IMMUNOGENETIC ADAPTATION TO AN EMERGENT AMPHIBIAN DISEASE

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Anna Evangeline Savage

January 2012

IMMUNOGENETIC ADAPTATION TO AN EMERGENT AMPHIBIAN DISEASE

Anna Evangeline Savage, Ph. D.

Cornell University 2012

The disease chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), emerged in the 1970s and has caused the decline and extinction of hundreds of amphibian species worldwide. Several pathogen and environmental factors have been identified that play critical roles in determining *Bd* disease dynamics. In comparison, host factors have been infrequently characterized and remain poorly understood. Here, I explore genetic responses to *Bd* in the lowland leopard frog (*Lithobates yavapaiensis*) to evaluate the hypothesis that host genetic factors contribute to *Bd* susceptibility across natural populations. I characterize disease prevalence, environmental variables, and measures of genetic variability in eleven natural populations to reveal spatial and temporal *Bd* dynamics. I also perform experimental *Bd* infections in lab-reared frogs collected from five natural populations. For both experimentally reared frogs and natural populations, I characterize allelic variation at an expressed Major Histocompatibility Complex (MHC) class IIB locus that encodes peptides that initiate acquired immunity. I find that infections are minimal in summer but abundant in winter, some populations are *Bd* infected without developing chytridiomycosis, and other populations are *Bd* infected and experience fatal bouts of chytridiomycosis. I identify an outlier locus that shows associations to *Bd* susceptibility, and find the best models predicting *Bd* dynamics include both genetic diversity and environmental variables. I show that MHC alleles associate with surviving *Bd* infection in both lab-infected frogs and naturally sampled

individuals. Individuals bearing MHC allele Q show significantly reduced risks of death, and I detect positive selection along the evolutionary lineage leading to allele Q. Further, in one *Bd* resistant population, I detect a significant signal of directional selection for allele Q. For lab-infected frogs only, MHC heterozygotes also have a significantly reduced risk of death. In summary, I find that population genetic and immunogenetic variation contributes to *Bd* susceptibility after controlling for environmental variation, demonstrating that host genetics significantly affect chytridiomycosis outcomes and may be a powerful tool for conserving global amphibian biodiversity.

BIOGRAPHICAL SKETCH

Anna Evangeline Savage was born on the fourth of July, 1982, in the town of Barrington, New Hampshire, USA. She grew up in Northwood, New Hampshire, and was homeschooled through elementary school, graduated from Coe-Brown Northwood Academy in 2000, and graduated *summa cum laude* from Amherst College in 2004 with a B.A. in Biology.

To my father Jack Savage

He knows what he did.

ACKNOWLEDGEMENTS

I thank my adviser Kelly Zamudio for seven years of guidance, support, and teaching me not to write passively. I also thank my two committee members: Rick Harrison, for giving me unconditionally good feedback and an excellent office, and Ton Schat, for keeping me on my toes, teaching me proper lab attire, and supporting me from the start. Harry Greene has been a tremendous friend, mentor, and inspiration. Jonathan Richmond and Karen Kiemneck-Tyburczy, two postdoctoral researchers in the Zamudio lab, did much of the legwork that made my thesis chapters possible, and were also incredible friends who made innumerable hours of PCR and cloning bearable. I would also like to thank the former and current Zamudio lab graduate students, each of whom enhanced my experience at Cornell: Lauren Chan, Jeanne Robertson, Kurt Galbreath, Elizabeth Kuperburg, Rayna Bell, Gui Becker, Ana Longo, and Ben Johnson. Melissa Lenker, Karen Tracy, Abby Duvall, Amanda Talaba, Katie Duryea, Iris Holmes, Guille Velo-Anton, Angie Stevenson (and family), and David Rodriguez also provided immense support and friendship. I thank Mike Sredl and Dennis Caldwell, who made my field work possible and successful, and the Arizona Game and Fish Department and The Nature Conservancy for providing me with incredible resources and support. Finally, I thank my mother, my brothers, my sisters, Jason, and the Cavatortas for loving and supporting me throughout this long process.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

AMPHIBIAN CHYTRIDIOMYCOSIS: AN INTRODUCTION

Unexplained amphibian declines began catching the attention of biologists as early as the 1970s. By 1989, population declines without obvious environmental or anthropogenic causes had become so abundant, conspicuous, and globally distributed that scientists convened for the first World Congress of Herpetology to discuss the mysterious frog and salamander disappearances (Barinaga 1990). Nine years of concerted research effort passed before the culprit was definitively identified to be the disease chytridiomycosis (Berger *et al.* 1998) caused by the chytrid fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*; Longcore *et al.* 1999). The pathology and progression of chytridiomycosis is now well understood, and biologists have identified numerous factors influencing *Bd* disease dynamics. However, epidemiological knowledge of *Bd* has yet to temper the effects of this emerging pathogenic disease. At present, a minimum of 350 amphibian species have been infected with *Bd* (Fisher *et al*. 2009), more than 200 species have declined or gone extinct (Skerratt *et al.* 2007), and *Bd* continues to be detected in new regions and species worldwide (Savage *et al.* 2011; Bell *et al*. 2011).

Chytridiomycosis is a single-pathogen, multi-host disease that occurs on every continent with amphibians (Europe [Bosch *et al.* 2001], Africa [Hopkins & Channing 2003], Australasia [Berger *et al.* 1998; Waldman *et al.* 2001], Central [Lips 1999; Lips *et al*. 2003], South [Bonaccorso *et al*. 2003; Ron *et al.* 2003], and North America [Bradley *et al.* 2002; Green *et al.* 2003]). This complex combination of interacting factors presents a challenge for isolating common causes and consequences of disease severity and pathogenicity. The disease triangle (Scholthof 2007) is a useful model for exploring interactions between the three components that contribute to all diseases: the host, the pathogen, and the environment. Studies investigating one corner of the triangle while

controlling for the other two can identify important elements that may be universal for chytridiomycosis. Conversely, studies exploring the interactions between all three disease components can provide a more realistic picture of the critical epidemiological factors for a specific combination of host, pathogen and environment. A summary of current knowledge for each corner of the chytridiomycosis disease triangle is presented below, followed by a discussion of the most critical areas needing more research.

Pathogen Contributions

The origin of *Bd* and causes for its emergence as an amphibian pathogen are still debated. Spatial-temporal patterns of outbreaks suggest that *Bd* is a novel introduced pathogen that is spreading regionally (Morgan *et al.* 2007; Lips *et al.* 2008) while other analyses indicate that climate-linked changes in pathogenicity may have facilitated localized outbreaks of endemic *Bd* strains in amphibian populations (Pounds *et al.* 2006). Consistent with both of these hypotheses, frog communities in some regions suffer chytridiomycosis die-offs immediately following *Bd* introduction (Lips 1999; Lips *et al.* 2003) and we now know that the virulence of *Bd* is highly temperature dependent (Piotrowski *et al*. 2004).

Bd is a diploid, single celled fungus with an infectious flagellated zoospore stage and a sessile thallus stage that produces reproductive bodies called zoosporangia (Longcore *et al.* 1999; Berger *et al.* 2005). Zoospores are free-living and will swim through water until encountering and encysting within amphibian skin (Longcore *et al.* 1999). New zoospores are produced clonally within each *Bd* zoosporangium and are released into the environment via discharge tubes that extend from the zoosporangium to the skin cell surface (Longcore *et al.* 1999). Sexual reproduction may rarely occur among *Bd* strains, but this process has never been confirmed, and low genetic diversity among worldwide *Bd* strains (Morgan *et al.* 2007) suggests that reproduction is generally

asexual. *Bd* only infects amphibian tissues containing keratin, which includes the outer skin layers of metamorphosed individuals (Longcore *et al.* 1999), and the mouthparts of tadpoles, which become deformed but do not lead to tadpole mortality (Rachowicz & Vredenburg 2004). The severity of disease is dependent on the amount of fungal growth (Vredenburg *et al.* 2010), and frogs with mild infections often show no signs or symptoms, while a high *Bd* load leads to skin swelling and shedding, redness, lethargy, seizures, and often death. Frogs have permeable skin that is important for respiration, hydration, and osmoregulation, and *Bd* causes mortality by severely disrupting electrolyte transport across the skin which leads to cardiac arrest (Voyles *et al.* 2009).

Temperature is the most important factor in determining *Bd* growth and pathogenicity. *Bd* grows optimally between 18-24ºC and poorly at 10ºC, but is killed in the laboratory when grown at temperatures above 28ºC (Piotrowski *et al.* 2004). Similarly, *Bd* pathogenicity is highest at temperatures ranging from 12-23°C and lowest at temperatures above 27°C (Berger *et al.* 1998, 2004; Longcore *et al.* 1999; Carey *et al.* 2006). *Bd* survives across pH values ranging from 4 to 8, but growth is optimal at a pH of 6-7 (Piotrowski *et al*. 2004). Zoospores require moisture to survive, but some strains survived for 12 weeks in soil with only 10% moisture (Johnson and Speare 2005). Together, these findings present a wide range of natural environmental conditions in which *Bd* can flourish.

Environmental Contributions

Bd and chytridiomycosis prevalence are affected by numerous ecological and environmental variables. Studies of natural populations have shown *Bd* to vary with latitude (Kriger *et al.* 2007), elevation (Brem & Lips 2008), precipitation (Puschendorf *et al.* 2009, Longo *et al*. 2010), temperature (Kriger *et al.* 2007, Puschendorf *et al*. 2009), habitat loss (Becker & Zamudio 2011) and host species richness (Searle *et al.* 2011).

However, there is not a single epidemiological pattern for all geographic regions or host species. *Bd* infections occur in a wide variety of habitats, ranging from high elevations in the Sierra Nevadas of California (Vredenburg *et al*. 2010) to lowland forests of Gabon (Bell *et al.* 2011) and tropical rainforests of Australia (Berger *et al.* 1998).

Chytridiomycosis outbreaks are generally more frequent and severe in tropical compared to temperate environments (Lips *et al.* 2003). Disease is most prevalent in upland tropical habitats, where cool moist conditions favor rapid proliferation and survival of the fungus (Lips *et al.* 2003, Houlahan *et al.* 2000). Roughly 70% of mid and high elevation tropical frog species are susceptible to chytridiomycosis, with over half of these going extinct (Lips *et al.* 2003; La Marca *et al.* 2005; Lips *et al.* 2006). The remaining species either decline and persist in low numbers (~20%) or are unaffected (~10%).

Temperate frog communities also experience disease outbreaks (Bradley *et al*. 2002; Green *et al.* 2003; Muths *et al*. 2003), but severity is often diminished due to seasonal abatement of *Bd* growth and/or pathogenicity (Kriger & Hero 2007a). The incidence and prevalence of *Bd* infections have been shown to be higher during cool months compared to warm months in numerous regions, including Australia (Retallick *et al.* 2004), Canada (Ouellet *et al*. 2005), the United States (Bradley *et al.* 2002), and Puerto Rico (Longo *et al.* 2010).

Taken together, studies of *Bd* across regions, habitats and seasonal conditions demonstrate that chytridiomycosis is a complex disease influenced not only by the environment, but by a unique combination of interactions among environmental variables. For example, increased habitat loss predicted lower disease risk in Australia, Brazil and Puerto Rico due to changes in host community structure and microclimate associated with habitat destruction (Becker & Zamudio 2011).

Host Contributions

Variation in host susceptibility to chytridiomycosis is the least explored corner of the *Bd* disease triangle. We know that chytridiomycosis decimates some amphibian species, while others in the same localities are seemingly unaffected (Retallick *et al.* 2004; Daszak *et al.* 2004; Lips *et al.* 2006). Interspecific studies show that certain species-level characteristics, including life history (Kriger & Hero 2007b) and innate (skin peptide) immune defenses (Woodhams *et al*. 2006; Woodhams *et al.* 2007), can partially predict which species are most at risk for chytridiomycosis. Intraspecific studies also demonstrate that, at least within some species, individuals vary in *Bd* susceptibility (Bradley *et al*. 2002; Kriger & Hero 2006). These findings suggest a role for host immune function in determining chytridiomycosis susceptibility, but this possibility remains underexplored. Experimental studies in the model frog genus *Silurana* show that under some conditions, *Bd* infection activates innate immune defenses (Ribas *et al.* 2009) or minimal immune responses of any kind (Rosenblum *et al*. 2009), while under other conditions, acquired immunity plays a role in host responses (Ramsey *et al*. 2010). Additionally, Major Histocompatibility Complex (MHC) immune regulatory genes were explored in a single species, *Bufo calamita*, and genotype frequencies among larval populations varied in a pattern consistent with directional selection in response to pathogen prevalence (May *et al.* 2011). Thus, amphibian immunogenetic resistance to *Bd* is a strong candidate mechanism for observed variation in susceptibility, but more functional and genetic studies are needed to confirm this pattern across host species, environmental conditions, and pathogen strains.

The Disease Triangle: Future Directions

To date, we have amassed an impressive body of literature on the epidemiology of *Bd*, from strain-specific differences in pathogenicity (Retallick & Miera 2007) to

complex models of pathogen dynamics under different environmental conditions (Briggs *et al.* 2010). However, poor knowledge of amphibian immunity is inhibiting our ability to fully elucidate disease dynamics in numerous host amphibians. Vertebrate immune systems are incredibly sophisticated and complex due to the necessity of having effective defense mechanisms for survival against the multitude of pathogenic organisms routinely encountered in the environment. Numerous immune pathways are likely to play a role in host responses to *Bd*, and rapid immunogenetic evolution may occur in response to strong selective pressure imposed by chytridiomycosis. To understand the extent, nature, and importance of these processes, further characterization of amphibian immune systems is sorely needed.

The best understood amphibian immune system is that of the model frog genus *Silurana* (Robert & Cohen 2011). Characterization of immune structure and function in *Silurana* reveals that amphibian immune systems are fundamentally similar to mammals (Du Pasquier *et al.* 1989). Frogs have a thymus where T cells differentiate and a spleen where B and T cells accumulate, and leukocytes such as neutrophils, basophils, eosinophils, monocytes, and macrophage-like cells are found in the blood (Robert & Ohta 2009). However, frogs lack lymph nodes, Peyer's patches, and germinal centers, and consequently, frogs show poor affinity maturation, the process by which B cells make antibodies with increased affinity for antigen during the course of a mammalian immune response (Du Pasquier *et al.* 2000). Studies of *Silurana* provide the best evidence of immune system conservation and differentiation among vertebrate taxa, yet *Silurana* is polyploid, fully aquatic, and resistant to *Bd*, making the genus a biological novelty among amphibians. With the exception of antimicrobial peptides, which are well characterized across diverse anuran taxa and in the context of chytridiomycosis (Woodhams *et al.* 2006; Woodhams *et al.* 2007; Rollins-Smith *et al.* 2011), basic knowledge of amphibian immunity is lacking across non-model species. Lab and field

studies of immune responses in non-model amphibians infected with *Bd* are thus the critical next steps in identifying host factors that may enhance survival in the face of chytridiomycosis.

The emergence of chytridiomycosis remains among the most serious threats to amphibian species worldwide. The 2008 Global Amphibian Assessment found that 42% of all amphibian species are declining, 159 species have likely gone extinct, and chytridiomycosis is the fastest growing threat to species persistence (IUCN 2008). Continued study of each corner of the chytridiomycosis disease triangle, with emphasis on host factors and their interaction with the pathogen and the environment, is our best approach for elucidating novel factors that promote *Bd* survival and mitigate the impact of this global pandemic.

REFERENCES

Barinaga M (1990) Where have all the froggies gone? *Science*, **247***,* 1033–34.

Becker CG, Zamudio KR (2011) Tropical amphibian populations experience higher disease risk in natural habitats *Proceedings: Biological Sciences*, **108**, 9893–9898.

Bell RC, Gata Garcia AV, Staurt BL, Zamudio KR (2011) High prevalence of the amphibian chytrid pathogen in Gabon. *EcoHealth*, DOI: 10.1007/s10393-010-0364-4

Berger L, Marantelli G, Skerratt LF, Speare R (2005) Virulence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* varies with the strain. *Diseases of Aquatic Organisms*, **68**, 47–50.

Berger L, Speare R, Daszak P *et al.* (1998) Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings: Biological Sciences,* **95**, 9031–9036.

Berger L, Speare R, Hines HB *et al.* (2004) Effect of season and temperature on mortality in amphibians due to chytridiomycosis. *Australian Veterinary Journal*, **82**, 31–36.

Bonaccorso E, Guayasamin JM, Méndez D, Speare R (2003) Chytridiomycosis as a possible cause of population declines in *Atelopus cruciger* (Anura: Burfonidae). *Herpetological Review,* **34**, 331–334.

Bosch J, Martinez-Solano I, Garcia-Paris M (2001) Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. *Biological Conservation*, **97**, 331–337.

Bradley GA, Rosen PC, Sredl MJ *et al.* (2002) Chytridiomycosis in native Arizona frogs. *Journal of Wildlife Diseases*, **38**, 206–212.

Brem FMR, Lips KR (2008) *Batrachochytrium dendrobatidis* infection patterns among Panamanian amphibian species, habitats and elevations during epizootic and

enzootic stages. *Diseases of Aquatic Organisms*, **81**, 189–202.

