus was introduced to Costa Rica more recently, but still a decade prior to amphibian declines. With this hypothesis being based on the novel pathogen

hypothesis (Rachowicz et al., 2005); amphibians may have undergone less noticeable declines or die-offs soon after the arrival of ranavirus, with a lack of detectability for diseased individuals exacerbated if disease predominantly affects larval life stages. Neither hypothesis rules out the involvement of ranavirus in declines from the late 1980s, with emergence of even endemic pathogens a known phenomenon (Warner 1968, Patz et al. 1996, Garamszegi 2011, Kock et al. 2018).

Environmental factors, such as the abnormal climate linked to Costa Rican declines (Pounds et al. 2006), could have driven emergence of ranavirus alongside chytrid. This hypothesised mechanism is especially pertinent, given that recent laboratory and *in situ* investigations have linked (FV3) ranavirus outbreaks to warming conditions, predicting climate change to drive ranavirus outbreaks (Price et al. 2018; Chapter 3). Similarly, although current patterns of amphibian population recoveries are positive (Nishida 2006, Abarca et al. 2010, González-Maya et al. 2013, Chaves et al. 2014), they may not indicate the threat of re-emergence to be overcome. Current climate predictions for Costa Rica align with those predicted to drive ranavirus outbreaks (Cai et al. 2014; Price et al. 2018; Chapter 3). Although I have been unable to determine the role ranaviruses played during population declines in this study, my study has paved the way for such questions to be investigated further. The volume of detection-based evidence for chytrid-driven declines is far higher than that for declines driven by other factors. However, this is potentially not a matter of which pathogen has single-handedly driven declines, as my study adds to the evidence that more than one pathogen was present in the system (Green & Sherman 2001, Merino et

al. 2005), during abnormal climatic events whilst herpetofauna were declining.

Chapter 6 Ranavirus detection in endemic and threatened amphibian populations of the Australian Wet Tropics

Abstract

The amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) has driven severe amphibian declines in the Australian tropics. These declines have resulted in species extirpations and extinctions, with many surviving in small, highly threatened populations. Despite the fragility of remaining populations, another group of lethal pathogens, ranaviruses, have rarely been investigated among native amphibians. Ranaviruses have previously been associated with fish, reptile and amphibian mortality events in Australia, yet remain poorly understood in this country, especially among amphibian hosts. Here, I used quantitative polymerase chain reaction assays to detect ranavirus presence from eight of 17 tested areas containing populations of endangered and critically endangered frog species. Infections, although present in these populations, seem to be at the lower bounds of detectability of the assay, which makes firm diagnosis at the individual level unreliable. Repeated detections of this pathogen however, are highly indicative of its presence at each area where it was detected. Therefore these populations are likely often exposed to ranavirus. The results of this study are not characteristic of populations experiencing rapid disease associated die-offs or declines, but further investigations should be undertaken to examine the potential drivers of these pathogens to predict future emergences and potential threats to endangered Australian amphibians.

Introduction

In Australia, enigmatic amphibian declines mirrored those from the Neotropics. Strikingly, despite habitat-related threats being present for hundreds of years, no amphibian extinctions were recorded in Australia until after the late 1970s (Hero et al. 2006). Spatially, Australian declines appeared particularly severe from the tropical rainforests of northern and eastern Queensland (Richards et al. 1994, Laurance et al. 1996, Schloegel et al. 2006). At the time these declines were hypothesized to be due to disease and eventually research investigating Australian and Central American declines led to the discovery of chytrid in the late 1990s (Laurance 1996, Berger et al. 1998). Chytrid is thought to have driven 43 Australian amphibian species to become extinct or vulnerable to extinction (Richards et al. 1994, Berger et al. 1998, IUCN 2017, Scheele et al. 2017). However, not all enigmatic Australian amphibian declines were attributed to chytrid (Burgin et al. 2005), and although several chytrid-affected populations appear to be recovering (Richards & Alford 2005, Puschendorf et al. 2011, Scheele et al. 2017), ranaviruses have also been detected in Australian amphibians.

Australian amphibian ranaviruses have received little attention in comparison to chytrid, but also when compared to ranaviruses of temporal regions. An amphibian ranavirus was first detected in Australia from native captive ornate burrowing frog (*Limnodynates ornatus*) metamorphs (Speare & Smith 1992). These frogs were collected from northern Queensland (Speare & Smith 1992), an area where extreme amphibian declines were ongoing (Richards et al. 1994, McDonald & Alford 1999). The virus was described as Bohle

iridovirus (BIV; Hyatt et al., 2000) and was once suggested to be a driving factor of enigmatic declines, as ranavirus-like signs of disease were observed in necropsied individuals from decline sites (Laurance et al. 1996). BIV has been detected from and confirmed to be lethal to other Australian amphibians (Speare & Smith 1992, Cullen et al. 1995, Cullen & Owens 2002, Weir et al. 2012), fish (Moody & Owens 1994, Ariel & Owens 1997) and reptiles (Ariel et al. 2015). Juvenile (or metamorph) life stages of all host taxa appear most susceptible to disease, with high rates of mortality rapidly occurring after infection (Moody & Owens 1994, Cullen et al. 1995, Ariel 1997, Ariel & Owens 1997, Cullen & Owens 2002, Ariel et al. 2015).

Little data exist on the geographic and species distributions of Australian ranaviruses. Although it is not known whether they are endemic, BIV and a fish ranavirus, epizootic hematopoietic necrosis virus (EHNV), were detected only from Australia until recently (Cheng et al. 2014). In 2010 a BIV-like ranavirus was isolated from a die-off of captive toads (*Anaxyrus boreas boreas*) in the USA (Whittington et al. 2010, Cheng et al. 2014). BIV is also thought to be closely related to a virus isolated from a captive gecko in Germany (Marschang et al. 2005, Hick et al. 2016), but all other reports are from Australia. In addition to the suspected native strains, a third seemingly non-native ranavirus has been detected in Australia: an FV3-like ranavirus was detected from ten confiscated moribund juvenile green tree pythons illegally imported from Papua New Guinea (Hyatt et al. 2002). The risk posed by the introduction of any non-native pathogen is potentially catastrophic (e.g. Warner, 1968), and especially so for FV3, as these ranaviruses are almost undoubtedly the driving force behind some amphibian population

declines (Teacher et al. 2010). Therefore, monitoring threatened Australian populations for emerging pathogens, especially those that have already been associated with declines, is an important part of their conservation.

In northern Queensland, where amphibian population declines occurred, reptiles have been surveyed for exposure and susceptibility to ranavirus (Ariel et al. 2015, 2017, Maclaine et al. 2018). Six species, including crocodiles, snakes and turtles, were found to be seropositive for BIV antigens (Ariel et al. 2017), indicating a widespread pattern of exposure. An apparent trend indicated frog-eating reptiles to show higher reactivity towards BIV, than those which consume homeothermic prey (Ariel 1997), inferring amphibians to be associated with pathogen transmission. Several reptile species have been infected experimentally and no histological changes were noted among infected adults, but juveniles often experienced high mortality (Ariel et al. 2015, 2017). Therefore, although it is unclear whether ranavirus poses a conservation concern in Australia, it is clear this pathogen can cause mortality among native species.

Increasingly, studies are detecting ranaviruses from populations that declined due to chytridiomycosis (Hoverman et al. 2012b; Souza et al. 2012; Whitfield et al. 2013; Warne et al. 2016; Rosa et al. 2017; Chapter 5), leading to the suggestion that ranaviruses are ubiquitous (Warne et al. 2016). Despite these new detections, the severe declines Australian amphibians have undergone, reports of ranavirus infecting native species and possible ranavirosis from within declining populations, no survey has been undertaken to investigate ranavirus among populations of threatened Australian amphibians. Therefore, I chose to follow up reports of high sero-positive

prevalence among native reptiles by investigating the hypothesis that ranavirus is similarly widespread among threatened Australian amphibian populations. Given the distribution of ranaviruses among reptile populations, I expect that amphibians that experienced declines are also exposed to this potentially lethal pathogen.