Briggs CJ, Knapp RA, Vredenburg VT (2010) Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. *Proceedings: Biological Sciences*, **107**, 9695–9700.

Carey C, Bruzgul JE, Livo LJ *et al.* (2006) Experimental exposures of boreal toads (*Bufo boreas*) to a pathogenic chytrid fungus (*Batrachochytrium dendrobatidis*). *EcoHealth*, **3**, 5–21.

Chatfield MW, Rothermel BB, Brooks CS, Kay JB (2009) Detection of *Batrachochytrium dendrobatidis* in amphibians from the Great Smoky Mountains of North Carolina and Tennessee, USA. *Herpetological Review*, **40**, 176–179.

Daszak P, Berger L, Cunningham AA *et al.* (2004) Experimental evidence that the bullfrog (*Rana catesbeiana*) is a potential carrier of chytridiomycosis, an emerging fungal disease of amphibians. *Herpetological Journal*, **14**, 201–207.

Du Pasquier L, Robert J, Courtet M, Mussmann R (2000) B-cell development in the amphibian *Xenopus*. *Immunological Reviews*, **17**, 201–213.

Du Pasquier, L, Schwager J, Flajnik MF (1989) The immune system of *Xenopus*. *Annual Review of Immunology*, **7**, 251–275.

Fisher MP, Garner TWJ, Walker SF (2009) Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. *Annual Review of Microbiology*, **63**, 291–310.

Green DE, Converse KA, Scgrader AK (2003) Epizootiology of sixty-four amphibian morbidity and mortality events in the USA, 1996-2001. *Annals of the New York Academy of Sciences,* **969**, 323–339.

Hopkins S, Channing A (2003) Chytrid fungus in Northern and Western Cape frog populations, South Africa. *Herpetological Review,* **34**, 334–336.

Houlahan JE, Findlay CS, Schmidt BR *et al.* (2000) Quantitative evidence for global

amphibian population declines. *Nature,* **404**, 752–755.

IUCN 2008. 2008 IUCN Red List of Threatened Species. <www.iucnredlist.org> Downloaded on 31 October 2011

Johnson ML, Speare R (2005) Possible modes of dissemination of the amphibian chytrid *Batrachochytrium dendrobatidis* in the environment. *Diseases of Aquatic Organisms,* **65**, 181–186.

Kriger KM, Pereoglou F, Hero JM (2007) Latitudinal variation in the prevalence and intensity of chytrid (*Batrachochytrium dendrobatidis*) infection in eastern Australia. Conservation Biology, **21**, 1280–1290.

Kriger KM, Hero JM (2007a) Large-scale seasonal variation in the prevalence and severity of chytridiomycosis. *Journal of Zoology,* **271**, 352–359.

Kriger KM, Hero JM (2007b) The chytrid fungus *Batrachochytrium dendrobatidis* is non-randomly distributed across amphibian breeding habitats. *Diversity and Distributions,* **13**, 781–788.

Kriger KM, Hero JM (2006). Survivorship in wild frogs infected with chytridiomycosis. *EcoHealth,* **3**, 171–177.

La Marca E, Lips KR, Lötters S *et al*. (2005) Catastrophic population declines and extinctions in neotropical harlequin frogs (Bufonidae: *Atelopus*). *Biotropica*, **37**, 190–201.

Lips KR (1999) Mass mortality and population declines of anurans at an upland site in western Panama. *Conservation Biology*, **13**, 117–125.

Lips KR, Green DE, Papendick R (2003) Chytridiomycosis in wild frogs from southern Costa Rica. *Journal of Herpetology,* **37**, 215–218.

Lips KR, Brem F, Brenes R *et al.* (2006) Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proceedings: Biological Sciences*, **103**, 3165–3170.

Lips KR, Reeve JD, Witters LR (2003) Ecological traits predicting amphibian population declines in Central America. *Conservation Biology*, **17**, 1078–1088.

Lips KR, Diffendorfer J, Mendelson JR 3rd, Sears MW (2008) Riding the wave: Reconciling the roles of disease and climate change in amphibian declines. *PLoS Biology,* **6**, e72

Longcore JE, Pessier AP, Nichols DK (1999) *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. *Mycologia*, **91**, 219–227.

Longo A, Burrowes PA, Joglar RL (2010) Seasonality of *Batrachochytrium dendrobatidis* infection in direct-developing frogs suggests a mechanism for persistence. *Diseases of Aquatic Organisms*, **92**, 253–60.

May S, Zeisset I, Beebee TJC (2011) Larval fitness and immunogenetic diversity in chytrid-infected and uninfected natterjack toad (*Bufo calamita*) populations. *Conservation Genetics*, **12**, 805–811.

Morgan JAT, Vredenburg VT, Rachowicz LJ *et al*. (2007) Population genetics of the frog killing fungus *Batrachochytrium dendrobatidis*. *Proceedings: Biological Sciences,* **104**, 13845–13850.

Muths E, Corn PS, Pessier AP, Green DE (2003) Evidence for disease related amphibian decline in Colorado. *Biological Conservation,* **110**, 357–365.

Ouellet M, Mikaelian I, Paul BD *et al*. (2005) Historical evidence of widespread chytrid infection in North American amphibian populations. *Conservation Biology*, **19**, 1431–1440.

Piotrowski JS, Annis SL, Longcore JE (2004) Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. *Mycologia*, **96**, 9–15.

Pounds AJ, Bustamante MR, Coloma L *et al*. (2006) Widespread amphibian extinctions from epidemic disease driven by global warming. *Nature*, **439**, 161–167.

Puschendorf R, Carnaval AC, Vanderwal,J *et al*. (2009) Distribution models for the

amphibian chytrid *Batrachochytrium dendrobatidis* in Costa Rica: Proposing climatic refuges as a conservation tool. *Diversity and Distributions*, **15**, 401–408.

Rachowicz LJ, Vredenburg VT (2004) Transmission of *Batrachochytrium dendrobatidis* within and between amphibian life stages. *Diseases of Aquatic Organisms*, **61**, 75–83.

Ramsey J, Reinert L, Harper L, Woodhams D, Rollins-Smith L (2010) Immune defenses against *Batrachochytrium dendrobatidis*, a fungus linked to global amphibian declines, in the South African Clawed Frog, *Xenopus laevis. Infection and Immunity*, **78**, 3981–3992.

Retallick RWR, Miera V (2007) Strain differences in the amphibian chytrid *Batrachochytrium dendrobatidis* and non-permanent, sub-lethal effects of infection. *Diseases of Aquatic Organisms*, **75**, 201–207.

Retallick R, McCallum H, Speare R (2004) Endemic infection of the amphibian chytrid fungus in a frog community post-decline. *PLOS Biology*, **2**, 1–7.

Ribas L, Li MS, Doddington BJ *et al.* (2009) Expression profiling the temperature-dependent amphibian response to infection by *Batrachochytrium dendrobatidis*. *PLoS ONE*, **4**, e8408.

Robert J, Cohen N (2011) The genus *Xenopus* as a multispecies model for evolutionary and comparative immunobiology of the 21st century. *Developmental and Comparative Immunology*, **35**, 916–923.

Robert J, Ohta Y (2009). Comparative and developmental study of the immune system in *Xenopus*. *Immunology Today*, **13**, 142–147.

Rollins-Smith LA, Ramsey JP, Pask JD, Woodhams DC (2011) Amphibian immune defenses against chytridiomycosis: Impacts of changing environments. *Integrative and Comparative Biology*, **51**, 552–562.

Ron SR, Duellman WE, Coloma LA, Bustamante MR (2003) Population decline of the

jambato toad *Atelopus ignescens* (Anura: Bufonidae) in the Andes of Ecuador. *Journal of Herpetology,* **37**, 116–126.

Rosenblum EB, Poorten TJ, Settles M *et al*. (2009) Genome-wide transcriptional response of *Silurana* (*Xenopus*) *tropicalis* to infection with the deadly chytrid fungus. *PLoS ONE*, **4**, e6494.

Savage AE, Grismer LL, Anuar S *et al.* (2011b) First record of *Batrachochytrium dendrobatidis* infecting four frog families from Peninsular Malaysia. *EcoHealth*, **7**, 165–170.

Scholthof KBG (2007) Essay: The disease triangle: pathogens, the environment and society. *Nature Reviews Microbiology*, **5**, 152–156

Searle CL, Biga LM, Spatafora JW, Blaustein AR (2011) A dilution effect in the emerging amphibian pathogen *Batrachochytrium dendrobatidis*. *Proceedings: Biological Sciences,* **108**, 16322–16326.

Skerratt LF, Berger L, Speare R *et al.* (2007) Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth*, **4**, 125–134.

Voyles J, Young S, Berger L *et al.* (2009) Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science*, **326**, 582–585.

Vredenburg VT, Roland A, Tate S, Briggs CJ (2010) Dynamics of an emerging disease drive large-scale amphibian population extinctions. *Proceedings: Biological Sciences,* **107**, 9689–9694.

Waldman B, van de Wolfshaar KE, Klena JD *et al.* (2001) Chytridiomycosis in New Zealand frogs. *Surveillance,* **28**, 9–11.

Woodhams DC, Voyles J, Lips KR, Carey C, Rollins-Smith LA (2006) Predicted disease susceptibility in a Panamanian amphibian assemblage based on skin peptide defenses. *Journal of Wildlife Diseases,* **42**, 207–218.

Woodhams DC, Ardipradja K, Alford RA *et al.* (2007). Resistance to chytridiomycosis

varies among amphibian species and is correlated with skin peptide defenses. *Animal Conservation*, **10**, 409–417.

CHAPTER 1:

DISEASE DYNAMICS VARY SPATIALLY AND TEMPORALLY IN A NORTH AMERICAN AMPHIBIAN[†]

Anna E. Savage[1], Michael J. Sredl[2], and Kelly R. Zamudio[1]

[1] Department of Ecology and Evolutionary Biology, E150 Corson Hall, Cornell University, Ithaca, New York 14853.

[2]Arizona Game and Fish Department, 5000 W. Carefree Highway, Phoenix, AZ 85086-5000

Abstract

Local environmental conditions are a primary factor influencing chytridiomycosis, an emerging disease caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*) that has affected over 200 amphibian species worldwide. In North America, seasonality and regional habitat differences predict considerable spatial and temporal disease variability, yet the sparse and opportunistic nature of most studies have provided insufficient data for understanding regional *Bd* epidemiology. We present a five-year field study that reveals spatial and temporal *Bd* dynamics across *Lithobates yavapaiensis* populations in Arizona, USA. Two populations showed no *Bd* infection or mortality, ten populations showed winter *Bd* infection, and five populations experienced winter mortality. Infection intensity decreased over winter sampling seasons, whereas mortality and infection prevalence did not change over time. Frogs dying from chytridiomycosis were significantly larger and had significantly higher infection intensities than survivors. We

conclude that conserving *L. yavapaiensis* and other native amphibians requires protection and management of riparian ecosystems to promote populations large enough to survive winter cycles of chytridiomycosis.

Introduction

Batrachochytrium dendrobatidis (*Bd*) is a fungal pathogen that has caused declines or extinction in over 200 amphibian species worldwide (Lips *et al.* 2006; Stuart *et al.* 2004). The consequences of *Bd* infection vary tremendously with local climatic conditions (Crawford *et al.* 2010; Kriger and Hero 2007; Longo *et al.* 2010). In upland tropical regions that experience little temperature variation across seasons, *Bd* infection often causes rapid declines in host amphibians from epidemics of the skin disease chytridiomycosis (Berger *et al.* 1998; La Marca *et al.* 2005). In contrast, amphibians in temperate regions with larger seasonal temperature fluctuations show variable responses to *Bd*, ranging from no observed effects (Garner *et al.* 2006; Longcore *et al.* 2007), to sub-lethal fitness costs (Retallick & Miera 2007), to episodic chytridiomycosis outbreaks and concomitant population declines (Kriger & Hero 2006; McDonald *et al.* 2005). These punctuated chytridiomycosis outbreaks usually occur in cool months, suggesting that cooler temperatures promote *Bd* growth, host susceptibility, or both.

With the exception of a few well-studied regions in the Sierra Nevada mountains (Briggs *et al.* 2010; Vredenburg *et al*. 2010), studies of local climatic determinants of chytridiomycosis in North America are patchy and conflicting. One retrospective study of North American museum specimens detected lower *Bd* prevalence in warmer months (Ouellet *et al.* 2005), while a similar contemporary study (Green *et al.* 2002) found chytridiomycosis outbreaks beginning in warm and cool months. In Canada, *Lithobates pipiens Bd* prevalence decreased as mean monthly temperatures increased (Voordouw *et al.* 2010), but survival did not differ among infected and uninfected individuals. In

Mexico, colder temperatures were associated with higher chytridiomycosis severity in four frog species (Hale *et al.* 2005). In contrast, Lips *et al.* (2004) documented chytridiomycosis mortalities in upland regions of Mexico during summer. These inconsistent patterns highlight the need to clarify the relationship between *Bd* infection, climate, local environmental conditions, and development of chytridiomycosis in North American amphibians.

Here, we present a 5-year field study of *Bd* infection dynamics in the lowland leopard frog (*Lithobates* [*Rana*] *yavapaiensis*), a stream-dwelling species inhabiting southwestern desert regions that has experienced chytridiomycosis die-offs since at least 1992 (Bradley *et al.* 2002). Documented chytridiomycosis outbreaks in *L. yavapaiensis* occur only in cooler months, but population surveys have been opportunistic (Sredl 2003). We conducted standardized, repeated surveys of twelve populations and measured *Bd* prevalence, intensity, and mortality in summer and winter. We used these data to determine whether (1) *Bd* infection and mortality vary among populations; (2) *Bd* infection and mortality vary seasonally; (3) *Bd* infection and mortality vary across years; and (4) *Bd* infection intensity, prevalence, and mortality co-vary within and across seasons, years and populations. Finally, we exploited the unique thermal dynamics in one region of Arizona to directly evaluate the relationship between water temperature, *Bd* infection, and chytridiomycosis.

Methods

We surveyed twelve *L. yavapaiensis* population localities in Arizona, USA, in summer (July–August) and winter (January–February) from 2006 to 2010 (Figure S1). All populations were surveyed within three weeks to limit within-season variation due to local environmental conditions. We gave equal survey time and effort across seasons, years, and localities, and used diurnal visual encounter surveys (Crump & Scott 1994) to

measure the abundance of adult and sub-adult *L. yavapaiensis* individuals. We toe-clipped and swabbed the epidermis of each individual using sterile fine-tip swabs (Medical Wire & Equipment Co. MW113) following standardized protocols (Hyatt *et al.* 2007). Frogs were handled with unused latex gloves to prevent pathogen transmission. Recapture rates were low (mean = 0.4%), thus we treated each sampling season as independent.

We used a Taylor 9842 temperature logger to record water temperatures. Within each locality, we took three mid-afternoon readings and retained the maximum recorded temperature. We separated Muleshoe Ranch (MR) into three sub-regions based on mean temperature variation across microhabitats: (1) MR_{HS} is a thermal spring >50°C at the source and >30°C throughout the frog sampling region, (2) MR_{SS} is a pond 0.5 km away fed by a thermal spring >30°C at the source and 20–30°C throughout the frog sampling region, and (3) MR_{BC} is a canyon stream 1.4 km away that has average winter water temperatures of 10°C. To evaluate the role of water temperature, we separated frog populations into thermal spring localities (MR_{HS} and MR_{SS}) and non-thermal spring localities (all other populations).

Eight of our 12 localities are Nature Conservancy properties with continual monitoring since the 1980s by on-site staff (MR_{SS}, MR_{HS} MR_{BC}, AC, HR), or sites that have been surveyed by Tucson Herpetological Society volunteers since the 1990s (AS, TV, CIC; Table S1.1). For each dead and dying frog, we recorded the date of death as the first date that our team, a volunteer, or a site manager observed the mortality event. We collected individuals with signs of chytridiomycosis (i.e., skin redness, lethargy, failure to seek cover, and loss of righting ability) for overnight observation; if death occurred within 24h and the individual tested positive for *Bd*, we categorized these frogs as chytridiomycosis mortalities. Individuals found dead during winter without other apparent causes of death (i.e., predation or injury) and testing positive for *Bd* were also

considered chytridiomycosis mortalities.

We extracted DNA from swabs using PrepMan Ultra (Applied Biosystems; Hyatt *et al.* 2007). Infection prevalence and intensity were determined using quantitative PCR (Boyle *et al*. 2004). Infection intensity was measured as the number of zoospore equivalents per swab, and individuals were considered *Bd*-positive at ≥1 zoospore equivalents. Values were not obtained for the January 2007 season. We calculated *Bd* infection as the number of positives divided by the total number of frogs swabbed and *Bd* mortality as the number of dead frogs divided by the total number of frogs observed. We calculated 95% Clopper-Pearson binomial confidence intervals from observed proportions of infection and mortality. We compared *Bd* infection, chytridiomycosis mortality, numbers of frogs, and water temperatures using two-tailed paired sample Wilcoxon signed-rank tests and rank sum tests. We compared *Bd* infection intensity among dead, dying and asymptomatic individuals using two-tailed Student's *t*-tests assuming unequal variances.

Results

We sampled 692 *L. yavapaiensis* individuals from 12 localities in Arizona, USA, with a mean pairwise population distance of 198 km (Table S1.1). No mortality and low *Bd* prevalence (1.6%) occurred in summer. In contrast, all populations were infected with *Bd* in winter except for House Spring (HS) and Aliso Spring (AS; Figure 1.1A), populations separated by 265 km. Of the ten infected populations, five experienced winter mortalities, and observed levels of *Bd* infection and mortality varied among localities (Figure 1.1A). *Bd* infection (P=0.005) and mortality (P=0.02) were significantly lower in summer compared to winter. *Bd* infection was significantly higher than mortality in summer (P=0.03) and winter (P=0.008).

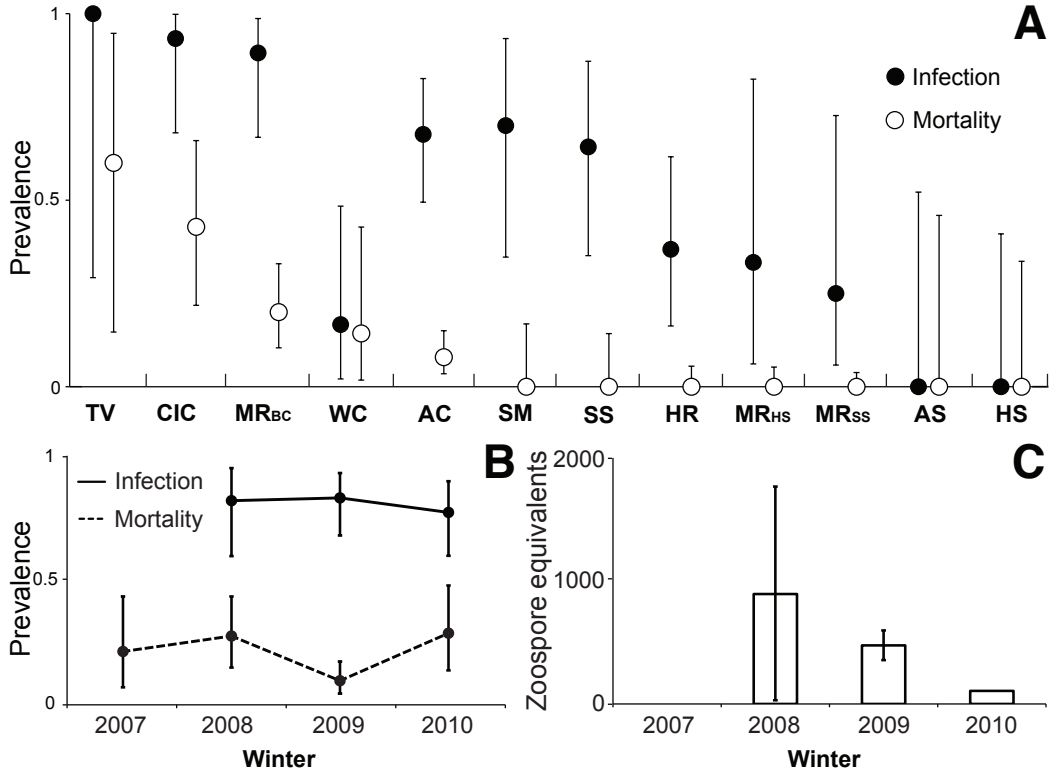

Figure 1.1: (A) Observed prevalence of winter *Lithobates yavapaiensis Bd* infection (closed symbols) and chytridiomycosis mortality (open symbols) across populations and years, with 95% Clopper-Pearson binomial confidence intervals indicated. Infection prevalence was measured as the proportion of swabbed individuals that harbored *Bd* infections, and chytridiomycosis mortality prevalence was measured as the proportion of frogs observed in winter that died with signs of chytridiomycosis. (B) Winter *Lithobates yavapaiensis* mean *Bd* infection prevalence (solid line) and chytridiomycosis mortality prevalence (dashed line) across sampling winters. Error bars show 95% Clopper-Pearson binomial confidence intervals. (C) Mean *Bd* infection intensity (measured as zoospore genome equivalents) across sampling winters. Error bars show standard

deviations.

Winter *Bd* infection prevalence did not significantly change across sampling years, nor did winter *Bd* mortality (Figure 1.1B). In contrast, infection intensity significantly decreased from 2008 to 2010 (P=0.02; Figure 1.1C). Across all years, *Bd* infection intensity limits were 0–4040 zoospore equivalents among the 139 individuals sampled in winter months. Notably, we detected little *Bd* on individuals found dead; mean infection intensity was 1280.6 for dying individuals, 276.3 for healthy individuals, and 15.6 for individuals found dead. Dead individuals laid out for a mean of 5.3 days (range: 0-16) without predation before we collected them. Individuals found alive but showing signs of chytridiomycosis all died within 24 hours, and these moribund individuals had significantly higher mean infection intensity compared to individuals found dead (P=0.02). Individuals sampled from populations with compared to without mortality did not differ in mean infection intensity (P=0.89). Among individuals sampled in winter, mean body mass was significantly higher for dead (28.5 g) and dying (33.7 g) individuals compared to asymptomatic individuals (18.2 g; P=0.01). *Bd* infection prevalence was highly seasonal, increasing in winter and decreasing in summer for all populations across the 5-year sampling period (Figure 1.2A). Across all localities, the mean number of frogs observed was significantly higher in summers compared to winters (P=0.001). At Tanque Verde Canyon (TV), the locality with the highest infection and mortality prevalence (Figure 1.1A), we did not observe any frogs after summer 2009.

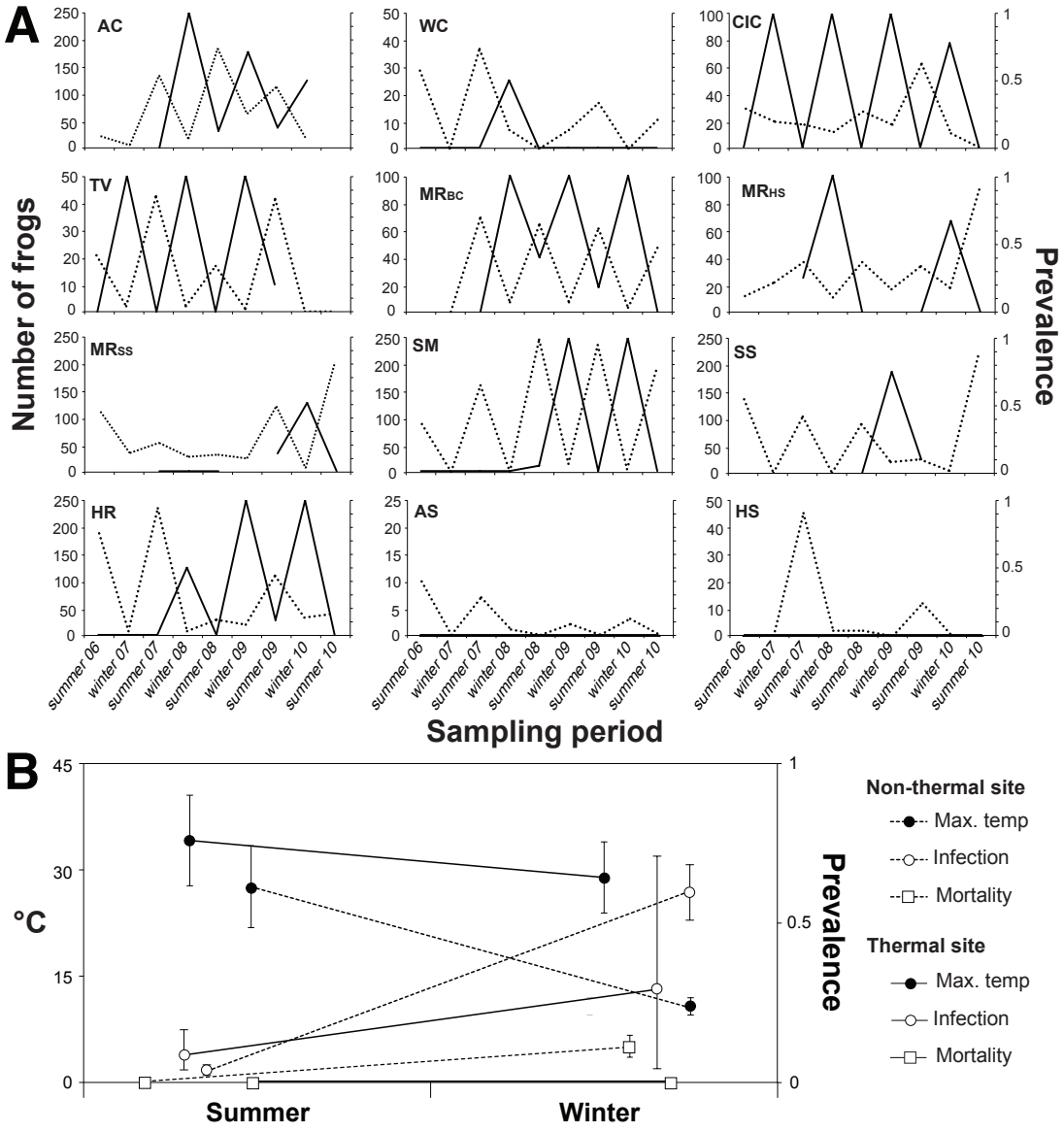

Figure 1.2: (A) Observed number of frogs (dashed line) and *Bd* infection prevalence (solid line) within each *Lithobates yavapaiensis* population in summer and winter from 2006 through 2010. (B) Mean maximum water temperature (filled circles), *Bd* infection prevalence (open circles), and chytridiomycosis mortality prevalence (open squares) for thermal spring (solid line; *N*=2) and non-thermal spring (dashed line; *N*=10) populations in winter versus summer.

We examined the effect of water temperature on *Bd* infection and chytridiomycosis mortality by comparing thermal springs to other localities. Among non-thermal spring localities, maximum water temperature was significantly higher in summer versus winter (P=0.002), whereas maximum water temperature at thermal springs did not differ significantly in summer versus winter (P=0.18; Figure 1.2B). In summer, maximum water temperature was not significantly different at thermal versus non-thermal spring sites (P=0.166). In contrast, maximum winter water temperature was significantly higher at thermal versus non-thermal spring sites (P=0.03; Figure 1.2B). Concordantly, chytridiomycosis mortality was significantly higher at non-thermal versus thermal spring localities in winter (P=0.05). However, *Bd* infection did not differ significantly at non-thermal spring versus thermal spring localities in winter (P=0.28) or summer (P=0.39).

Discussion

Seasonal and spatial variation in disease prevalence are common in human and wildlife systems (Hosseini *et al.* 2004; Pascual and Dobson 2005) and can arise from numerous factors, such as pathogen thermal requirements (Van Riper *et al.* 1986), host immunity changes over seasons or temperatures (Cheng *et al.* 2009), and variable host population genetic diversity (Pearman & Garner 2005). However, *Bd* modeling suggests that intraspecific variation in infection outcomes do not require differences in susceptibility, virulence, or environment, but can instead result from epidemic versus endemic dynamics of the same host-pathogen interaction (Briggs *et al.* 2010). Understanding the intraspecific dynamics of *Bd* in *L. yavapaiensis,* a species that continues to persist despite high chytridiomycosis susceptibility, may provide critical knowledge for the recovery of other, more susceptible species.

The higher infection and disease prevalence in cooler seasons we detected in *L.*

yavapaiensis is consistent with other studies of *Bd* in natural amphibian populations (Berger *et al.* 2004; Kriger & Hero 2006). Schlaepfer and colleagues (2007) estimated *Bd* infection prevalence in Arizona *L. yavapaiensis* from March through October and found no seasonal variation, no infection at three localities, and concluded *Bd* was likely excluded from these localities. We sampled two of the same three localities (MR_{HS} and HR) and found 33% and 37% winter *Bd* prevalence, respectively. This finding highlights the need for sampling protocols to cover the range of seasons and temperatures to assure that prevalence estimates are not biased.

Our study is the first to report *Bd* infection intensity in an amphibian of the southwestern United States, and we recovered two surprising patterns. First, we found that chytridiomycosis susceptible individuals were significantly larger and had significantly higher infection intensities than individuals that survived winters, contradicting other studies of *Bd* across age and size classes. For example, juvenile *Dendrobates tinctorius* were more likely to die from chytridiomycosis than adults (Lamirande & Nichols 2002), lower mass was associated with higher mortality in *Alytes obstetricans* (Garner *et al.* 2009), and metamorphs had significantly higher fungal loads than adults in *Lithobates sierrae* (Briggs *et al.* 2010). Very few *L. yavapaiensis* juveniles are captured in winter, thus our demographic infection patterns may result from the seasonal comparison of different sizes of adult frogs. In contrast, we saw no difference in infection between juveniles and adults during summer, thus we can exclude the possibility that juveniles emerging in the summer uninfected was skewing our summer prevalence estimates. The second surprising pattern was that we found low *Bd* intensities on frogs found dead, whereas dying frogs swabbed within 24h of death had the highest *Bd* loads. This pattern suggests that that other microbes displace *Bd* or that *Bd* zoospores disperse from frog carcasses after death, but to our knowledge these phenomena are undocumented in the literature. Alternately, some of the dead frogs we

found may have died from other causes; however, the fact that they were laying out in the open without signs of predation at localities where other frogs were dying of chytridiomycosis makes this explanation unlikely.

We found invariant *Bd* dynamics at thermal spring sites, where water perennially averages >30°C, suggesting that seasonal chytridiomycosis differences at all other Arizona localities result from cooler winter water temperatures. Analysis of disease dynamics throughout Australia found strong negative effects on *Bd* when air temperatures averaged >30°C (Drew *et al.* 2006), laboratory studies identify 30°C as the upper threshold for *Bd* viability (Piotrowski *et al.* 2004), and water temperature was negatively correlated with *Bd* infection intensity in North American newt populations (Raffel *et al.* 2011). Our study corroborates that temperature contributes to *Bd* infection outcomes, but we cannot confirm whether this pattern results from effects on the host, the pathogen, or both. Host immunity may play a role, as amphibian immune defenses decrease with temperature (Raffel *et al*. 2006) independent of season (Jozkowicz & Plytycz 1998), and in the laboratory, innate immune defenses against *Bd* occur at 26°C but not 18°C (Ribas *et al*. 2009). What remains uncertain is the relative importance of increased pathogen virulence compared to decreased host immune defenses under cooler environmental conditions.

In the United States*, Bd* infects over 50 amphibian species in the Southeast (Chatfield *et al.* 2009), Northeast (Hossack *et al.* 2010), Northwest (Pearl *et al*. 2007), and Rocky Mountains (Muths *et al*. 2008) with no reports of chytridiomycosis outbreaks. However, if die-offs occur during winter months when temperate-zone amphibians are inconspicuous and rarely monitored, chytridiomycosis declines may have gone unnoticed. The extreme seasonality of chytridiomycosis observed in *L. yavapaiensis* suggests that similar *Bd* dynamics may be occurring throughout North America. Consequently, better monitoring efforts should be in place for temperate-zone

amphibians that have experienced enigmatic declines, such as *Lithobates pipiens* (Lanoo 2005).

Amphibians of southwestern North America face a multitude of environmental threats (Witte *et al.* 2008). In Arizona and Mexico at least 13 native species are infected with *Bd* (Hale *et al.* 2005; Sredl 2003), and 36% of 324 known ranid frog populations became extirpated from Arizona during 1986–2001 (Witte *et al.* 2008). We focused on *L. yavapaiensis* because it remains widespread compared to congeners such as *L. chiricahuensis*, which has declined precipitously (Clarkson & Rorabaugh 1989), and *L. tarahumarae*, which has been extirpated from the United States (Hale & Jarchow 1988). However, chytridiomycosis continues to exert a toll on *L. yavapaiensis* populations, and habitat destruction and introductions of nonnative animals present additional threats (Witte *et al*. 2008). Our study population with the highest measures of winter mortality (TV) was possibly extirpated, highlighting the fragility of these populations in the face of disease. *Bd* infects numerous amphibian species and may survive in temperate aquatic systems outside of amphibian hosts (Walker *et al*. 2007) making pathogen eradication a difficult solution. Management for *Bd* should thus be added to eliminating other threats, such as bullfrogs, crayfish, non-native fish, and land use practices that increase erosion and alter pools required by *L. yavapaiensis* (Wallace *et al.* 2010), in order to promote populations large enough to persist through winter cycles of chytridiomycosis.

Acknowledgements: We thank D. Caldwell, M. Haberstitch, M. Lawrence, M. Lenker, A. Longo, J. Richmond, M. Schlaepfer, and R. Rogers. This work was supported by an NSF-DDIG (DEB-0909013) to A.E.S. and a Population and Evolutionary Process NSF Grant (DEB-0815315) to K.R.Z.

REFERENCES

Berger L, Speare R, Daszak P *et al.* (1998) Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings: Biological Sciences,* **95**, 9031–9036.

Berger L, Speare R, Hines HB *et al.* (2004) Effect of season and temperature on mortality in amphibians due to chytridiomycosis. *Australian Veterinary Journal*, **82**, 31–36.

Boyle DG, Boyle DB, Olsen V *et al.* (2004) Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real- time Taqman PCR assay. *Diseases of Aquatic Organisms*, **60**, 141–148.