Methods

Amphibians were collected under permit by Robert Puschendorf through visual encounter surveys whilst walking transects along 17 sites comprised of streams and rivers in Queensland, Australia, between 2008 and 2013. Sites were based within three broad habitat types, which included dry sclerophyll, wet sclerophyll and rainforest. Toes were clipped from live, post-metamorphic individuals at the first interphalangeal joint of the second digit of the right hind foot using sterile scissors. Scissors were flame-disinfected and vinyl gloves changed between individuals to limit pathogen transmission. Animals were examined for external signs of disease (Bertelsen & Crawshaw 2003, OIE 2016), which were recorded if present. Animals were released at the site of capture and all equipment was disinfected upon leaving the site.

DNA was extracted at James Cook University, Australia, using Qiagen DNeasy Blood and Tissue kits following the manufacturer's protocol, and extractions were stored at -80°C until their export to the UK in 2017. Upon their arrival in Plymouth, UK, samples were tested for ranavirus in duplicate using the qPCR assay outlined in Chapter 4 and in Leung et al. (2017). Standards consisting of 300, 30,000 and 3,000,000 ranavirus copies and negative controls comprising of nuclease-free water were used. Samples

were considered positive for ranavirus if sigmoidal amplification occurred in both replicates, and where no sigmoidal amplification was detected, the sample was considered negative. Where amplification occurred in only one well, samples were conservatively considered not to represent ranavirus presence. Positive samples were run on a nested PCR (Chapter 4; Mao et al., 1997; Meng et al., 2013) with the aim of amplifying and sequencing ranavirus DNA.

Results

Overall 510 individual amphibians were sampled and tested for ranavirus from the 17 sites. A total of 369 individuals were sampled from dry sclerophyll, 20 from rainforest and 121 from wet sclerophyll habitats. Ranavirus was detected at eight (47%) of the tested sites (Table 6.1, Figure 6.1), from 14 individuals. All three habitat types sampled were represented by positive samples. The mean cycle threshold (C_t) score for positive samples was 43.4 (mean standard deviation 1.5).

Species collected comprised 407 *Litoria nannotis*, five *L. rheocola*, three *L. serrata*, two *L*. *lorica,* one *L. jungguy* and 92 unidentified *Litoria* species. These species have IUCN red list classifications ranging from Least Concern (*L. serrata*) to Near Threatened (*L. jungguy*), Endangered (*L. nannotis, L. rheocola*) and Critically Endangered (*L. lorica*), and all except *L. jungguy* have experienced population declines (McDonald & Alford 1999, IUCN 2017). The ranavirus positive samples were comprised of two highly threatened species: thirteen *L. nannotis* and one *L. lorica* samples were positive (Table 6.1).

Of the fourteen positive samples, none showed bands using the nested PCR; therefore, I was unable to collect sequence information on the type of ranavirus being detected.

Table 6.1. Habitats, sites and species from which samples positive for ranavirus were found. Site 'ID' corresponds to those shown in Figure 6.1 (B2, 3, 4). '+' is the count of ranavirus positives from the focal site. 'N' indicates the total number of individuals sampled per site. '+ species' indicates the species found to be ranavirus positive from the focal site.

Site and species	ID	Latitude	Longitude	+	N	+ species
Dry sclerophyll						
Blencoe Falls	8	-18.223992	145.54014	3	26	*L. nannotis*
Bottom McLeod	4	-16.4616	145.1214	1	24	*L. lorica*
Reedy Creek	2	-16.416886	145.161839	3	44	*L. nannotis*
Rainforest						
Bridge 10	7	-18.210696	145.807519	1	18	*L. nannotis*
Wet sclerophyll						
Daintree falls	1	-16.307858	145.164207	1	20	*L. nannotis*
Spurgeon 'Dry'	5	-16.463082	145.181943	1	104	*L. nannotis*
Spurgeon Wet	3	-16.451782	145.190801	3	46	*L. nannotis*
Upper Cow Creek	6	-16.471774	145.224951	1	19	*L. nannotis*

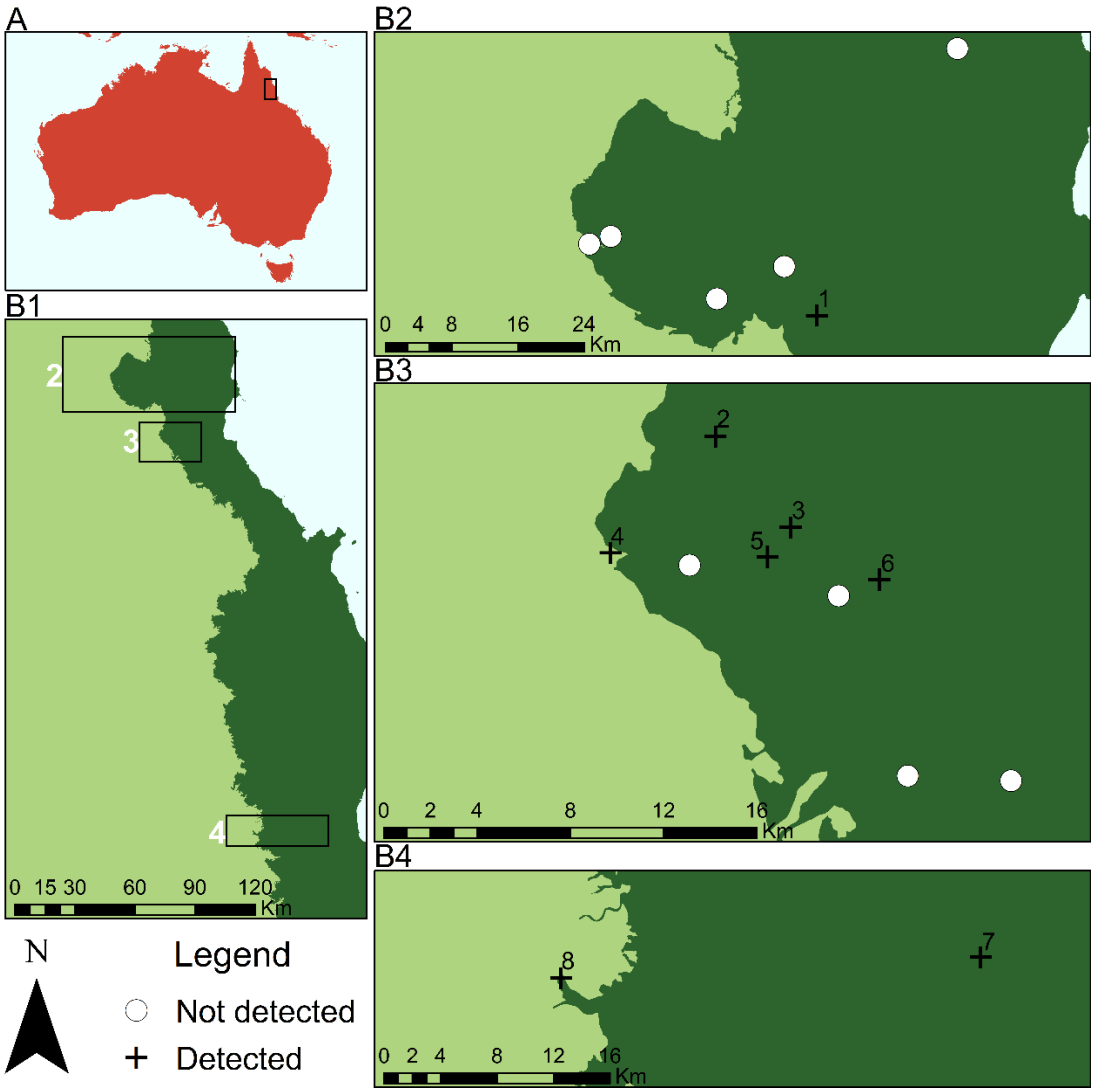


Figure 6.1. Distribution of sites and their ranavirus detection status. A) Australia with broad extent of sampling area shown. B1) Extent shown in A, showing three broad sampling areas, numbers correspond to figure B numbers of the three maps on the right-hand side. B2, B3 and B4 show the exact sites sampled and whether ranavirus was detected or not detected from the site. Details of numbered sites can be found in Table 6.1. Map was downloaded from TNCMAPS (TNCMAPS 2009) and ranavirus information was added using ArcMap 10.5.1.

Discussion

Ranavirus has the ability to cause morbidity and mortality in Australian host species (Ariel et al. 2015), yet few studies have been undertaken to investigate the distributions of these pathogens in the wild. Here, I have found ranavirus to be present in eight sites around the wet tropics region of northern Queensland, with positives detected from populations of endangered and critically endangered species. All positive samples were detected very late into the qPCR assays, suggesting low viral loads to be present in these samples. However, the results of a recent comparison of diagnostic techniques indicate this assay to have extremely high specificity and sensitivity (Chapter 4; Leung et al., 2017). This suggests that these results are an artefact of the assay's high sensitivity (Chapter 4). As false-positives were low in previous studies testing this ranavirus qPCR (Chapter 4; Leung et al., 2017), it is more likely that the virus is truly present, but in extremely low loads. Such low loads may not be expected to amplify using conventional PCR, which is up to two magnitudes less sensitive than this qPCR (Chapter 4) and they may reflect low intensity ranavirus infections in the wild. These low-intensity results may also reflect the sampling techniques used (Hyatt et al. 2007). It is likely that some positive samples from these amphibian populations have been missed due to the use of toe-clips, as ranavirus targets internal organs (Gray et al. 2012). However, it is also possible that the detection of ranavirus could stem from environmental contact with this pathogen, instead of infection. This would still indicate the individuals sampled to, at minimum, have been exposed to the pathogen and have the potential to subsequently become infected. Either way, none of the

animals sampled exhibited external signs of ranavirosis. These results are also not characteristic of individuals experiencing disease – for which ranavirus would likely be detected much earlier in the qPCR assay. Such results could indicate this pathogen to be more likely an endemic strain, as opposed to a non-native virus such as FV3, which could be expected to result in disease outbreaks similar to those in the UK and USA (Green et al. 2002, Teacher et al. 2010), although sequencing is essential to confirm this.

The results of this study combined with previous reports of high prevalences of ranavirus sero-positive species and the detection of sero-reactivity may provide support to the hypothesis that ranaviruses are widespread in Australia (Ariel et al. 2017). The results found here also provide more evidence that native species are regularly exposed to (and possibly have some resistance to) ranavirus (Ariel et al. 2017). I detected ranavirus from samples of two species which have experienced severe population declines associated with the emergence of chytridiomycosis (McDonald & Alford 1999). Yet current populations of these species, and others, appear to be recovering (Richards & Alford 2005, Puschendorf et al. 2011, Scheele et al. 2017, McKnight et al. 2017), making it unlikely that ranavirus is currently having a negative impact upon affected populations. However, it is unknown whether this pathogen could have been involved in historic population declines.

Due to the extremely late ranavirus qPCR amplifications, it is likely that some samples containing ranavirus were not detected due to low pathogen loads, which has limited the understanding of the distribution of this virus among species. Repeatedly running the assay is unlikely to resolve this issue, as

results at these lower bounds of detectability are inconsistent and usually not replicable (Miller et al. 2015). Therefore, I have not established population prevalence or attempted to understand the broader patterns of infection in this system; I was simply able to detect this virus in the system. In addition, although positive ranavirus status was based on the detection of one positive sample for five of the eight positive sites, this low number of detections per site is likely explained by the overall low sample sizes collected at all but one of these sites (see Table 6.1).

With such low intensity infections, and a current lack of information on the effects of ranaviruses among tropical amphibians, it is difficult to understand the conservation significance of these results. What is clear is that further investigations are necessary given that other ranaviruses have the ability to cause population declines, and that native Australian ranaviruses have been linked to die-off events (Speare & Smith 1992, Cullen et al. 1995, Cullen & Owens 2002, Weir et al. 2012). Useful studies may include the examination of museum records for evidence of ranavirus-related die-offs and the testing of archived internal tissues, instead of toe-clips, to clarify whether ranavirus is systemic or an environmental contaminant. This is especially important for sites where declines were not associated with chytrid (Burgin et al. 2005). Surveys of ranavirus along environmental gradients should be undertaken to investigate whether this pathogen is driven by environmental factors, and hence could pose a threat under changing environmental conditions as has been reported for other ranaviruses (Price et al. 2018). Ongoing surveillance should be conducted to determine whether the presence of this pathogen could represent a current threat towards native hosts.

Chapter 7 General Discussion

Amphibian declines from Costa Rica are infamous within the field of amphibian conservation, yet many affected populations remain poorly investigated and unmonitored. My chapters tie together to improve the understanding of host and pathogen ecologies in the tropics, and can be used to progress and inform conservation actions. The candidate species identified in Chapter 2 have previously been categorised as part of widespread species. The population sizes and geographic ranges of these new candidate species are unknown, but the division of known species into two or more candidate species indicates these traits could be comparatively vastly reduced (Nicholls & Racey 2006). Some of these candidate species have already been extirpated from parts of their range (Bolaños 2002, Puschendorf et al. 2006), thus if they represent true species, these units of cryptic diversity could be candidates for protection, if still in existence. However, if the goal of conservation is to preserve biodiversity, taxonomic inventories, as they stand, are not inclusive of all biodiversity (DeWeerdt 2002). To receive protection, it should not matter whether these individuals meet the technical definition of species or instead represent important units of biological diversity below the species level – existing as evolutionarily significant units of diversity.

One pattern of Anuran variation was consistently shown among samples from Costa Rica and two Panamanian sites. This repeated pattern among taxa could indicate a speciation mechanism, such as historic climatic changes (Smith et al. 2012), which may indicate a widespread pattern

among Central American taxa as a whole. Further investigations of cryptic diversity should be undertaken to determine whether this geographic pattern is repeated beyond amphibians.

My phylogenetic findings are supported by the generation of cladistic structuring within recently assessed species in my phylogeny that mirrors the structures found by other researchers (Wang et al. 2008, Hertz et al. 2012, Batista et al. 2014, 2016). This congruence indicates my analyses to be robust enough to indicate potential cryptic biodiversity. However, mitochondrial DNA alone should not be used to categorically delineate species. Differences in methods can result in over- and underestimation of species diversity, which can mislead interpretations (Sites & Marshall 2003). Further investigation is required to confirm and describe the six additional cryptic species identified here. Analyses should include nuclear DNA, bioacoustics and morphometric lines of evidence (Vences & Wake 2007, Padial et al. 2010). If confirmed as true species, further surveys must be undertaken to determine the ranges and population sizes of both the newly and traditionally described species.