Briggs CJ, Knapp RA, Vredenburg VT (2010) Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. *Proceedings: Biological Sciences*, **107**, 9695–9700.

Bradley GA, Rosen PC, Sredl MJ *et al.* (2002) Chytridiomycosis in native Arizona frogs. *Journal of Wildlife Diseases*, **38**, 206–212.

Chatfield MW, Rothermel BB, Brooks CS, Kay JB (2009) Detection of *Batrachochytrium dendrobatidis* in amphibians from the Great Smoky Mountains of North Carolina and Tennessee, USA. *Herpetological Review*, **40**, 176–179.

Cheng AC, Cheng SA, Chen YY, Chen JC (2009) Effects of temperature change on the innate cellular and humoral immune responses of orange-spotted grouper *Epinephelus coioides* and its susceptibility to *Vibrio alginolyticus*. *Fish and Shellfish Immunology*, **26**, 768–72.

Clarkson RW, Rorabaugh JC (1989) Status of Leopard Frogs (*Rana pipiens* Complex: Ranidae) in Arizona and southeastern California. *Southwestern Naturalist*, **34**, 531–538.

Crawford AJ, Lips KR, Bermingham E (2010) Epidemic disease decimates amphibian

abundance, species diversity, and evolutionary history in the highlands of central Panama. *Proceedings: Biological Sciences,* **107**, 13777–13782.

Crump ML, Scott NJ (1994) Visual encounter surveys. In: Heyer WR, Donnelly MA, McDiarmid RW, Hayek LC, Foster MS (Eds.), Measuring and monitoring biological diversity: standard methods for amphibians. Smithsonian Institution Press, Washington, DC, pp. 84–92.

Drew A, Allen EJ, Allen LJS (2006) Analysis of climatic and geographic factors affecting the presence of chytridiomycosis in Australia. *Diseases of Aquatic Organisms*, **68**, 245–250.

Garner TWJ, Walker S, Bosch J *et al.* (2009) Life history trade-offs influence mortality associated with the amphibian pathogen *Batrachochytrium dendrobatidis*. *Oikos*, **118**, 783–791.

Garner TWJ, Perkins M, Govindarajulu P *et al.* (2006) The emerging amphibian pathogen *Batrachochytrium dendrobatidis* globally infects introduced populations of the North American bullfrog, *Rana catesbeiana*. *Biology Letters*, **2**, 455–459.

Green DE, Converse KA, Schrader AK (2002) Epizootiology of sixty-four amphibian morbidity and mortality events in the USA, 1996–2001. *Annals: New York Academy of Sciences*, **969**, 323–339.

Hale SF, Rosen PC, Jarchow JL, Bradley GA (2005) Effects of the chytrid fungus on the Tarahumara frog (*Rana tarahumarae*) in Arizona and Sonora, Mexico, in: USDA Forest Service Proceedings RMRS-P-36, pp. 407–411.

Hale SF, Jarchow RJD (1988) The status of the Tarahumara frog (*Rana tarahumarae*) in the United States and Mexico Part II. Arizona Game and Fish Department and Region. Office of Endangered Species. U.S. Fish & Wildlife Service.

Hossack BR, Adams MJ, Campbell Grant EH *et al.* (2010) Low prevalence of chytrid fungus (*Batrachochytrium dendrobatidis*) in amphibians of U.S. headwater

streams. *Journal of Herpetology*, **44**, 253–260.

Hosseini PR, Dhondt AA, Dobson A (2004) Seasonality and wildlife disease: how seasonal birth, aggregation and variation in immunity affect the dynamics of Mycoplasma gallisepticum in house finches. *Proceedings: Royal Society B*, **271**, 2569–2577.

Hyatt AD, Boyle DG, Olsen V *et al*. (2007) Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms,* **73**, 175–192.

Jozkowicz A, Plytycz B (1998) Temperature but not season affects the transplantation immunity of anuran amphibians. *Journal of Experimental Zoology*, **28**, 58–64.

Kriger KM, Hero JM (2006) Large-scale seasonal variation in the prevalence and severity of chytridiomycosis. *Journal of Zoology*, **271**, 352–359.

Kriger KM, Hero JM (2007) The chytrid fungus *Batrachochytrium dendrobatidis* is non-randomly distributed across amphibian breeding habitats. *Diversity and Distributions*, **13**, 781–788.

La Marca E, Lips KR, Lötters S *et al*. (2005) Catastrophic population declines and extinctions in neotropical harlequin frogs (Bufonidae: *Atelopus*). *Biotropica*, **37**, 190–201.

Lamirande EW, Nichols DK (2002) Effects of host age on susceptibility to cutaneous chytridiomycosis in blue- and-yellow poison dart frogs (*Dendrobates tinctorius*), in: McKinnell, R.G., Carlson, D.L. (Eds), Proceedings of the sixth international symposium on the pathology of reptiles and amphibians, Saint Paul, Minnesota, pp. 3–13.

Lannoo M (Editor), 2005. Amphibian Declines: The Conservation Status of United States Species. University of California Press, Berkeley, California.

Lips KR, Mendelson III, JR, Muñoz-Alonso A *et al.* (2004) Amphibian population declines

in montane southern Mexico: resurveys of historical localities. *Biological Conservation,* **119**, 555–564.

Lips KR, Brem F, Brenes R *et al.* (2006) Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proceedings: Biological Sciences*, **103**, 3165-3170.

Longcore JR, Longcore JE, Pessier AP, Halteman WA (2007) Chytridiomycosis widespread in anurans of northeastern United States. *Journal of Wildlife Management*, **71**, 435–444.

Longo AV, Burrowes PA, Joglar RL (2010) Seasonality of *Batrachochytrium dendrobatidis* infection in direct-developing frogs suggests a mechanism for persistence. *Diseases of Aquatic Organisms*, **92**, 253–260.

McDonald KR, Mendez D, Muller R *et al.* (2005) Decline in the prevalence of chytridiomycosis in frog populations in North Queensland, Australia. Pacific *Conservation Biology*, **11**, 114–120.

Morgan JA, Vredenburg VT, Rachowicz LJ *et al*. (2007) Enigmatic amphibian declines and emerging infectious disease: population genetics of the frog killing fungus *Batrachochytrium dendrobatidis*. *Proceedings: Biological Sciences*, **104**,1385–1390.

Muths E, Pilliod DS, Livo LJ (2008) Distribution and environmental limitations of an amphibian pathogen in the Rocky Mountains, USA. *Biological Conservation*, **141**, 1484–1492.

Oullet M, Mikaelian I, Pauli BD *et al.* (2005) Historical evidence of widespread chytrid infection in North American amphibian populations. *Conservation Biology*, **19**, 1431–1440.

Pascual M, Dobson A (2005) Seasonal patterns of infectious diseases. *PLoS Medicine*, **2**, 18–20.

Piotrowski JS, Annis SL, Longcore JE (2004) Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. *Mycologia*, **96**, 9–15.

Pearl CA, Bull EL, Green DE *et al.* (2007) Occurrence of the amphibian pathogen *Batrachochytrium dendrobatidis* in the Pacific Northwest. *Journal of Herpetology*, **41**, 145–149.

Pearman PB, Garner TWJ (2005) Susceptibility of Italian agile frog populations to an emerging strain of Ranavirus parallels population genetic diversity. *Ecology Letters*, **8**, 401–408.

Piotrowski JS, Annis SL, Longcore JE (2004) Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. *Mycologia*, **96**, 9–15.

Pounds AJ, Bustamante MR, Coloma L *et al*. (2006) Widespread amphibian extinctions from epidemic disease driven by global warming. *Nature*, **439**, 161–167.

Raffel TR, Michel PJ, Sites EW, Rohr JR (2011) What drives chytrid infections in newt populations? Associations with substrate, temperature, and shade. *EcoHealth*, **7**, 526–36.

Raffel TR, Rohr JR, Kiesecker JM, Hudson PJ (2006) Negative effects of changing temperature on amphibian immunity under field conditions. *Functional Ecology*, **20**, 819–828.

Retallick RWR, Miera V (2007) Strain differences in the amphibian chytrid *Batrachochytrium dendrobatidis* and non-permanent, sub-lethal effects of infection. *Diseases of Aquatic Organisms*, **75**, 201–207.

Ribas L, Li MS, Doddington BJ *et al.* (2009) Expression profiling the temperature-dependent amphibian response to infection by *Batrachochytrium dendrobatidis*. *PLoS ONE*, **4**, e8408.

Schlaepfer MA, Sredl MJ, Rosen PC, Ryan MJ (2007) High prevalence of *Batrachochytrium dendrobatidis* in wild populations of lowland leopard frogs *Rana yavapaiensis* in Arizona. *EcoHealth*, **4**, 421–427.

Sredl MJ (2003) Understanding and mitigating effects of chytrid fungus to amphibian populations in Arizona. Technical Report No. 208, in: Sredl, M.J. (Ed.)., Ranid frog conservation and management, Nongame and Endangered Wildlife Program, Arizona Game and Fish Department, Phoenix, Arizona, pp. 1–20.

Stuart SN, Chanson JS, Cox NA *et al.* (2004) Status and trends of amphibian declines and extinctions worldwide. *Science*, **306**, 1783–1786.

Van Riper C, Van Riper SG, Goff LM, Laird M (1986) The epizootiology and ecological significance of malaria in Hawaiian land birds. *Ecological Monographs*, **56**, 327–344.

Voordouw MJ, Adama D, Houston B *et al.* (2010) Prevalence of the pathogenic chytrid fungus, *Batrachochytrium dendrobatidis*, in an endangered population of northern leopard frogs, *Rana pipiens*. *BMC Ecology*, **10**, e6.

Vredenburg VT, Roland A, Tate S, Briggs CJ (2010) Dynamics of an emerging disease drive large-scale amphibian population extinctions. *Proceedings: Biological Sciences,* **107**, 9689–9694.

Walker SF, Salas MB, Jenkins D *et al.* (2007) Environmental detection of *Batrachochytrium dendrobatidis* in a temperate climate. *Diseases of Aquatic Organisms*, **77**, 105–112.

Wallace JE, Steidl RJ, Swann DE (2010) Habitat characteristics of lowland leopard frogs in mountain canyons of southeastern Arizona. *Journal of Wildlife Management*, **74**, 808–815.

Witte CL, Sredl MJ, Kane AS, Hungerford LL (2008) Epidemiologic analysis of factors associated with local disappearances of native ranid frogs in Arizona. *Conservation Biology*, **22**, 375–383.

CHAPTER 2:

GENETIC AND ENVIRONMENTAL PREDICTORS OF POPULATION RESPONSES TO AN EMERGENT AMPHIBIAN PATHOGEN

Anna E. Savage[1], C. Guilherme Becker[1], and Kelly R. Zamudio[1]

[1] Department of Ecology and Evolutionary Biology, E150 Corson Hall, Cornell University, Ithaca, New York 14853.

Abstract

A central aim of evolutionary biology is understanding how geography and environmental variation shape genetic differentiation in natural populations. The disease chytridiomycosis, caused by the pathogen *Batrachochytrium dendrobatidis* (*Bd*), has led to population die-offs in the lowland leopard frog (*Lithobates yavapaiensis*) since the 1970s, therefore this pathogen has potentially shaped genetic diversity among surviving populations. Here, we used microsatellite loci to characterize genetic differentiation in 11 *L. yavapaiensis* populations with variable disease dynamics. We estimated migration and explored the selective and demographic conditions that may permit populations to adapt to *Bd* at varying levels of gene flow. We found that two *Bd*-uninfected populations belonged to the same genetic deme, whereas each *Bd*-infected population was genetically unique. We detected one outlier locus that deviated from neutral expectations and had a signal of directional selection, indicating possible linkage with a genetic region under selection. Within populations, alleles of this outlier locus were significantly correlated with chytridiomycosis mortality among individuals. Across populations, neutral genetic diversity and environmental variables predicted *Bd* infection and mortality

prevalence, whereas the outlier locus showed no significant patterns. We conclude that genetic isolation across a variable disease landscape in the presence of adaptive genetic diversity has permitted each population to independently evolve resistance to *Bd*, despite similarities among populations in the environmental factors controlling pathogen growth and survival. Continued integration of genetic and environmental contributions to disease dynamics can help resolve *Bd* spatial epidemiology and generate more effective conservation management strategies for mitigating global chytridiomycosis.

Introduction

Infectious diseases are potent agents of natural selection (Darwin 1871) that can impact population demography and have significant effects on population genetic variation, even at ecological timescales (Tishkoff & Verrelli 2003; Campbell *et al.* 2010). Host-pathogen systems are particularly useful for studies of natural selection over short timescales because the potentially strong selective pressure imposed by some infectious agents has the potential to drive rapid host adaptation (Thrall & Jarosz 1994; Dwyer *et al.* 1997; Duffy & Sivars-Becker 2007; Cavatorta *et al.* 2008). While the genetic mechanisms of host resistance, host tolerance, and pathogen virulence are well known in human disease systems (Feng *et al.* 2004; Råberg *et al.* 2007; Barreiro & Quintana-Murci 2010) and in plants (Flor 1956; Fineblum & Rausher 1995; Qihan *et al.* 2010), less is known about the genetic basis for evolving disease resistance in natural animal populations (but see Bernatchez & Landry 2003). One common pattern emerging from studies of natural populations is a positive correlation between host genetic diversity and resistance to disease (Meagher 1999; Zhu *et al.* 2000; Pearman & Garner 2005), a pattern that has been attributed to higher adaptive potential in genetically diverse populations or species (Frankham 2005).

Chytridiomycosis is an emerging infectious disease caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) that has caused population declines or extinction in over 200 amphibian species worldwide (Skerratt *et al.* 2007). In many tropical and sub-tropical regions, chytridiomycosis has spread in an epidemic wave (Lips *et al.* 2006; Cheng *et al.* 2010), causing sudden population and/or species extinctions (La Marca *et al.* 2005) and in some cases, becoming enzootic in host populations that persist (Murray *et al.* 2009; Longo & Burrowes 2010). In other regions, species are infected by *Bd* but have no known history of chytridiomycosis outbreaks (Lane *et al.* 2003; Garner *et al.* 2006; Longcore *et al*. 2007; Savage *et al*. 2011b), while others show intraspecific variation in *Bd* susceptibility among individuals and populations (Savage *et al.* 2011a; Tobler & Schmidt 2010; Kriger *et al.* 2007). These regional epidemiological differences have often been attributed to differences in climatic factors that affect *Bd* growth and/or persistence (Rohr *et al.* 2008). Although lab and field studies now confirm *Bd* dependence on environmental variables such as latitude, elevation, precipitation, and temperature (Carey *et al.* 2006; Kriger *et al.* 2007; Brem & Lips 2008; Rohr *et al.* 2011), few studies have examined host genetic variability underlying chytridiomycosis tolerance or resistance, and how the evolution of genetically resistant populations may occur in a climatically variable landscape. To date, genetic studies of host susceptibility focus on patterns of gene expression (Rosenblum *et al.* 2009) and variation in innate (Woodhams *et al.* 2006, 2007) or acquired (Savage & Zamudio 2011) immune genes, but those studies are either derived from laboratory experiments or do not explicitly consider population genetic changes that may have occurred during or after initial outbreaks of chytridiomycosis. Thus, to fully understand disease outcome in natural populations, we need more information about the distribution of genetic variability in natural populations, and how it relates to the evolutionary potential for disease resistance under naturally variable environmental conditions.

Identifying loci linked to adaptive phenotypic divergence presents a challenge in non-model species with limited genomic resources. However, recent approaches that focus on genetic divergence among natural populations have greatly improved our ability to detect markers under selection using a limited number of loci (Pritchard *et al*. 2000b; Beaumont & Balding 2004; Excoffier *et al.* 2009). Assuming that all loci have experienced the same demographic history, mutation rates, and migration among populations, locus-specific estimates of F_{ST} can be used to identify genomic regions that have been under selection. Specifically, loci with exceptionally high levels of differentiation are more likely to have experienced directional selection, whereas loci with low levels of differentiation were likely constrained by stabilizing selection (Beaumont & Balding 2004; Holsinger & Weir 2009). Associations between environmental variables and allele frequencies often arise when the intensity of selection varies spatially across populations (Novembre & Di Rienzo 2009). We can therefore identify populations experiencing local adaptation by combining data from population disease variables and unlinked genetic markers and comparing them across populations. Chytridiomycosis is a primary cause of worldwide amphibian declines, thus identifying genetic regions potentially correlated with differences in *Bd* exposure outcomes across populations would provide important insights into host responses to varying selective pressure imposed by this emergent pathogen.

Population genetic theory predicts that pathogens can both influence, and be influenced by, vertebrate host genetic diversity (Haldane 1949). However, while numerous studies document individual-level associations between infection and genetic polymorphism (Acevedo-Whitehouse *et al.* 2003; Ortego *et al.* 2007a), the impact of population level genetic diversity on pathogen dynamics remains unresolved. Some studies correlate host population genetic diversity to pathogen prevalence (Meagher 1999; Pearman & Garner, 2005). In contrast, other studies of host-pathogen systems

detect no relationship between genetic variability of the host and prevalence of the pathogen (Ortego *et al.* 2007b; Hawley *et al.* 2010). A simple relationship between host population genetics and pathogen dynamics may be unlikely, given the multitude of environmental factors that can influence disease in natural populations (Osnas & Lively 2004). Indeed, epidemiological researchers highlight the need for studies integrating genetic, spatial, and environmental processes influencing pathogen dynamics and host population genetics (Balkenhol *et al.* 2009; Biek & Real 2010).