Chapter 3 was conceptualised because the drivers of ranavirus had not been examined from tropical systems before. In addition, despite experiencing extreme amphibian declines, Costa Rican amphibian populations have often lacked scientific investigation. The ACG was chosen as my focal sampling area as it contains populations that are anecdotally considered to have been reduced due to chytrid (Puschendorf et al. 2006). As with knowledge of diversity among amphibian species in the ACG, very little is known about their pathogens. I detected chytrid from the wet forests of the ACG,

confirming this pathogen to be present. This presence provides evidence that chytrid could have been in the ACG when amphibians declined, hence provides some support to the hypothesis that amphibians from this protected area declined due to chytridiomycosis. I was also able to confirm the presence of an FV3-like ranavirus within all sampled populations. Similarly, Whitfield et al. (2012) found FV3-like ranaviruses to be infecting amphibians in La Selva Biological Station, north-east Costa Rica. However, whilst two previous surveys for ranavirus have been focused in La Selva Biological Station (Whitfield et al. 2012, 2013), I investigated this pathogen throughout central and northern Costa Rica and consistently found it to have a presence in samples collected between 2014 and 2017 and 1976 and 1987. However, I was unable to provide any evidence to support previous findings that infections with chytrid and ranavirus are significantly associated (Whitfield et al. 2013), as I found only nine coinfections.

Furthermore, although ranavirus has been detected from Central American amphibians (Whitfield et al. 2012, 2013, Stark et al. 2014), reptiles of this region had never been tested before. In Chapters 3 and 5, I have shown this class to be susceptible to infection in Central America, as in other regions (Duffus et al. 2015). However, systematic surveys focused on reptile species should be undertaken to extend the known host range of ranavirus. As this pathogen also has the capacity to infect fish (Duffus et al. 2015), future work could also focus on testing this taxon.

The samples used in Chapter 3 were tested for ranavirus prior to the development of the highly sensitive qPCR (by Leung et al. 2017) that was used in subsequent analyses. Thus, ranavirus was detected using a nested

PCR and sequencing. This method was demonstrated in the following chapter to be less sensitive than the newly developed qPCR. Therefore, some infections at the lower bounds of detectability were likely missed. Samples for this chapter still would have been tested using the nested PCR, regardless of whether the qPCR had been available. This is because sequencing is recommended in confirming ranavirus from areas it has not previously been tested from (OIE 2017). With the addition of this qPCR future surveys may expect prevalence estimates to be slightly higher than those found here. However, due to this increase in sensitivity, the likelihood of detecting ranavirus from individuals that have virions on their skin, but are not infected, would increase (Smith 2007). This is likely still useful information, as an increase in detection (hence amphibian exposure or infection) may be used to reflect differences in the prevalence of ranavirus among sampled environments, at a high resolution (Kriger et al. 2007). Due to my use of toe-clips, the detection of ranavirus in Chapter 3 may not always reflect infection. However, my use of visceral tissues, which had not been tested from Costa Rican hosts before, indicated the capacity for these hosts to become infected. Additionally, these infections do not necessarily reflect the presence of disease. But they do explicitly show Costa Rican hosts to have the capacity to be infected with a potentially lethal virus (e.g. Stark et al. 2014).

In 2015 the strongest El Niño event since 1997 occurred (McPhaden 2015). El Niño events are normal events driven by natural oceanic variations, however their intensity and frequency has been linked to climate change (Cai et al. 2014). In the ACG, the effects of the 2015 El Niño events included a

reduction in precipitation, with Santa Rosa receiving 236mm of rain – the lowest recorded volume since 1979 (range, exc. 2015: 880-3038mm). This lack of precipitation was reflected by the drying up of waterbodies, such as the Rio Calera, which is close to Santa Rosa (details found in Chapter 3). These areas are important for amphibian conservation, as they are thought to have provided species refugia from chytridiomycosis (Puschendorf et al. 2005, 2009, Zumbado-Ulate et al. 2014). Species such as the Critically Endangered *Craugastor ranoides*, which was once widespread between Nicaragua and Panama, now exists in one relictual population, recorded from six river localities between sectors Santa Rosa and Murcielago of the ACG (Puschendorf et al. 2005, Puschendorf et al. in review). Individuals of this population were recorded from the Rio Calera in 2014 (Puschendorf et al. in review), but it is unknown how they have been affected by the drying of this river.

Using Costa Rican samples, I found ranavirus detection prevalence to increase with temperature. This finding aligns with previous laboratory studies, which have indicated FV3 ranaviruses to replicate at optimal temperatures within the upper range of those found from my study systems (Chinchar 2002, Echaubard et al. 2014). These findings will facilitate future studies in identifying unexamined areas in which ranavirus is most likely to be found. Importantly, this association with higher temperatures could lead to a steady increase in prevalence as Costa Rica warms, due to the effects of climate change, and sudden peaks in prevalence as short-term El Niño events increase in intensity (Cai et al. 2014). The combination of disease and climate change has previously been shown to be catastrophic for amphibians

(Pounds et al. 2006). As Costa Rica warms (IPCC 2001), outbreaks of ranavirus could occur within the same species that have already been pushed to the edge of extinction by chytrid (e.g. Solís et al. 2008). I have shown ranavirus to currently be present in the Critically Endangered *C. ranoides*, in habitats that are predicted to become warmer and drier due to the effects of climate change (IPCC 2001). Thus the link between ranavirus and temperature should be of high conservation concern for the persistence of species such as *C. ranoides*. Additionally, I have shown that ranavirus was present among ACG populations of *C. ranoides* that have since been extirpated. Ranaviruses have been shown to cause severe amphibian declines in other study systems (Teacher et al. 2010, Price et al. 2014, Rosa et al. 2017) and this finding raises the possibility that ranavirus disease played a role in Costa Rican amphibian declines. Together, these findings merit urgent monitoring of this species. Initially, surveys should be undertaken to establish the continued presence of one population at the Rio Calera, but continued monitoring should be used to determine any population-level effects ranavirus and climate change could be having.

One die-off has already been attributed to ranavirus in Central America (Stark et al. 2014), and another, unexamined event, occurred in the ACG in 2017. This latter die-off was unable to be investigated as no carcasses were collected, and when staff visited the location the following day, all carcasses had disappeared. It is unknown whether local ranavirus associated die-offs are part of cyclical endemic disease dynamics or are abnormal and possibly impacting populations. Although disease was not observed among live sampled amphibians here, this does not exclude the possibility that it is

common among populations. For example, some populations thought to have been driven to extinction by chytrid were never observed with signs of disease (e.g. Savage et al. 2008). In Chapter 5, I detected some of the earliest investigated ranavirus infections from wild individuals and provided the first evidence of this pathogen's presence during Costa Rican amphibian declines. Thus, its relatively long presence in Costa Rica may indicate ranavirus not to be of immediate conservation concern, but without further evidence, this conclusion cannot be assumed. Even after relatively long periods of presence pathogens are capable of still reducing host populations (Benning et al. 2002, Pilliod et al. 2010, Vredenburg et al. 2018) and persisting at relatively high virulence (Lenski & May 1994). Of the 16 species in which ranavirus was detected, 14 have been assessed through the IUCN Red List and half are threatened with extinction or known to have undergone declines (IUCN 2017). This figure includes two species that are thought to be extinct (Bolaños et al. 2008, Pounds, Bolaños, & Chaves 2008). Addistionally, the samples in Chapter 5 were collected for taxonomic research, thus the sampling pool is likely biased towards individuals that appeared physically healthy and towards species of taxonomic interest at the time. Thus, the number of ranavirus detections from this study likely underrepresent the true number of infections.

Another important implication of this thesis is that I have demonstrated the importance of testing and comparing an assay prior to its use in a project. Comparison of the diagnostic methods used in ranavirus detection was not initially a primary aim of this thesis. However, during my work for Chapter 3 the difficulties in detecting ranaviruses when infection burden was low were

highlighted. In Chapter 4 I identified general issues with the use of popular molecular techniques and this study can be used as the template for assessment of assays prior to use. Here, two assays commonly used in the published literature (Black et al. 2017) were found to be relatively less sensitive than a newly developed assay. Consequently, low burdens of ranavirus might have been frequently missed during surveys by other researchers utilising these methods, thus having implications upon the known global distribution of ranaviruses. The biological relevance of low pathogen loads and the usefulness of highly sensitive assays has previously been questioned (Smith 2007). But, although some detections may reflect exposure as opposed to infection, this information is still highly valuable (Kriger et al. 2007). For instance, in Chapter 6, I interpreted consistent detections of ranavirus at low loads with caution. Nevertheless, even cautious interpretation of these results indicates threatened species to be exposed to a potentially lethal pathogen. Had less sensitive methods been used, ranavirus may not have been detected at all, leaving researchers unaware of its presence amongst the focal populations. The consequences of this could be catastrophic for conservation programmes, which may unwittingly move unknown pathogens into immunologically naïve populations (Walker et al. 2008).

My consistent detection of ranavirus amongst populations that had faced extreme declines led me to question whether this pathogen could also be present amongst other populations that declined due to chytrid. The species within the focal populations of Chapter 6 have already been driven towards extinction by an emerging infectious disease (Berger et al. 1998, McDonald

& Alford 1999). These species are the focus of current conservation actions, including translocations and any future movements between populations should be fully assessed (Sainsbury & Vaughan-Higgins 2012). Similarly, translocations have been proposed for one critically endangered species from the ACG (Puschendorf et al. in review). The combination of Chapters 3, 5 and 6 highlight the presence of a potentially lethal pathogen in areas containing species of high conservation importance and provide information to assist in the creation of disease risk assessments (Sainsbury & Vaughan-Higgins 2012). Additionally, the results of Chapter 6 indicate ranavirus to be widespread among the focal Australian amphibian populations, which supports the hypothesis that native hosts are frequently exposed to ranavirus (Ariel et al. 2017). These results also provide some evidence towards the hypothesis that ranavirus is ubiquitous (Warne et al. 2016) and during my study, I have detected it from every area tested.

In Australia, research to determine the effect of ranavirus on endangered species should make up an important part of their monitoring for conservation. The capacity of ranavirus-induced mortality could be examined by the capture or tracking of infected individuals, to determine disease progression, followed by necropsy of deceased individuals. Ranavirus strain should also be determined, and monitored for the introduction of non-native strains. As a priority in Costa Rica, further testing of cryptic diversity is required to identify species with factors such as low range sizes, which can make them more susceptible to extinction driven by pathogen outbreaks (Earl & Gray 2014). This should be accompanied by population monitoring

with regular testing of ranavirus to determine whether this pathogen is associated with die-off or decline events.

Appendices

Chapter 2: Supplementary materials

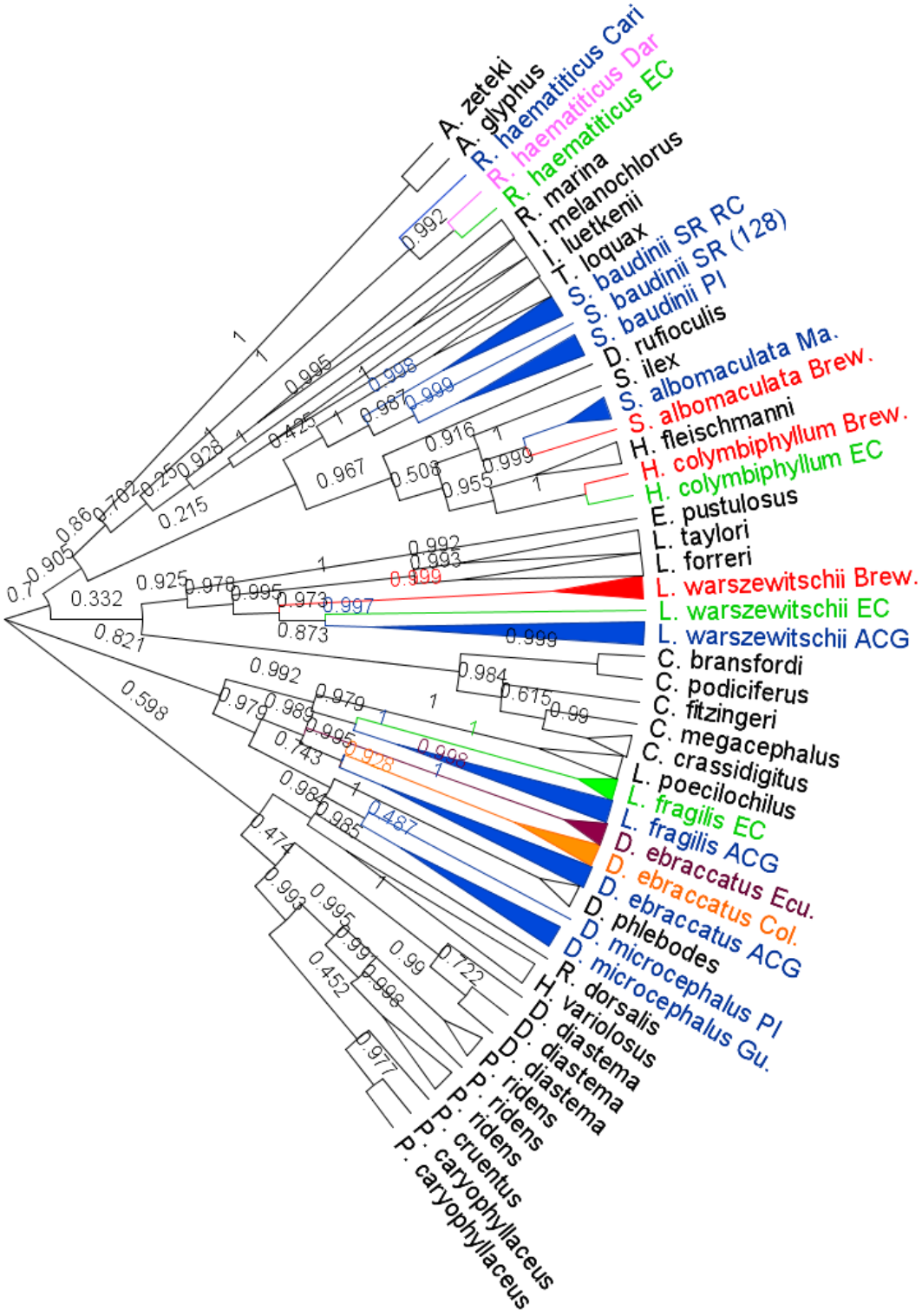


Figure S 2.1. Phylogeny of Costa Rican amphibians generated from 16s rRNA gene sequences.