The lowland leopard frog, *Lithobates yavapaiensis*, is a stream-dwelling species occurring in desert regions of southwestern North America. A recent range-wide phylogeography based on mitochondrial markers demonstrated that *L. yavapaiensis* populations expanded historically from northwestern Arizona into northern Mexico during the Pleistocene (Oláh-Hemmings *et al.* 2010). However, in recent decades *L. yavapaiensis* has experienced population declines and range contractions (Clarkson & Rorabaugh 1989; Scott 1993) due in part to chytridiomycosis outbreaks (Bradley *et al.* 2002; Savage *et al.* 2011a). Currently, *L. yavapaiensis* persists in the Sonoran Desert of Arizona, USA and Sonora, Mexico and in western New Mexico where it is nearly extirpated (Platz & Frost 1984; Jennings & Hayes 1994; Sredl 2005). The persistence of multiple isolated populations following dramatic chytridiomycosis declines in the 1990s (Bradley *et al.* 2002) and *Bd* infection since at least 1974 (Hale *et al.* 2005; Schlaepfer *et al.* 2007) indicates *Bd* resistance may have evolved in some populations, making this species an ideal candidate for quantifying the evolutionary genetic consequences of an emerging infectious disease.

In this study, we characterize population genetic structure among *L. yavapaiensis* populations and look for associations between genetic variation, environmental variation, and *Bd* disease dynamics. We previously described the prevalence and infection

intensity of *Bd* in Arizona *L. yavapaiensis* populations (Savage *et al.* 2011a) and confirmed that chytridiomycosis varies spatially among populations and temporally among seasons, causing mortality only in winter. Here, we use winter *Bd* infection and mortality estimates, multi-locus host genotypes, and a suite of environmental variables to explore the relationship between host, pathogen, and environment in shaping *Bd* dynamics. We identify whether each genetic locus has a signature of neutral evolution or selection, and analyze each selective category separately. Within populations, where individuals face equivalent environmental regimes, we look for disease associations between individual genetic markers and *Bd* mortality. At a unique thermal spring site with locally variable environmental regimes, we evaluate the evolutionary potential for local adaptation to *Bd* between subpopulations in a case where gene flow counteracts distinct selective regimes. Finally, among populations, where individuals may face distinct environmental conditions, we consider the relative roles of host population genetics and environmental variables in predicting *Bd* dynamics. Together, our analyses provide insight into past, present and future interactions of host population genetics with pathogen dynamics and environmental variation in a declining amphibian species.

Methods

Pathogen sampling

We collected epidermal swabs from *L. yavapaiensis* individuals at eleven localities in Arizona, USA, in winters (January – February) of 2006 through 2011 to characterize *Bd* prevalence, *Bd* infection intensity and *Bd*-associated mortality at each of these localities (Savage *et al.* 2011a). All samples included in this study were collected during winter months because chytridiomycosis outbreaks in Arizona *L. yavapaiensis* populations occur during cool months when temperatures are pathogen-optimal (Savage *et al.* 2011a). *Bd* infection intensity, measured as the number of *Bd* genome equivalents

per swab, was determined by extracting *Bd* DNA from swabs with Prepman Ultra (Applied Biosystems) and using quantitative PCR (qPCR) methods developed for *Bd* quantification (Hyatt *et al.* 2007). We measured winter *Bd* infection prevalence as the number of *Bd* positive swabs divided by the total number of swabs collected (N=145), and *Bd* mortality prevalence as the number of frogs found *Bd* positive and dead or dying divided by the total number of frogs observed (N=483). We calculated 95% Clopper-Pearson binomial confidence intervals for prevalence from the observed proportions of infection and mortality in each population sample.

The Muleshoe Ranch (MR) locality included three neighboring sub-populations with variable environmental conditions affecting disease prevalence: (1) Muleshoe Ranch Hot Spring (MR_{HS}) is a thermal spring that is >50°C at the source and >30°C throughout the region where frogs were sampled, (2) Muleshoe Ranch Secret Spring (MR_{SS}) is a nearby pond fed by a small thermal spring that is >30°C at the source but below 30°C elsewhere, and (3) Muleshoe Ranch Bass Canyon (MR_{BC}) is a nearby canyon stream that is not fed by a thermal spring (Schlaepfer *et al.* 2007). Frogs from MR_{HS} and MR_{SS} were *Bd*-infected but demonstrated no winter chytridiomycosis mortality, whereas some frogs from MR_{BC} were both *Bd*-infected and died from chytridiomycosis in winter months (Savage *et al.* 2011a). For purposes of analyses, the MR locality was therefore divided into the two populations, $MR_{HS/SS}$ and MR_{BC}, for comparisons of disease status.

Host population sampling and microsatellite genotyping

We collected toe tips from frogs at each of the 11 study populations (mean=46 ± 21 individuals per site) in summers (July-August) and winters (January-February) of 2006-2010. In total we genotyped 509 individuals at fourteen unlinked microsatellite loci (Savage & Jaeger 2009), including all of the individuals swabbed in winter to infer *Bd*

infection prevalence and intensity. Template DNA for microsatellite genotyping was extracted using a 5% Chelex 100 solution (Bio-Rad Laboratories) with 0.5 μg proteinase K per sample. Chelex extractions were incubated at 55°C for 120 minutes, and 99°C for 10 minutes. Supernatants from these extractions were used directly as template for PCR amplification, performed under the following conditions: 5 min initial denaturation at 94°C; 35 cycles of 1 min denaturing at 94°C, 1 min annealing at primer-specific annealing temperatures, 1 min extension at 72°C; and a final extension of 75°C for 5 min. Amplified products with different fluorescent labels or non-overlapping size ranges were multiplexed and electrophoresed on a 3730 Genetic Analyser (Applied Biosystems). Fragments were sized by comparison to the LIZ-500 standard using GeneMapper version 3.5 (Applied Biosystems). We used MICRO-CHECKER 2.2.3 (van Oosterhout *et al.* 2004) to test for the presence of scoring errors and null alleles.

Population genetic structure and diversity

We used STRUCTURE version 2.1 (Pritchard *et al.* 2000a) to identify the most likely number of genetic demes (*K*) present in our sample and the coefficient of membership to those demes for each *L. yavapaiensis* individual genotyped. STRUCTURE uses a Bayesian algorithm to cluster genotypes in the absence of any geographic information. We used a model allowing admixture and assuming uncorrelated gene frequencies, and ran 3,000,000 Markov chain Monte Carlo (MCMC) iterations after a burn-in of 1,000,000 iterations. We assessed adequacy of run parameters by examining convergence of key summary statistics (Pritchard *et al.* 2000a). We conducted 20 independent runs for each value of *K* and retained the run with the highest likelihood, and then used the second-order rate of change to determine the most likely value of *K (*Evanno *et al*. 2005).

We used GENEPOP 3.4 (Raymond & Rousset, 1995) to calculate observed and

expected heterozygosities and test for deviations from Hardy-Weinberg equilibrium (HWE) at each locus and population locality using a Monte Carlo chain method (1000 dememorizations, 100 batches, 1000 iterations; Guo &Thompson 1992) and a Bonferroni correction for multiple tests for a table-wide significance level of α=0.05 (adjusted *P*=0.0002). We estimated *D*, a measure of differentiation (Jost 2008), across all population pairs using Software for the Measurement of Genetic Diversity (SMOGD) version 1.2.5 (Crawford 2009). We used FSTAT 2.1 (Goudet 1995) to test for linkage disequilibrium at each locus over all populations (adjusted *P*=0.00004) and to compute pairwise F_{ST} values and their significance among localities (adjusted *P*=0.0005). We calculated genetic diversity indices for each population using the program GENALEX version 6 (Peakall & Smouse 2006).

Inter-population migration and the potential for adaptive immunity

We characterized migration among populations using BAYESASS version 1.3 (Wilson & Rannala 2003), which uses Bayesian analysis and MCMC to estimate mean immigration rates among populations and their confidence intervals (CI). We assumed negligible drift over the last two generations, and that immigrants in a population did not exceed 33% per generation. Further, loci were assumed to be in linkage equilibrium and free of null alleles. We performed 9,000,000 iterations (following a 1,000,000 burn-in) and sampled every 2000 iterations.

To explore the dynamics of gene flow, drift and selection among neighboring populations differing in disease epidemiology, we focused on one locality, the Muleshoe Ranch (MR), where populations with different disease dynamics occur in close proximity and migration could thus potentially have large effects on the potential evolution of *Bd* resistance. We followed the model employed by Adkison (1995) and McCairns & Bernatchez (2008) to define the necessary demographic conditions leading to adaptive

divergence among the MR populations MR_{HS} and MR_{BC}. The model is based on a numerical approximation of Slatkin's (1973) characteristic length scale of variation in gene frequency (l_c), which defines the minimal cline distance at which populations cannot respond to environmental variation. Predictions of three alternative scenarios – genetic homogenization (H), differentiation due to random drift (R), or adaptive divergence (A) – are based on two derived variables: β, the ratio of migration to drift, and *k*, the ratio of the geographical scale at which selection favors a given allele (*j*) relative to l_c (Nagylaki & Lucier 1980). Given that MR_{HS} and MR_{BC} fall into different disease categories, we took the stringent view that these localities represent independent populations with different selection acting in each (*j*=1). We used a range of estimates of effective population size (N_e) and strength of selection (*s*) to infer the conditions likely to lead to adaptive divergence (A: β>1.1; *k*>1.1), random differentiation (R: β<1), or genetic homogeneity (H: β>1.1; *k*<1) across the 95% confidence interval of estimated migration among MR_{HS} and MR_{BC}.

Loci under selection

Loci that deviate from neutral evolutionary expectations, hereafter termed 'outlier' loci, potentially carry a signature of natural selection. We detected outlier loci in our study taxon using the Beaumont & Nichols (1996) Fdist approach implemented in LOSITAN (Antao *et al.* 2008). This method is based on the theoretical expectation that, for a range of population structures and demographic histories, genetic differentiation among populations in contrasting environments should be different for loci under selection when compared to neutral loci. LOSITAN uses coalescent simulations to generate a null distribution of F_{ST} values based on an infinite island model, and loci with unusually high F_{ST} values are putatively under directional selection, while loci with low F_{ST} values are potentially under stabilizing selection. We simulated the neutral F_{ST}

distribution with 100,000 iterations and used a conservative significance threshold of $P < 0.005$. We then calculated all pairwise population F_{ST} values and compared them to population heterozygosity measures to identify F_{ST} outliers. Runs were performed using two possible mutation models: the stepwise mutation model, most commonly applied to microsatellite markers (Valdés *et al.* 1993) and the infinite allele model.

Genetic predictors of disease within populations

We tested for associations between individual microsatellite genotypes and chytridiomycosis susceptibility among *Bd*-infected *L. yavapaiensis* individuals using STRAT (STRuctured population Association Test; Pritchard *et al*. 2000b). STRAT controls for spurious disease-allele associations that arise due to the presence of common alleles with unequal frequencies across genetic groups by examining disease associations independently for each genetic deme identified using Structure (Pritchard *et al*. 2000a). The test statistic Λ represents the likelihood of association between allele frequencies and disease phenotype within genetic groups. We assigned disease phenotypes as 0 if the individual was found alive without disease signs, and 1 if the individual was found dead or dying. *Bd* mortality (i.e., dead infected frogs) is an unambiguous assignment, whereas frogs sampled alive could potentially have died at future time points, therefore this analysis is designed to detect genetic associations with *Bd* susceptibility, but not *Bd* resistance. We generated Λ for each of the 14 microsatellite loci and inferred significance probabilities by comparison to 10,000 random simulations of genotype frequencies within genetic groups.

Environmental and genetic predictors of disease across populations

We used Generalized Linear Models (GLMs) to test for associations of disease variables (*Bd* mortality prevalence, infection prevalence, and infection intensity) with

genetic and environmental variables across populations. For environmental variables, we obtained canopy density information from Global Forest Resource Assessment [1000m resolution; coverage period 1995-1996 (USGS-FAO 2000)] and elevation data (90m resolution) from CGIAR Consortium for Spatial Information (Jarvis *et al.* 2009). Additionally, nineteen bioclimatic variables were extracted using Worldclim/Bioclim layers (1000m resolution) available at <http://www.worldclim.org/bioclim> (Hijmans *et al.* 2005). These metrics of temperature and precipitation are averaged from fifty-year records (1950-2000) from a dense network of climatic stations throughout the world (e.g. precipitation records from 47,554 locations, temperature from 24,542 locations). All environmental variables were measured at a 1km diameter buffer from sampling locations to maintain a consistent scale across all variables in the analyses. We also included maximum water temperature for each site, averaged across winter field surveys (3-5 measurements per visit). We used ArcGIS 9.3.1 in all analyses (ESRI 2009). For genetic variables, we used GENALEX version 6 (Peakall & Smouse 2006) to calculate population measures of observed heterozygosity, expected heterozygosity, mean number of effective alleles, and mean number of private alleles for the 13 neutral loci combined and for the outlier locus (C110).

We identified important genetic factors predicting each of the response variables (*Bd* mortality prevalence, infection prevalence, and infection intensity) using linear regressions. To test for an association between genetic factors and both *Bd* mortality prevalence and *Bd* infection prevalence*,* we used GLMs with binomial distribution and logit link. To test for an association between genetic factors and *Bd* infection intensity we used GLMs with normal distribution and identity link. After this first univariate assessment, we used stepwise regressions (forward method; cutoff $P < 0.20$) to screen for environmental factors that potentially predict each of the response variables to be included in model selection procedures. For each response variable,

we screened a total of 23 explanatory variables including canopy density, latitude, elevation, mean winter maximum temperature, and the 19 bioclimatic temperature and precipitation metrics. We confirmed lack of spatial autocorrelation within each response variable using Moran's I correlograms (Rangel *et al*. 2010). We used GLM model selections including environmental and genetic variables to explain each response variable and tested all possible models without interactions. Competing models were ranked based on Akaike Information Criterion (AICc), and we report the best-fit model for each run.

Results

Bd *infection and chytridiomycosis mortality in* L. yavapaiensis

We collected tissue samples and skin swabs from 509 *L. yavapaiensis* individuals from 11 localities in Arizona, USA (Figure 2.1). Observed levels of *Bd* infection and chytridiomycosis mortality vary significantly within and among these localities in winter months (Savage *et al.* 2011a). Our focal populations occupy a narrow range of elevations, and variation in elevation did not explain the observed disease patterns (Table S1.1). All populations were infected with *Bd* in winter months except for House Spring (HS) and Aliso Spring (AS). Of the infected populations, five experienced winter chytridiomycosis mortalities and five did not (Figure 2.2A). Mean *Bd* infection intensity was not significantly different between populations with and without winter mortality (Figure 2.2B). It is therefore unclear whether adaptation to *Bd* in *L. yavapaiensis* occurs via a mechanism of disease resistance (limiting pathogen burden) or disease tolerance (limiting the damaging effects of a given pathogen burden; Råberg *et al.* 2007), and mechanisms of survival may be variable across populations. For clarity, we hereafter refer to any process of surviving *Bd* infection as *Bd* resistance, although we are aware that tolerance may play a role.

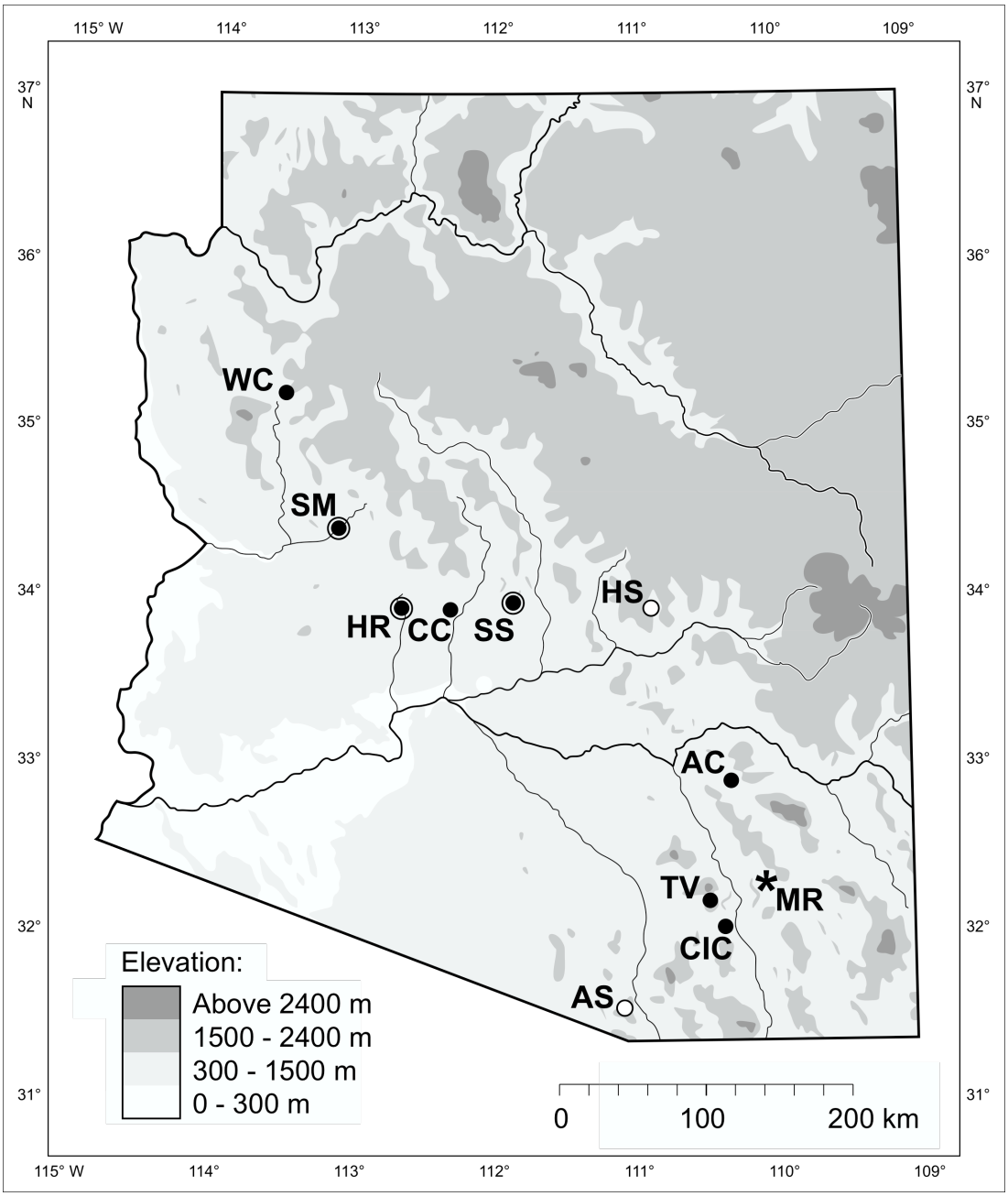

Figure 2.1: Sampled *Lithobates yavapaiensis* populations in Arizona, USA. White symbols denote *Bd* uninfected populations, black symbols denote *Bd* infected populations, single circles denote populations without mortality, and double circles denote populations with mortality. The asterisk marks the location of the Muleshoe Ranch thermal spring population. Locality abbreviations are as follows: AS = Aliso

Spring; AC = Aravaipa Canyon; CC = Cottonwood Canyon; CIC = Cienega Creek; HS = House Spring; HR = Hassayampa River; MR = Muleshoe Ranch; SM = Santa Maria River; SS = Seven Springs; TV = Tanque Verde Canyon; WC = Willow Creek.

Table 2.1: Pairwise estimates of F_{ST} (upper) and *D* (lower) for each collection locality. All values are significant except those in italics.

	CC	AC	TV	AS	CIC	MR_{BC}	SM	WC	HR	SS	HS
CC	–	0.400	0.564	*0.565*	0.473	0.411	0.377	0.440	0.335	0.268	0.604
AC	0.713	–	0.315	0.318	0.237	0.161	0.269	0.301	0.244	0.275	0.328
TV	0.825	0.541	–	*0.393*	0.248	0.275	0.405	0.479	0.406	0.386	0.483
AS	0.891	0.674	0.500	–	*0.275*	0.239	0.312	*0.395*	*0.336*	0.348	*0.412*
CIC	0.785	0.526	0.337	0.507	–	0.178	0.312	0.356	0.335	0.299	0.357
MR_{BC}	0.764	0.427	0.502	0.534	0.447	–	0.253	0.256	0.257	0.247	0.328
SM	0.749	0.735	0.859	0.764	0.764	0.699	–	0.204	0.159	0.221	0.356
WC	0.755	0.773	0.885	0.844	0.816	0.634	0.492	–	0.180	0.227	*0.452*
HR	0.608	0.597	0.804	0.776	0.825	0.684	0.414	0.501	–	0.194	0.392
SS	0.505	0.719	0.784	0.838	0.729	0.674	0.610	0.624	0.571	–	0.366
HS	0.869	0.640	0.600	0.556	0.597	0.687	0.779	0.840	0.817	0.732	–

Genetic diversity and differentiation by disease category

Our 14 microsatellite markers were highly polymorphic across sampled populations (Figure 2.3). The total number of alleles per locus ranged from 10 to 33 with a mean value of 19; within populations, the mean number of alleles ranged from 2.7 to 10.0 (Figure 2.3). Nine populations had at least one locus with a significant deviation from HWE, but no locus showed deviation in a majority of populations (Table S2.2), thus we included all data in further analyses. Pairwise estimates of F_{ST} and Jost's *D* indicated high genetic differentiation among all populations except for the three neighboring MR populations (Table 2.1). Excluding comparisons among the MR populations, mean

pairwise F_{ST} was 0.32 (range 0.17-0.60) with 90% of comparisons significant (adjusted *P* = 0.000549) and mean *D* was 0.64 (range 0.41-0.91) with all comparisons significant (95% confidence intervals did not include zero).

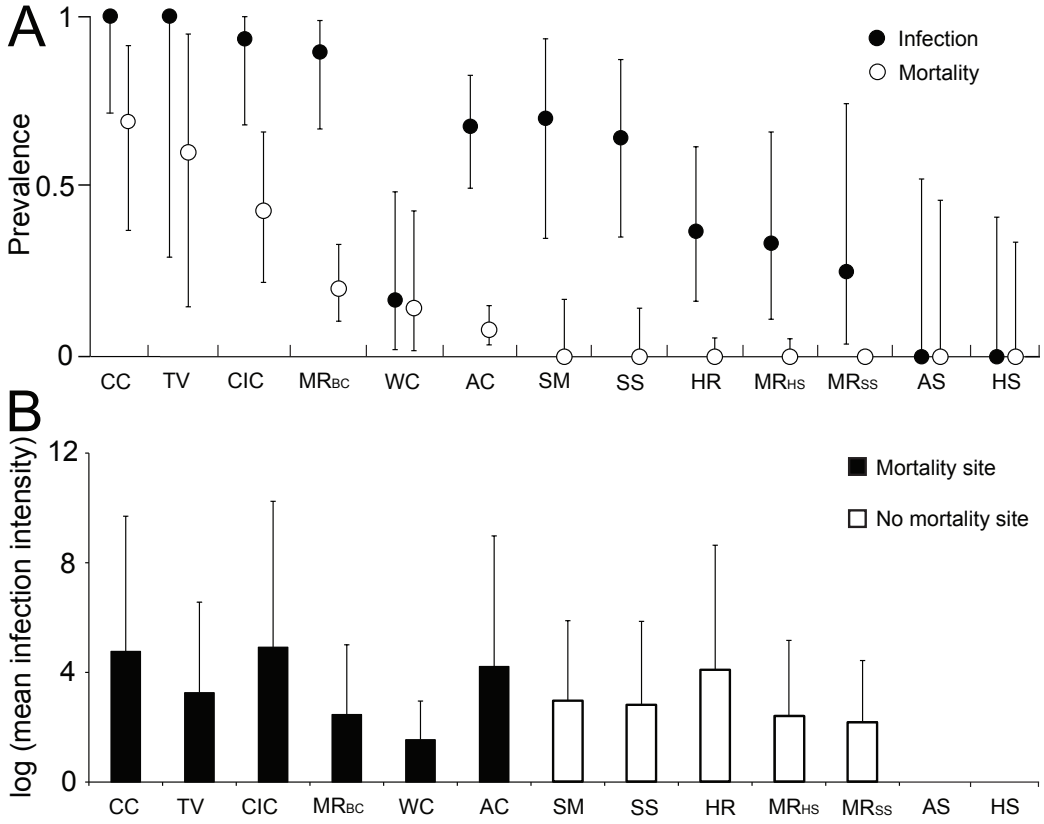

Figure 2.2: (A) *Lithobates yavapaiensis* observed winter *Bd* infection (closed symbols) and chytridiomycosis mortality (open symbols) by locality, with 95% Clopper-Pearson binomial confidence intervals. (B) Logarithm of mean population winter *Bd* infection intensity measured as the average number of genome equivalents recovered per animal.

The two *Bd* uninfected populations had lower heterozygosity, number of alleles, and number of effective alleles compared to all other populations (Figure 2.3). *Bd* tolerant populations (SM, HR, SS, MR) showed higher diversity, number of alleles, and number of effective alleles than *Bd* susceptible populations (MR, CIC, CC, AC, TV, WC); however, across *Bd* susceptible populations these measures varied considerably. Few private alleles were detected in any population, but MR, AC, and SM had the highest values (Figure 2.3).

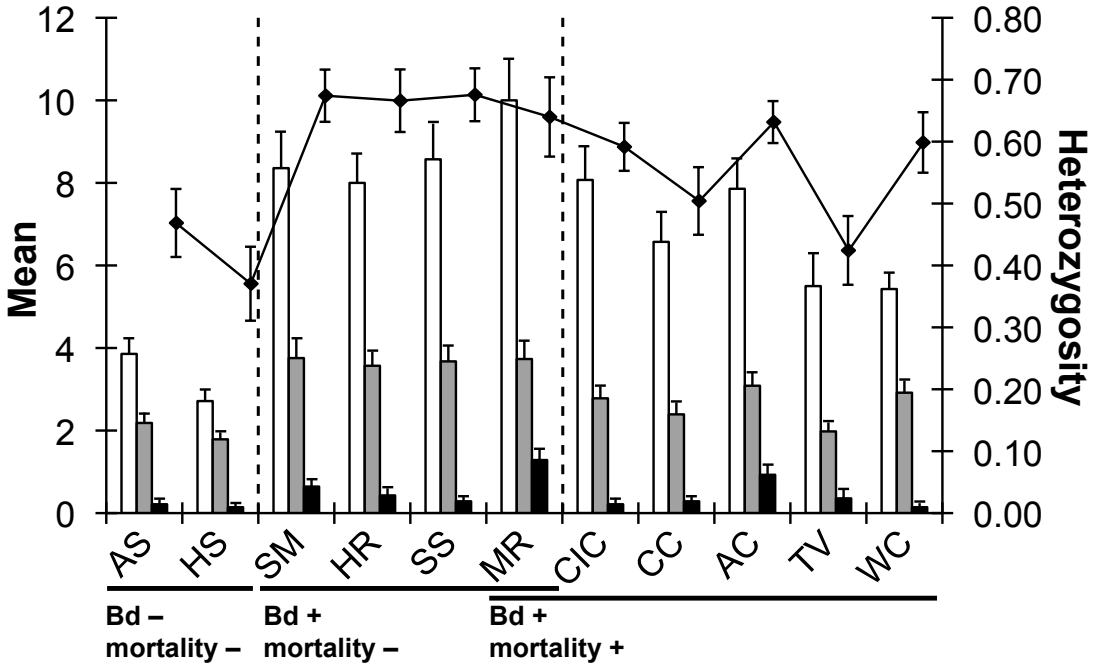

Figure 2.3: Patterns of allelic richness and heterozygosity in 11 sampled populations of *Lithobates yavapaiensis* genotyped at 14 microsatellite loci. Bars represent mean ± SD number of alleles (white bars), mean ± SD number of effective alleles (grey bars), and mean ± SD number of private alleles (black bars). Mean ± SD heterozygosities for each population (across all loci) are represented by the black line. Populations are arranged according to *Bd* infection dynamics, ranging from no infection or mortality to infection only to infection with mortality.

Structure analyses revealed 10 genetic clusters or demes that corresponded to the 11 geographic sampling localities with one exception: individuals from localities House Spring (HS) and Aliso Spring (AS) were assigned to the same genetic deme despite the large geographic distance separating these populations (265 km) and the presence of four genetically distinct populations in the intervening region (Figure 2.4). Notably, these two populations were assigned to the same genetic deme for values of *K* ranging from 6-12, indicating a strong signal of genetic ancestry. Individuals from all other geographic populations were assigned to independent genetic demes with an average *q* of 0.89 (range 0.74-0.95). Hereafter, locality names will thus refer to both genetic and geographic populations, with the label HS/AS for the House Spring/Aliso Spring shared deme.

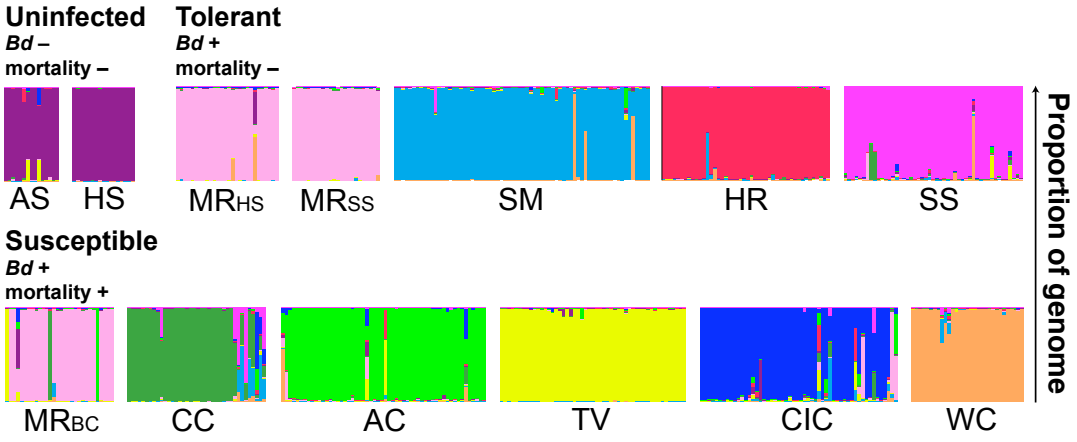

Figure 2.4: Structure analysis results for *K* = 10 with individuals grouped by locality and disease status. Vertical bars show the proportion of membership to each genetic deme for 509 *Lithobates yavapaiensis* individuals genotyped at 14 microsatellite loci. Population abbreviations follow Figure 2.1. The 10 genetic demes are represented as follows: AS/HS = purple; MR = light pink; SM = light blue; HR = red; SS = pink; CC = dark green; AC = light green; TV = yellow; CIC = blue; WC = tan.

Migration and the potential for adaptive immunity

BAYESASS estimates indicated negligible contemporary migration (*m*) among all pairs of populations (*m* = 0-6% immigrant ancestry, mean *m* = 0.02%, all values non-significant) except among the sub-populations within the MR locality (Figure 2.5; Table S2.3). Migration among these sub-populations was considerable, statistically significant, and unidirectional from the thermal springs locality (MR_{HS}) into both the pond (MR_{SS}; *m* = 27% immigrant ancestry from MR_{HS}) and the canyon (MR_{BC}; *m* = 21% immigrant ancestry from MR_{HS}). Migration was not detected between MR_{SS} and MR_{BC}, or from either of these localities back to MR_{HS}.

We modeled the potential for the MR_{BC} subpopulation to evolve chytridiomycosis resistance given the high migration rate from the thermal springs, which environmentally shelters frogs from chytridiomycosis due to perennially high temperatures unfavorable to *Bd* (Savage *et al.* 2011a; Schlaepfer *et al.* 2007). We parameterized adaptive divergence models with a broad range of effective population sizes (N_e) ranging from 10-10,000 individuals, and included migration rates spanning the 95% confidence interval of estimated dispersal from MR_{HS} into MR_{BC} (14 – 29% per generation). Assuming that *Bd*-imposed selection is zero at the thermal springs, the model predicts that the strength of selection (*s*; range 0-1) for chytridiomycosis resistance in the canyon must be greater than 0.07 for the MR_{BC} frogs to adapt at the lower 95% CI of migration, independent of N_e (Table 2.2). If migration rates are closer to the estimated mean value of 21%, *s* must be greater than 0.11 for the canyon frogs to evolve *Bd* resistance, regardless of N_e.

Table 2.2: Predicted values of effective population size (N_e) and strength of selection (s) leading to adaptive divergence (A), genetic homogenization (H), or random differentiation (R) across the 95% confidence interval range of migration rates (m) estimated from site MRHS into site MRBC. Predictions are based on the model of Nagylaki & Lucier (1980).

N_e	m	s = 0.001 - 0.07	s = 0.071 - 0.10	s = 0.11 - 1
10	0.14	R	A	A
100	0.14	**H**	A	A
1000	0.14	**H**	A	A
10,000	0.14	**H**	A	A
100,000	0.14	**H**	A	A
10	0.21	R	**H**	A
100	0.21	**H**	**H**	A
1000	0.21	**H**	**H**	A
10,000	0.21	**H**	**H**	A
100,000	0.21	**H**	**H**	A
10	0.29	R	**H**	A
100	0.29	**H**	**H**	A
1000	0.29	**H**	**H**	A
10,000	0.29	**H**	**H**	A
100,000	0.29	**H**	**H**	A

Detection of a locus under selection and disease associations within populations

The LOSITAN F_{ST} analysis identified one of the 14 microsatellite loci as an outlier in 121 pairwise comparisons ($P < 0.005$). Locus *Ro*C110 was found to be under directional selection with an exceptionally high F_{ST} value (Figure 2.6A). All other loci fell within the expected range for neutrally evolving genetic markers.