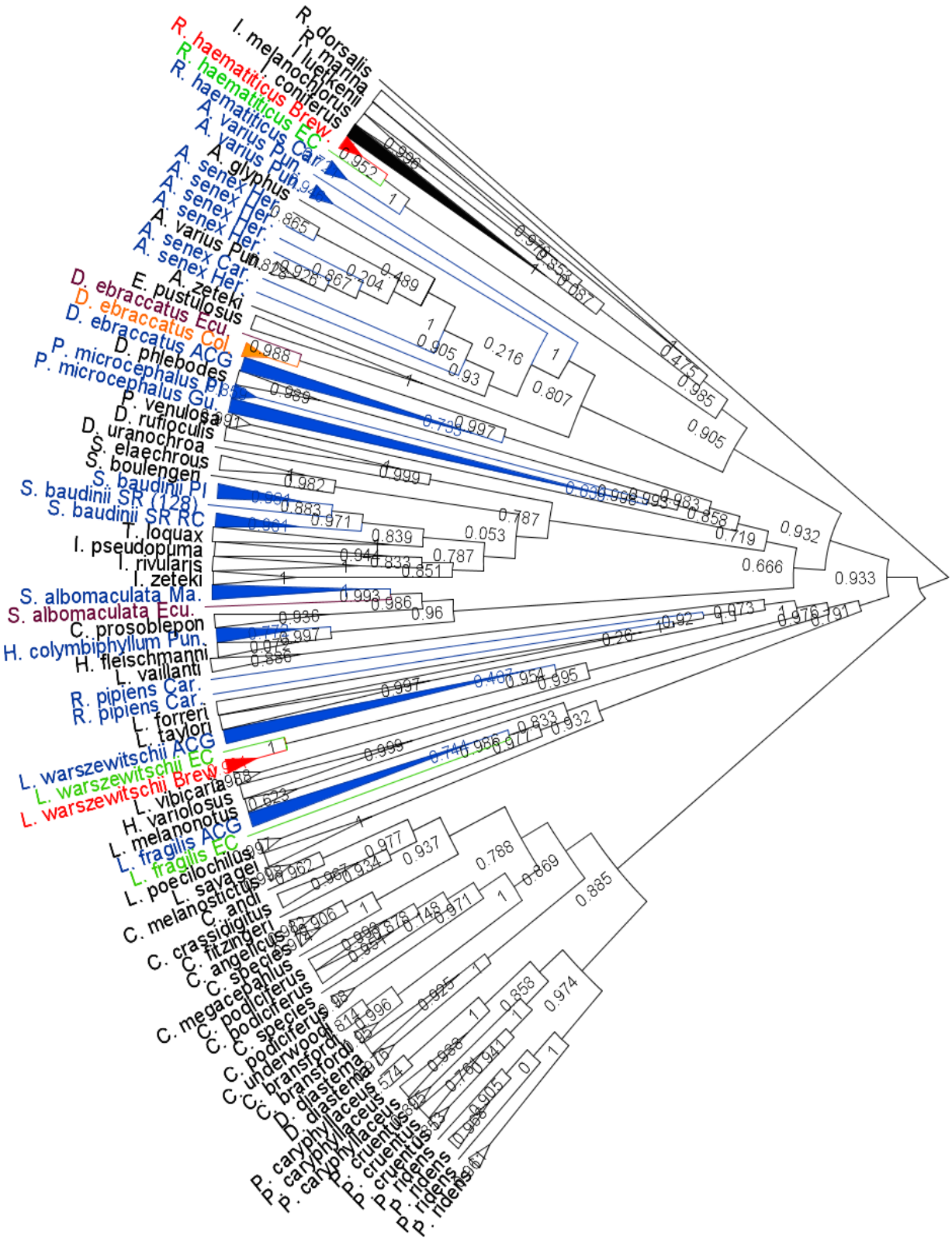


Figure S 2.2. Phylogeny of Costa Rican amphibians generated using cytochrome oxidase subunit 1 (CO1) sequences.

Table S 2.1. Sequences downloaded from GenBank that were used to generate phylogenies.

Species	Voucher number	Collection locality	Latitude	Longitude	Genbank 16S	Genbank CO1
Atelopus glyphus	AJC 1855	Panama	7.762 N	77.724 W	KR863128	KR863128
Atelopus varius	MVZ 223279	Costa Rica	8.938931 N	-82.834387	AY325996	
Atelopus varius	MVZ AG29	Costa Rica	8.95108 N	-82.84056	U52779	
Atelopus zeteki	UMFS 11492	Panama	8'39.99'N	80'0.249'W	DQ283252	DQ283252
Bufo coniferus	TOE_151	Panama	8.667 N	80.592 W	FJ784601	
Centrolene prosoblepon	SIUC H-7053	Panama			AY843574	
Cochranella albomaculata	USNM 534151	Honduras	15°19′10″ N	85°17′30″ W	EU663003	
Craugastor bransfordii	AJC 1921	Panama	9.32N	79.289W	KR863145	KR862890
Craugastor crassidigitus	AJC 1785	Panama	9.217 N	79.422 W	KR863153	KR862898
Craugastor crassidigitus	KRL 0665	Panama	8.667 N	80.592 W	FJ784328	FJ766642
Craugastor crassidigitus	MVZ:207248	Costa Rica	10.3 N	-84.81667	EU186715	
Craugastor megacephalus	KRL 0711	Panama	8.667 N	80.592 W	KR863195	KR862940
Craugastor podiciferus	UCR<CRI>:22201	Costa Rica	9.713 N	83.949 W	KT950299	
Craugastor underwoodi	UCR<CRI>:20203	Costa Rica	9.813 N	83.168 W	KT950298	
Dendropsophus ebraccatus	AJC 3502	Colombia	7.0759	73.5481	KP149426	KP149221
Dendropsophus ebraccatus	AJC 3504	Colombia	7.0759	73.5481	KP149355	KP149156
Dendropsophus ebraccatus	QCAZA40847	Ecuador			KY406451	KY406676
Dendropsophus ebraccatus	26297	Panama	9°89 N	79°46	AF308100	
Dendropsophus ebraccatus	RdS 790	Belize			AY843624	
Dendropsophus microcephalus	UTA A-50632	Honduras			AY843643	
Diasporus diastema	AB637	Panama			KT186624	KT186572
Diasporus diastema	AB675	Panama			KT186617	KT186563
Diasporus diastema	CH 6802	Panama	9.231 N	79.419 W	KR863215	KR862960

Duellmanohyla rufioculis	MVZ207197	Costa Rica	10.9 N	-85.5	DQ388749	
Duellmanohyla uranochroa	MVZ 203908	Costa Rica	9.614 N	-83.78616	DQ388750	
Engystomops pustulosus	LW96	Costa Rica			DQ337244	
Engystomops pustulosus	AJC 3875	Colombia	7.3962 N	73.4955 W	KP149340	KP149143
Hyalinobatrachium fleischmanni	AJC 1792	Panama	9.168 N	79.414 W	KR863260	KR863005
Hyalinobatrachium fleischmanni	KRL 1316	Panama	8.667 N	80.592 W	KR863263	KR863008
Hyalinobatrachium fleischmanni	CH 6649	Panama	9.168 N	79.414 W	KR863267	KR863012
Hyla loquax	UTA-A 55119	Guatemala			DQ055822	
Hyalinobatrachium colymbiphyllum	317323	Panama	9.231 N	79.403 W	KF604302	KF604297
Hyalinobatrachium colymbiphyllum	KRL 1556	Panama	8.667 N	80.592 W	FJ784561	FJ766711
Hypopachus variolosus	JHM 666	Costa Rica			KC180061	
Hypopachus variolosus	UTA A-50968	Guatemala			KC179978	
Hypopachus variolosus	UTA:A-51790	Nicaragua			JF836986	
Hypopachus variolosus	UCR 17777	Costa Rica			JF836987	
Hypopachus variolosus	KU:291270	El Salvador			JF836985	KU985750
Incilius coniferus	SIUC 6913	Panama			DQ283166	
Incilius melanochlorus	MVZ229635	Costa Rica	10.43333 N	-84	AY680255	JN867981
Isthmohyla rivularis	MVZ 149750	Costa Rica			AY843659	
Leptodactylus fragilis	KRL 0869	Panama	8.667 N	80.592 W	FJ784416	
Leptodactylus melanonotus	USNM 535964	Belize			AY943237	
Leptodactylus pentadactylus	USNM 347153	Panama			AY943238	
Leptodactylus poecilochilus	KRL 0118	Panama	8.667 N	80.592 W	FJ784321	FJ784416
Leptodactylus poecilochilus	KR 0118	Panama	8.67 N	80.59 W		FJ766747
Leptodactylus savagei	USNM 298079	Panama			AY947866	