The 10 genetic demes identified using Structure were further analyzed to test for associations between microsatellite allele frequencies and two chytridiomycosis phenotypes: (1) alive, meaning the individual was observed to be alive and healthy regardless of *Bd*-infection status, or (2) dead, meaning the individual was *Bd*-infected and found dead or dying with signs of chytridiomycosis. Locus *Ro*C110 showed a significant association between allele frequencies and *Bd* infection phenotype within

genetic groups (Λ=21.09, df=11, *P*=0.009). In populations with *Bd* mortality, allele frequency distributions for this locus were significantly different between dying and surviving individuals (Figure 2.6B). However, the same alleles were not associated with chytridiomycosis phenotype across different genetic demes.

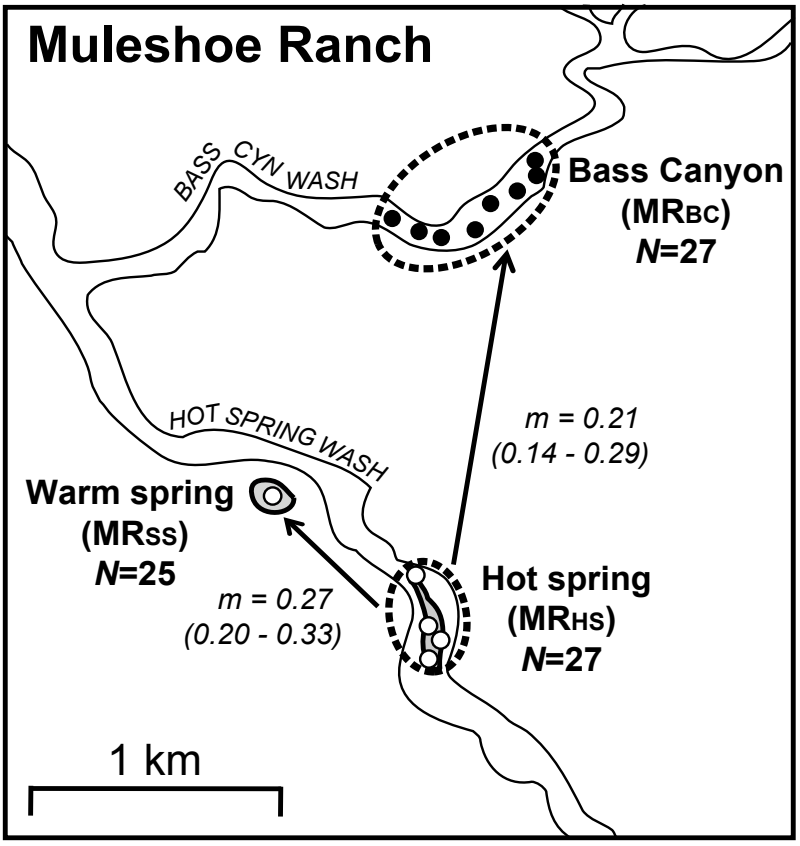

Figure 2.5: BAYESASS estimates of migration (*m*) among Muleshoe Ranch (MR) *Lithobates yavapaiensis* subpopulations (*N*=sample size). Open circles indicate sampling from localities with *Bd* infection but no chytridiomycosis mortality. Filled circles indicate sampling from localities with *Bd* infection and chytridiomycosis mortality. Arrows show the presence and direction of migration. For each subpopulation, 95% confidence intervals for the proportion of individuals with immigrant ancestry are indicated in parentheses.

Genetic and environmental predictors of disease

Among all population genetic measurements, the best predictors of population disease status were observed heterozygosity and number of private alleles for the neutral loci, and observed heterozygosity for the outlier locus. For the neutral loci, observed heterozygosity significantly predicted mortality prevalence (β = -9.699, χ^2 = 19.067, P < 0.001), but was not associated to *Bd* infection prevalence (β = -1.951, χ^2 = -0.733, P = 0.392) or *Bd* infection intensity (β = 2.735, χ^2 = 0.309, P = 0.578). We also found a positive association between number of private alleles, *Bd* infection prevalence (β = 1.620, χ^2 = 11.463, P < 0.001), and *Bd* infection intensity (β = 0.693, χ^2 = 0.354, P = 0.552), but not *Bd* mortality prevalence (β = 0.410, χ^2 = 0.736, P = 0.391). For the outlier locus, we did not find significant associations of heterozygosity with mortality prevalence (β = 1.240, χ^2 = 1.827, P = 0.176) or *Bd* infection intensity (β = 1.053, χ^2 = 0.205, P = 0.650), although we found a marginal association with *Bd* infection prevalence (β = 1.737, χ^2 = 3.745, P = 0.053).

Our initial stepwise screening for meaningful environmental variables selected four variables to explain mortality prevalence, two variables to explain *Bd* infection prevalence, and eight variables to explain *Bd* infection intensity. When jointly considering these effects in a model selection approach including all possible models, genetic factors remained as strong predictors of *Bd* infection prevalence and mortality prevalence, but not *Bd* infection intensity (Table S2.4). Among the best models, *Bd* mortality prevalence had a strong negative association with neutral observed heterozygosity, mean diurnal temperature range, and annual precipitation (Table 2.3). Likewise, *Bd* infection prevalence had a strong negative association with neutral observed heterozygosity. In the same model, the number of private alleles and minimum temperature of the coldest month positively predicted *Bd* infection intensity (Table 2.3). None of the genetic factors were included in the best models predicting *Bd*

infection intensity (Table S2.4). In the most likely model, three temperature and rainfall metrics were the best explanatory variables (Table 2.3).

Table 2.3: Generalized Linear Models simultaneously testing the effects of genetic and environmental factors on *Bd* mortality prevalence, infection prevalence, and infection intensity among 11 populations of *L. yavapaiensis* in Arizona.

***Bd* Mortality Prevalence**

Term	*β*	*SE*	χ^2	*P*
Constant	*64.121*	*14.197*	*48.016*	*<0.001*
Observed Heterozygosity (Ho)	-13.335	2.839	27.772	<0.001
Mean Diurnal Temperature Range	-0.324	0.072	46.067	<0.001
Annual Precipitation	-0.157	0.006	10.929	0.001

***Bd* Infection Prevalence**

Term	*β*	*SE*	χ^2	*P*
Constant	*5.031*	*1.854*	*8.379*	*0.004*
Observed Heterozygosity (Ho)	-12.441	4.073	11.077	0.001
Number of Private Alleles	3.355	0.780	25.197	<0.001
Min Temperature of Coldest Month	0.060	0.017	20.161	<0.001

***Bd* Infection Intensity**

Term	*β*	*SE*	χ^2	*P*
Constant	*35.323*	*3.075*	*28.205*	*<0.001*
Temperature Annual Range	-0.072	0.007	24.597	<0.001
Precipitation of Wettest Month	-0.089	0.006	31.858	<0.001
Precipitation of Driest Quarter	-0.038	0.011	7.741	0.005

Whole model tests: mortality prevalence (χ^2 = 65.328, DF = 3, P < 0.001); *Bd* infection prevalence (χ^2 = 44.767, DF = 3, P < 0.001); *Bd* infection intensity (χ^2 = 34.719, DF = 3, P < 0.001). Final models chosen based on AICc.

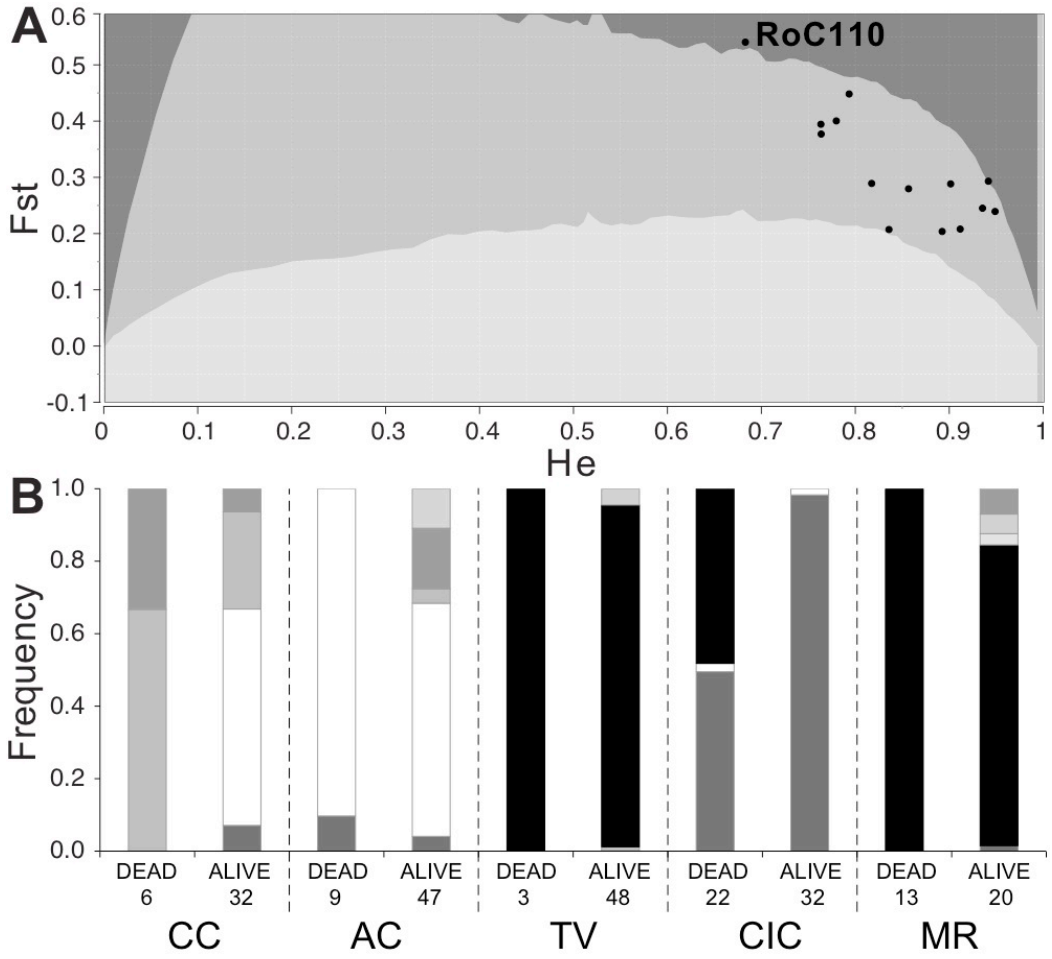

Figure 2.6: (A) F_{ST} versus heterozygosity for fourteen microsatellite loci. The dark gray area shows the upper 99.5% confidence interval and the white area shows the lower 99.5% confidence interval with 10,000 simulations from the stepwise mutation model. (B) Allele frequencies of the outlier locus *Ro*C110 that showed significant associations with Bd mortality within genetic demes. Numbers of individuals and allele frequencies among alive and dead *L. yavapaiensis* individuals are shown for the five genetic demes that experience *Bd*-associated mortality. Each shade of grey represents a different allele.

Discussion

Host population genetics and infectious diseases interact spatially and temporally, producing epidemiological patterns that can both shape current host population structure and be shaped by historical host population demography (Boots *et al.* 2004, Biek *et al.* 2006). Our study demonstrates the utility of combining measures of disease dynamics with environmental variables and genetic differentiation at neutral and outlier loci to disentangle the effects of environment, demography, gene flow, and selection on *Bd* epidemiological patterns among populations. We identified an outlier locus and compared it with neutral loci, permitting the detection of spatial genetic correlations with disease variables while excluding historical, demographic, or other population processes. To date this approach has been used infrequently (Luikart *et al*. 2008; Gebremedhin *et al.* 2009; Richter-Boix *et al*. 2011), but it has immense potential to elucidate genetic adaptation in locally varying selective environments (Lowry 2010). Our study is also the first, to our knowledge, that models genetic and environmental chytridiomycosis covariates simultaneously, revealing that host population genetics are significant predictors of disease dynamics when considering the entire selective, genetic, and environmental landscape.

Population structure and disease

Pairwise differentiation measures, genetic clustering analysis, and gene flow estimates each demonstrated that *L. yavapaiensis* populations are highly genetically differentiated. This extensive genetic structure differs from a recent mitochondrial phylogeographic study that showed all *L. yavapaiensis* populations except one belonged to a nearly undifferentiated group with haplotypes varying at most by two nucleotides (Oláh-Hemmings *et al.* 2010). This discordance is expected, because microsatellite markers with more rapid mutation rates, reflect more recent demographic processes,

and offer the resolution to infer the limits to gene flow in this system. The number and size of *L. yavapaiensis* populations have declined considerably in the last half-century due to severe fragmentation, alteration of aquatic habitats, introduced species, and chytridiomycosis (Sredl 2005). While the mitochondrial analysis revealed a strong signature of species expansion thousands of years before any of these factors were present, our results indicate that recent fragmentation and declines may have resulted in the observed loss of genetic connectivity among populations.

Structure analyses revealed a common genetic background for *Bd* uninfected populations, in stark contrast to the distinct genetic background for each *Bd* infected population (Figure 2.4). Membership to the same genetic deme for the two populations (HS and AS) that have likely never been exposed to *Bd* may arise due to a common genetic signature of *L. yavapaiensis* population structure in the absence of *Bd*. However, these populations also had the lowest measures of heterozygosity, number of alleles, and effective number of alleles (Figure 2.3), and both have exceptionally small population sizes (Savage *et al.* 2011a). Thus, isolation and drift are the likely forces shaping the distribution of genetic differentiation in these two populations, and further identification and sampling of *Bd*-uninfected populations will be necessary to determine the precise effects of the presence or absence of *Bd* infection on *L. yavapaiensis* genetic structure. Due to the current lack of migration and genetic distinctiveness of each *Bd* infected population, we conclude that *L. yavapaiensis* populations with *Bd* are evolving independently and each may adapt to disease pressure in novel and unique ways.

Disease dynamics among sub-populations

A recent review of associations between genetic variation and fitness traits across 34 amphibian studies found that genetic-fitness correlations were documented in 65% of cases (Allentoft & O'Brien 2010). In contrast, a second review focusing on longer-term

studies across organisms with good understanding of the traits under selection indicated that selection may exhaust genetic variance, thereby limiting a host's response (Blows & Hoffmann 2005). Both reviews concluded that determining the extent of and constraints on genetic-fitness correlations would require more knowledge of the ecological settings and genetic background of natural populations than is currently available for most systems.

We exploited the environmental variation present at Muleshoe Ranch (MR) due to the thermal spring (Savage *et al*. 2011a) to dissect the role of fine-scale host evolutionary genetic processes versus environmental variation in constraining or allowing populations to adapt to *Bd*. The thermal spring water temperatures at MR_{HS} and MR_{SS} exceed the limits for optimal *Bd* growth (Piotrowski *et al.* 2004; Schlaepfer *et al.* 2007) or pathogenicity (Carey *et al*. 2006), whereas MR_{BC} winter water temperatures are cool enough to promote *Bd* proliferation (Savage *et al.* 2011a). All three MR subpopulations were infected with *Bd* but experienced distinct selective pressures due to this environmental variation. We predicted that MR_{BC} individuals were under selection for chytridiomycosis resistance, while the other subpopulations were environmentally sheltered from this selective pressure. However, MR_{BC} belongs to the same genetic deme as MR_{HS} and MR_{SS} and was not significantly differentiated based on pairwise F_{ST} and *D* values. This lack of differentiation occurred because 14-29% of the MR_{BC} subpopulation consisted of first- or second-generation immigrants from MR_{HS} (Figure 2.5). Modeling the parameters of migration, drift, and selection necessary to create this scenario showed that the selection coefficient imposed by *Bd* would need to be high ($s > 0.11$) for MR_{BC} frogs to adapt given the annual influx of susceptible genotypes from MR_{HS}. A selection coefficient of 0.11 corresponds to an 11% increase in fitness of individuals with the advantageous genotype when compared to individuals lacking that genotype. In genome-wide studies of polymorphisms in *Drosophila*, estimated selection

coefficients for single mutations range from 0.000012 to 0.02 (Jenson *et al.* 2008). In studies of human HIV, a highly virulent pathogen that imposes strong selection for resistance, whole-genome analyses detect selection coefficients for positively selected genotypes ranging from 8.0E−3 (Neher & Leitner 2010) to 0.09 (Liu *et al.* 2002). Thus, the threshold of 0.11 for MR_{BC} frogs is unlikely to be met, even when selection for chytridiomycosis resistance is strong. Because the evolution of host resistance is most likely a fitness trade-off (Anderson & May 1982) and MR_{BC} frogs only face punctuated selection for disease resistance in cooler months when *Bd* is most virulent (Carey *et al.* 2006), it is unlikely that selection will be strong enough for this population to become fixed for resistance to chytridiomycosis.

Disease associations within populations

We found one candidate locus, *Ro*C110, that was an F_{ST} outlier and showed significant allelic associations to *Bd* mortality within genetic groups. Comparing allele frequencies among dead and live frogs showed that this pattern is largely explained by mortality within population CIC. In this population, sample sizes were large enough to determine that a significant proportion of dead frogs had an allele that was never found in live frogs (Figure 2.6B, black fill). Notably, this allele was nearly fixed in dead and live frogs from population MR, the locality where gene flow and environmental sheltering likely overwhelms selection and prevents sub-populations from adapting to *Bd*. This allele was also nearly fixed in population TV, a locality with high winter *Bd* mortality that has possibly been extirpated since 2009 (Savage *et al.* 2011a). Taken together, these patterns suggest that within-population *Bd* susceptibility may include a functional genetic component linked to allelic variation at locus *Ro*C110.