Lithobates taylori	TCWC 55963	Nicaragua			AY779244	
Lithobates vaillanti	KU 195299	Mexico			AY779214	
Lithobates warszewitschii	KRL 1567	Panama	8.667 N	80.592 W	KR911918	KR911915
Lithobates warszewitschii	CH 6659	Panama	9.231 N	79.403 W	KR863284	KR863028
Lithobates warszewitschii	AJC 1798	Panama	9.32 N	79.289 W	KR863282	KR863026
Phyrnohyas venulosa	AMNH-A 1411427	Guyana			AY549362	
Phyrnohyas venulosa	KU 217753	Ecuador			AY819514	
Pristimantis caryophyllaceus	KRL 0898	Panama	8.667 N	80.592 W	FJ784421	FJ766770
Pristimantis caryophyllaceus	AJC 1936	Panama	9.32 N	79.289 W	KR863301	KR863044
Pristimantis cruentus	AJC 1767	Panama	9.231 N	79.403 W	KR863316	KR863061
Pristimantis ridens	AMNH A124551	Panama			EF493355	
Pristimantis ridens	AJC 0126	Costa Rica			JN991466	JN991400
Pristimantis ridens	ENS 10722	Honduras			JN991464	JN991398
Pristimantis ridens	KRL 0846	Panama	8.667 N	80.592 W	FJ784399	FJ766805
Pristimantis ridens	AJC 1766	Panama	9.231 N	79.403 W	KR863318	KR863061
Lithobates warszewitschii	JSF 1127	Panama			AY779209	
Lithobates warszewitschii	AJC 1985	Panama	9.265 N	79.508 W	KR863281	KR863025
Rhaebo haematiticus	TOE_120	Panama	8.667 N	80.592 W	FJ784593	
Rhaebo haematiticus	KRL 0965	Panama	8.667 N	80.592 W	FJ784452	FJ766815
Rhaebo haematiticus	AJC 1847	Panama	7.756 N	77.684 W	KR863342	KR863085
Rhinella marina	AJC 3877	Colombia	7.3962 N	73.4955 W	KP149357	KP149158
Rhinella marina	AJC 3852	Colombia	5.903 N	73.695 W	KP149422	KP149217
Sachatamia albomaculata	QCAZ:40815	Ecuador			KY611466	
Sachatamia albomaculata	AJC 1755	Panama	9.231 N	79.403 W	KR863349	KR863092
Sachatamia ilex	AJC 1956	Panama	9.32 N	79.289 W	KR863358	KR863101
Smilisca baudinii	JJW871	Mexico			DQ388762	
Smilisca baudinii	MZFC 15828	Mexico	16° 29.76' N	94° 27.93'W	DQ830819	

Chapter 3*:* Supplementary materials

Table S 3.1. List of species infected with chytrid and ranavirus and the sites individuals were collected at. Cac = Cacao, Car = Caribe, Ma = Maritza, Mu = Murcielago, Pi = Pitilla, RC = Rio Calera, SR = Santa Rosa.

Species	Sites	*+Bd*		*+Rv*	
		+	**n**	**+**	**n**
Bufonidae					
Incilius luetkenii	Mu,RC,SR	0	124	21	103
Incilius coccifer	SR	-	-	0	2
Incilius coniferus	Pi	-	-	0	1
Incilius melanchlorus	Ma, Car	1	4	0	7
Incilius valliceps	Car	0	2	0	2
Rhaebo haematiticus	Car	0	3	0	8
Rhinella marina	Car,Ma,Mu,Pi,RC,SR	2	120	8	112
Total		**3**	**253**	**29**	**236**
Centrolenidae					
Sachatamia albomaculata	Ma	1	6	2	6
Colubridae					
Elaphe triaspis	SR	-	-	0	1
Oxybelis aeneus	SR	-	-	0	1
Tantilla armilata	SR	-	-	0	2
Trimorphodon biscutatus	SR	-	-	0	3
Sibon anthracops	SR	-	-	0	1
Leptodeira nigrofasciata	SR	-	-	0	1
Total		**-**	**-**	**0**	**9**
Corytophanidae					
Corytophanes cristatus	Ma,Pi	-	-	0	5
Total		-	**-**	**0**	**5**

Craugastoridae					
Craugastor fitzingeri	Car,Pi,RC,Ma	0	5	1	4
Craugastor podiciferus	Cac	0	1	0	1
Craugastor ranoides	Mu	0	19	1	8
Pristimantis ridens	Cac,Ma	0	4	0	4
Total		**0**	**29**	**2**	**58**
Dactyloidae					
Anolis oxylophus	Cac	-	-	0	1
Dactyloa insignis	Cac	-	-	0	1
Total		**-**	**-**	**0**	**3**
Gekkonidae					
Coleonyx mitratus	SR	-	-	0	1
Hemidactylus frenatus	SR	-	-	0	2
Total		**-**	**-**	**0**	**3**
Geomydidae					
Rhinoclemmys pulcherrima	SR	-	-	1	0
Hylidae					
Dendropsophus ebraccatus	Ca,Pi	8	30	5	39
Dendropsophus microcephalus	Ca,Pi	16	28	2	46
Dendropsophus phlebodes	Ca,Pi	21	26	4	45
Duelmanohyla rufioculis	Cac	12	7	2	25
Trachycephalus typhonius	SR	0	10	0	13
Scinax elaeochrous	Car	0	15	1	20
Scinax staufferi	Ma,SR	0	3	0	3
Smilisca baudinii	Pi,RC,SR	0	11	0	14
Tlacohyla loquax	Car,Pi	36	17	3	58
Smilisca sordida	Pi	-	-	0	11
Total		**93**	**147**	**17**	**274**

Iguanidae					
Ctenosaura similis	SR	-	-	0	8
Kinosternidae		**-**	**-**	**0**	**2**
Kinosternon leucostomum	SR	-	-	0	2
Leptodactylidae					
Leptodactylus fragilis	Pi,RC	4	11	3	14
Leptodactylus melanonotus	Mu	0	1	0	4
Leptodactylus savagei	Car,Pi	1	5	0	7
Leptodactylus poecilochilus	Car,Pi	0	23	2	22
Leptodactylus sp	Car	0	1	0	1
Physalaemus pustulosus	Mu,RC,SR	0	10	3	28
Total		**5**	**51**	**8**	**76**
Microhylidae					
Hypopachus variolosus	Pi,SR	0	33	11	24
Phyllodactylidae					
Phyllodactylus tuberculosus	SR	-	-	0	1
Thecadactylus rapicauda	Ma	-	-	0	1
Total		**-**	**-**	**0**	**2**
Polychrotidae					
Norops capito	Cac	-	-	0	6
Norops cupreus	SR	-	-	0	6
Norops sp	SR	-	-	0	4
Norops biporcatus	Ma,Pi	-	-	0	2
Norops humilis	Cac,Ma	-	-	0	4
Norops limifrons	Pi	-	-	0	2
Norops oxylophus	Cac,Ma,Pi	-	-	0	10
Norops polylepis	Ma,Pi	-	-	0	2

Total		**-**	**-**	**0**	**36**
Ranidae					
Lithobates forreri	Car,Ma,RC,SR	1	15	3	15
Lithobates taylori	Car,Pi	3	8	4	11
Lithobates warszewitschii	Cac,Car,Ma,Pi	7	26	2	45
Lithobates vaillanti	Pi	-	-	0	8
Total		**11**	**49**	**9**	**79**
Rhinophrynidae					
Rhinophrynus dorsalis	SR	0	2	1	1
Scincidae					
Mabuya unimarginata	Cac	-	-	0	1
Scincella cherriei	Cac	-	-	0	1
Total		-	**-**	**0**	**2**
Sphaerodactylidae					
Gonatodes albogularis	SR	-	-	0	3
Viperidae					
Bothrops asper	Car	-	-	0	1
Porthidium ophrynomegas	Mu,SR	-	-	2	7
Total		**-**	**-**	**2**	**8**
Unidentified amphibian	Car	0	4	0	4
Unidentified reptile	SR	**-**	-	0	2


A
B
C
D
E
F

◄Figure S 3.1. Frog mortality event at Sendero Los Patos, Parque Nacional Santa Rosa. A) *Incilius* species, B) *Rhinophrynus dorsalis* individual 1, C) *Rhinophrynus dorsalis* individual 2, D) *Rhinophrynus doralis* individual 3. E & F) surrounding location dead frogs found at. All photos taken by Melissa Espinoza.

Figure S 3.2. *Leptodeira annulata* consuming road-killed anoline lizard in Sector Santa Rosa (10.8509°N 85.6093°W), Área de Conservación Guanacaste, Costa Rica.

Chapter 4: Supplementary materials

Table S 4.1. Ranavirus isolates with published whole genome sequences that were used for estimating Mao and Meng amplicon phylogenies. Full isolate name, abbreviated names or acronyms used, GenBank accession numbers are included.

Name	Abbreviation	NCBI Genbank accession	Citation
Short-finned eel ranavirus	SERV	KX353311	(Subramaniam et al. 2016)
European sheatfish virus	ESV	JQ724856	Mavian et al. 2012a
Epizootic haemotopoietic necrosis virus	EHNV	FJ433873	(Jancovich et al. 2010)
Ambystoma tigrinum virus	ATV	AY150217	(Jancovich et al. 2003)
Ranavirus maximus isolate SMA15001	Rmaximus	KX574343	(Ariel et al. 2016)
Testudo hermanni ranavirus isolate CH8/96	THRV	KP266741	(Stöhr et al. 2015)
Andrias davidianus ranavirus isolate 1201	ADRV1201	KC865735	(Chen et al. 2013)
Andrias davidianus ranavirus isolate 2010SX	ADRV2010	KF033124	(Jiang et al. 2011)
Chinese giant salamander iridovirus isolate CGSIV-HN1104	CGSIV	KF512820	(Li et al. 2014)
Common midwife toad ranavirus isolate Mesotriton	CMTV	JQ231222	Mavian et al. 2012b

alpestris/2008/E			
Common midwife toad ranavirus isolate Pelophylax kl. Esculentus/2013/NL	CMTV_NL	KP056312	(van Beurden et al. 2014)
Pike perch iridovirus isolate SLU14001	PPIV	KX574341	(Holopainen et al. 2016)
Tiger frog virus	TFV	AF389451	(He et al. 2002)
Tortoise ranavirus isolate 1 (882/96)	ToRV1	KP266743	(Stöhr et al. 2015)
Soft-shelled turtle iridovirus	STIV	EU627010	(Huang et al. 2009)
Rana grylio iridovirus	RGV	JQ654586	(Lei et al. 2012)
Frog virus 3 isolate SSME	SSME	KJ175144	(Morrison et al. 2014)
Frog virus 3	FV3	AY548484	(Tan et al. 2004)
German gecko ranavirus isolate 2000/99	GGRV	KP266742	(Stöhr et al. 2015)
Bohle iridovirus isolate BIV-ME 93/35	BIV	KX185156	(Hick et al. 2016)

Table S 4.2. Costa Rican amphibian and reptile species and sample numbers used for comparing assay performance.

Species	**Total**
Ctenosaura similis	1
Craugastor ranoides	1
Dendropsophus ebraccatus	3
Dendropsophus phlebodes	8
Duelmanohyla rufioculis	4
Elaphe triaspis	1
Hypopachus variolosus	8
Incilius luetkenii	27
Leptodactylus fragilis	3
Leptodactylus poecilochilus	2
Lithobates forreri	4
Lithobates taylori	1
Lithobates warszewitschii	4
Rhinella marina	10
Rhinophrynus dorsalis	1
Sachatamia albomaculata	1
Tlacohyla loquax	1
Total	80

Chapter 5: Supplementary materials

Table S 5.1. Identification (ID) of samples tested for ranavirus from the Museum of Vertebrate Zoology.

Museum ID	Species
MVZ:Herp:179536	*Gymnopis multiplicata*
MVZ:Herp:149736	*Atelopus senex*
MVZ:Herp:149739	*Atelopus senex*
MVZ:Herp:164818	*Atelopus varius*
MVZ:Herp:149864	*Anolis intermedius*
MVZ:Herp:175967	*Rana pipiens*
MVZ:Herp:203819	*Eleutherodactylus caryophyllaceus*
MVZ:Herp:203990	*Anolis altae*
MVZ:Herp:203974	*Anolis humilis*
MVZ:Herp:207330	*Rana warszewitschii*
MVZ:Herp:207198	*Duellmanohyla rufioculis*
MVZ:Herp:207255	*Eleutherodactylus andi*
MVZ:Herp:207304	*Physalaemus pustulosus*
MVZ:Herp:207320	*Rana pipiens*
MVZ:Herp:207283	*Eleutherodactylus rugulosa*
MVZ:Herp:207324	*Rana pipiens*
MVZ:Herp:207325	*Rana pipiens*
MVZ:Herp:207327	*Rana pipiens*
MVZ:Herp:207211	*Isthmohyla tica*
MVZ:Herp:207285	*Eleutherodactylus rugulosa*
MVZ:Herp:207260	*Eleutherodactylus melanostictus*
MVZ:Herp:207294	*Leptodactylus melanonotus*

Table S 5.2. Samples in which amplification occurred in a single well from qPCR. Any sample which subsequently amplified using nested PCR and for which a sequence was generated is excluded from this list.

Species	**Number**
Reptilia	23
Anolis altae	3
Anolis capito	2
Anolis humilis	4
Anolis intermedius	1
Anolis limifrons	3
Anolis microtus	1
Anolis pachypus	1
Anolis tropidolepis	2
Bothriechis lateralis	1
Geophis hoffmanni	2
Geophis zeledoni	1
Gonatodes albogularis	1
Imantodes inornatus	1
Amphibia - anura	37
Craugastor andi	1
Craugastor bransfordii	1
Craugastor caryophyllaceus	3
Craugastor podiciferus	1
Craugastor ranoides	2
Dendropsophus ebraccatus	2
Dendropsophus microcephalus	2
Duellmanohyla rufioculis	5
‘Eleutherodactylus sp.’	3
Hyalinobatrachium colymbiphyllum	1
Isthmohyla pseudopuma	1
Isthmohyla rivularis	5
Lithobates forreri	1

	Lithobates vibicaria	1
	Physalaemus pustulosus	5
	'Rana pipiens'	3
Total		60

Permits

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Museum samples were cleared for export by the United States Fish and Wildlife Service.

Export permits for Australian samples were granted by the Australian Government's Department of the Environment and Energy. Australian sampling permits were granted to Robert Puschendorf by the Queensland Government Environment Protection Agency and sample collection was approved by James Cook University's Animal Ethics Committee.

All import permits were provided by the Animal and Plant Health Agency.

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