Molecular adaptation to local ecological or environmental factors (including pathogens) is commonly explored in model species or non-model taxa with extensive

genomic resources (De La Vega *et al.* 2002). In contrast, genetic signatures of adaptation are rarely explored in wildlife populations, likely due to the limited number of molecular markers available. Indeed, for traits under weak selection or for quantitative traits determined by multiple loci with small effects, significant patterns are unlikely to be detected from a limited set of loci. However, as the strength of selection on a given trait increases, so does the extent of linkage disequilibrium (Lewontin & Kojima 1960; Schork 2002). Thus, for populations facing strong selective pressure – for example, from a disease such as chytridiomycosis that causes massive population die-offs – selection may be sufficiently strong to create genetic associations detectable by a smaller number of unlinked genomic markers. Indeed, the 'local-effects' hypothesis, in which genotype-fitness associations result from a physical association between a neutral marker and a locus under selection (David 1998), has gained recent empirical support from studies showing that natural populations can show high levels of linkage disequilibrium (Yan *et al.* 1999; Reich *et al.* 2001) and that some loci contribute more than others to fitness associations (Hanson *et al.* 2004; Acevedo-Whitehouse *et al.* 2006).

The functional genetic region linked to *Ro*C110 that may be responsible for producing significant associations with disease dynamics is not known; however, we suspect an immunity gene or haplotype, which could have large enough effects on survival to generate broad linkage disequilibrium with a microsatellite marker. Given the modest sample sizes and number of loci used in our study, further work is necessary to determine whether a functional genetic basis exists for the disease associations we detected. However, even if this association is ultimately determined to have a non-selective basis, the separation of purportedly neutral loci into outlier and neutral-conforming categories that are analyzed separately is critical for accurate assessment of neutral population structure, especially when genetic differentiation is inferred from a

limited number of loci.

Disease associations among populations

Several studies of *Bd* disease dynamics have analyzed ecological and environmental predictors of disease (Briggs *et al.* 2010, Becker & Zamudio 2011, Rohr *et al.* 2011), while other studies have identified genetic-fitness associations (May *et al.* 2011, Savage & Zamudio 2011). To our knowledge, this study is the first to jointly consider environmental and genetic factors contributing to *Bd* dynamics. Of all genetic factors tested, observed heterozygosity showed the strongest effect on population disease measures when also considering environmental factors, with higher heterozygosity predicting significantly lower *Bd* infection and mortality prevalence. This pattern is consistent with the 'general effect' hypothesis (David 1998), where heterozygosity across multiple microsatellite markers reflects genome-wide heterozygosity and is thus an indirect measurement of a population's average fitness. In contrast, the outlier locus showed no significant patterns with disease across populations when also considering environmental factors, an expected result given that intra-population associations will likely be erased when only considering inter-population genetic measurements. Interestingly, the mean number of private alleles showed a positive relationship with *Bd* infection prevalence, suggesting that a higher proportion of unique alleles increases a population's disease risk. This pattern likely occurs because populations with more private alleles tend to be smaller, more isolated populations with higher genetic drift (Slatkin & Takahata 1985) that are less able to genetically adapt to *Bd*. Finally, our analyses show that *Bd* infection intensity is completely environmentally determined, with no host genetic contribution. This result is concordant with the high variation we observed in mean infection intensities across populations; environmental conditions dictate *Bd* growth and therefore the magnitude of infection, but some

populations tolerate high infection intensities with no apparent consequences (e.g., HR) while others show mortality at fairly low infection intensities (e.g., WC). Thus, the functional consequence of infection intensity is locality-specific and should be compared within rather than among *L. yavapaiensis* populations.

In summary, we found a single outlier locus that showed significant association with *Bd* mortality within populations, whereas only neutral measures of heterozygosity and genetic drift were significant predictors of *Bd* mortality across populations. This pattern is consistent with the lack of gene flow we detected among sampled populations; because populations are completely isolated, they evolve as independent units based upon standing measures of genetic diversity and, in the case of the outlier locus, the presence and frequency of susceptibility-associated alleles at the time of initial *Bd* emergence. Our analyses of the Muleshoe Ranch hot spring and adjacent populations highlight the possible alternative outcomes if gene flow continued to unite all populations; selection against *Bd* susceptibility would need to be strong to overwhelm the effects of migration and drift, given that environmental conditions vary across populations and significantly contribute to *Bd* dynamics.

Conclusions and conservation implications

In this study, we demonstrated the utility of integrating genetic analyses of differentiation at neutral and outlier loci, individual disease assays, and environmental variables in natural wildlife populations to better understand potential evolutionary responses to disease pressure within and among populations. Our findings may prove useful in predicting the fate of populations facing pathogen selective pressure and designating important evolutionary lineages containing genetic variation for local adaptation to chytridiomycosis. *Bd* currently infects amphibians on every continent where they occur (Lips 1999, Bosch *et al.* 2001, Waldman *et al.* 2001, Hopkins &

Channing 2003, Ron *et al.* 2003, Green *et al.* 2003, Goka *et al.* 2009), and chytridiomycosis threatens the persistence of numerous species worldwide (Lips *et al.* 2006, Skerratt *et al.* 2007). Understanding the precise ecological and evolutionary dynamics that allow or prevent populations from persisting with *Bd* will be critical for accurate planning and implementation of species conservation efforts (Woodhams *et al*. 2011). For populations with the potential to evolve *Bd* resistance, this may entail management actions that promote genetic diversity and increase effective population sizes. Paradoxically, the dramatic fragmentation and isolation of *L. yavapaiensis* populations in recent decades (Witte *et al.* 2008) has prevented ongoing gene flow from erasing local adaptation to variable disease dynamics, heightening the evolutionary potential for remaining populations to overcome chytridiomycosis susceptibility. Preserving and promoting genetic diversity is therefore likely to be the most effective management strategy to increase the long-term probability of *L. yavapaiensis* species persistence in the face of ongoing chytridiomycosis outbreaks.

Acknowledgements: We thank the Zamudio lab and three anonymous reviewers for constructive comments on earlier versions of the manuscript; D. Caldwell, A. Martin, M. Booth, J. Richmond, M. Schlaepfer, R. Rogers, M. Lawrence and M. Haberstitch for assistance in the field; and Steve Bogdanowicz, the Evolutionary Genetics Core Facility, and the Computational Biology Service Unit for help with data collection and analyses. This work was supported by a National Science Foundation (NSF) Graduate Research Fellowship, a National Geographic Society Young Explorers Grant, and an NSF Doctoral Dissertation Improvement Grant (number) to AES, and an NSF Population Evolutionary Processes grant (DEB-0815315) to KZ.

REFERENCES

Acevedo-Whitehouse K, Gulland F, Grieg D, Amos W (2003) Disease susceptibility in California sea lions. *Nature*, **422**, 35.

Acevedo-Whitehouse K, Spraker TR, Lyons E *et al.* (2006) Contrasting effects of heterozygosity on survival and hookworm resistance in California sea lion pups. *Molecular Ecology*, **15**, 1973–1982.

Adkison MD (1995) Population differentiation in Pacific salmon: local adaptation, genetic drift, or the environment? *Canadian Journal of Fisheries and Aquatic Sciences,* **52**, 2762–2777.

Allentoft ME, O'Brien J (2010) Global amphibian declines, loss of genetic diversity and fitness: a review. *Diversity,* **2**, 47–71.

Anderson RM, May RM (1982) Coevolution of hosts and parasites. *Parasitology*, **85**, 411–426.

Antao T, Lopes A, Lopes R, Beja-Pereira A, Luikart G (2008) LOSITAN: a workbench to detect molecular adaptation based on a Fst-outlier method. *BMC Bioinformatics*, **9**, 323.

Balkenhol N, Gugerli F, Cushman SA *et al*. (2009) Identifying future research needs in landscape genetics: where to from here? *Landscape Ecology*, **24**, 455–463.

Barreiro LB, Quintana-Murci L (2010) From evolutionary genetics to human immunology: how selection shapes host defence genes. *Nature Reviews Genetics*, **11**, 17–30.

Beaumont M, Nichols R (1996) Evaluating loci for use in the genetic analysis of population structure. *Proceedings: Biological Sciences*, **263**, 1619–1626.

Beaumont MA, Balding DJ (2004) Identifying adaptive genetic divergence among populations from genome scans. *Molecular Ecology*, **13**, 969–980.

Becker CG, Zamudio KR (2011) Tropical amphibian populations experience higher disease risk in natural habitats. *Proceedings: Biological Sciences* **108**, 9893–9898.

Bernatchez L, Landry C (2003) MHC studies in nonmodel vertebrates: what have we learned about natural selection in 15 years? *Journal of Evolutionary Biology,* **16**, 363–377.

Biek R, Real LA (2010) The landscape genetics of infectious disease emergence and spread. *Molecular Ecology*, **19**, 3515-3531.

Blanchet S, Rey O, Berthier P *et al.* (2009) Evidence of parasite-mediated disruptive selection on genetic diversity in a wild fish population. *Molecular Ecology*, **18**, 1112-1123.

Bradley G, Rosen P, Sredl M, Jones T, Longcore J (2002) Chytridiomycosis in native Arizona frogs. *Journal of Wildlife Diseases*, **38**, 206–212.

Brem FMR, Lips KR (2008) *Batrachochytrium dendrobatidis* infection patterns among Panamanian amphibian species, habitats and elevations during epizootic and enzootic stages. *Diseases of Aquatic Organisms*, **81**, 189–202.

Briggs CJ, Knapp RA, Vredenburg VT (2010) Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. *Proceedings: Biological Sciences, **107**,* 9695-9700.

Biek, R, Drummond AJ, Poss M (2006) A virus reveals population structure and recent demographic history of its carnivore host. *Science*, **311**, 538–540.

Blows MW, Hoffmann AA (2005*)* A reassessment of genetic limits to evolutionary change*. Ecology*, **86**, 1371**–**1384.

Boots M, Hudson PJ, Sasaki A (2004) Large shifts in pathogen virulence relate to host population structure. *Science*, **303**, 842**–**845.

Bosch J, Martinez-Solano I, Garcia-Paris M (2001) Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. *Biological Conservation,* **97**, 331–337.

Campbell GG, Noble LR, Rollinson DD *et al.* (2010) Low genetic diversity in a snail

intermediate host (Biomphalaria pfeifferi Krass, 1848) and schistosomiasis transmission in the Senegal River Basin. *Molecular Ecology*, **19**, 241–256.

Carey C, Bruzgul JE, Livo LJ *et al*. (2006) Experimental exposures of boreal toads (*Bufo boreas*) to a pathogenic chytrid fungus (*Batrachochytrium dendrobatidis*). *EcoHealth*, **3**, 5–21.

Cavatorta JR, Savage AE, Yeam I, Gray SM, Jahn MM (2008) Positive darwinian selection at single amino acid sites conferring plant virus resistance. *Journal of Molecular Evolution*, **67**, 551–559.

Cheng TL, Rovito SM, Wake DB, and Vredenburg VT (2011) Coincident mass extirpation of neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis*. *Proceedings: Biological Sciences,* **108**, 9502–9507.

Clarkson RW, Rorabaugh JC (1989) Status of Leopard Frogs (*Rana pipiens* Complex: Ranidae) in Arizona and Southeastern California. *Southwestern Naturalist*, **34**, 531–538.

Crawford NG (2009) SMOGD: Software for the Measurement of Genetic Diversity. *Molecular Ecology Resources*, **10**, 556–557.

Darwin C (1871) *The Descent of Man, and Selection in Relation to Sex*. John Murray, London.

David P (1998) Heterozygosity–fitness correlations: new perspectives on old problems. Heredity, 80, 531–537.

De La Vega FM, Dailey D, Zielgle J *et al.* (2002) New generation pharmacogenomic tools: a SNP linkage disequilibrium map, validated SNP assay resource, and high-throughput instrumentation system for large-scale genetic studies. *Biotechniques Supplement*, 48–54.

Duffy MA, Sivars-Becker L (2007) Rapid evolution and ecological host–parasite dynamics. *Ecology Letters*, **10**, 44–53.

Dwyer G, Elkinton JS, Buonaccorsi JP (1997) Host heterogeneity in susceptibility and disease dynamics: tests of a mathematical model. *American Naturalist*, **150**, 685–707.

Excoffier L, Hofer T, Foll M (2009) Detecting loci under selection in a hierarchically structured population. *Heredity*, **103**, 285–298.

Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources,* **10**, 564–567.

Feng ZL, Smith DL, McKenzie FE *et al.* (2004) Coupling ecology and evolution: malaria and the S-gene across time scales. *Mathematical Biosciences*, **189**, 1–19.

Fineblum WL, Rausher MD (1995) Tradeoff between resistance and tolerance to herbivore damage in a morning glory. *Nature*, **377**, 517–520.

Flor HH (1956) The complementary genetic systems in flax and flax rust. *Advances in Genetics*, **8**, 29–54.

Frankham R (2005) Stress and adaptation in conservation genetics. Journal of Evolutionary Biology 18: 750–755.

Garner TWJ, Perkins MW, Govindarajulu P *et al*. (2006) The emerging amphibian pathogen *Batrachochytrium dendrobatidis* globally infects introduced populations of the North American bullfrog, *Rana catesbeiana*. *Biology Letters*, **2**, 455–459.

Gebremedhin B, Ficetola GF, Naderi S *et al.* (2009) Combining genetic and ecological data to assess the conservation status of the endangered Ethiopian walia ibex. *Animal Conservation*, **12**, 89–100.

Green DE, Converse KA, Scgrader AK (2003) Epizootiology of sixty-four amphibian

morbidity and mortality events in the USA, 1996-2001. *Annals of the New York Academy of Sciences*, **969**, 323–339.

Goka K, Yokoyama, J, Une Y *et al.* (2009) Amphibian chytridiomycosis in Japan: distribution, haplotypes and possible route of entry into Japan. *Molecular Ecology*, **18**, 4757–4774.

Haldane JBS (1949) Disease and evolution. *La Ricerca Scientifica*, **19** (Suppl.), 68–76.

Hale SF, Rosen PC, Jarchow JL, Bradley GA (2005) Effects of the chytrid fungus on the Tarahumara frog (*Rana tarahumarae*) in Arizona and Sonora, Mexico. In: *USDA Forest Service Proceedings RMRS-P-36*, 407–411.

Hansson B, Westerdahl H, Hasselquist D, Åkesson M, Bensch S (2004) Does linkage disequilibrium generate heterozygosity–fitness correlations in great reed warblers? Evolution, 58, 870–879.

Hawley DM, Dhondt KV, Dobson AP *et al.* (2010) Common garden experiment reveals pathogen isolate but no host genetic diversity effect on the dynamics of an emerging wildlife disease. *Journal of Evolutonary Biology*, **3**, 1680-1688.

Holsinger KE, Weir BS (2009) Genetics in geographically structured populations: defining, estimating and interpreting F. *Nature Reviews Genetics*, **10**, 639–650.

Hopkins S, Channing A (2003) Chytrid fungus in Northern and Western Cape frog populations, South Africa. *Herpetological Review*, **34**, 334–336.

Hyatt AD, Boyle DG, Olsen V *et al.* (2007) Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms*, **73**, 175–192.

Jennings MR, Hayes MP (1994) Decline of native ranid frogs in the desert southwest. In: *Herpetology of the North American Deserts, proceedings of a symposium*, Southwestern Herpetologists Society Special. Pub. No. 5, 183–211.

Jensen JD, Thornton KR, Andolfatto P (2008) An approximate Bayesian estimator

suggests strong, recurrent selective sweeps in Drosophila. *PLoS Genetics*, **4**, e1000198.

Jost L (2008) G_{ST} and its relatives do not measure differentiation. *Molecular Ecology*, **17**, 4015–4026.

Kriger KM, Pereoglou F, Hero JM (2007) Latitudinal variation in the prevalence and Intensity of chytrid (*Batrachochytrium dendrobatidis*) infection in Eastern Australia. *Conservation Biology*, **21**, 1280–1290.

Lane EP, Weldon C, Bingham J (2003) Histological evidence of chytridiomycete fungal infection in a free-ranging amphibian, *Afrana fuscigula* (Anura: Ranidae), in South Africa. *Journal of the South African Veterinary Association,* **74**, 20–21.

Lewontin RC, Kojima K (1960) The evolutionary dynamics of complex polymorphisms. *Evolution*, **14**, 458–472.

Lips KR (1999) Mass mortality and population declines of anurans at an upland site in western Panama. *Conservation Biology*, **13**, 117–125.

Lips KR, Brem F, Brenes R *et al.* (2006) Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proceedings: Biological Sciences,* **103**, 3165–3170.

Liu SL, Mittler JE, Nickle DC *et al.* (2002) Selection for human immunodeficiency virus type 1 recombinants in a patient with rapid progression to AIDS. *Journal of Virology*, **76**, 10674–84.

Longcore JR, Longcore JE, Pessier AP, Halteman WA (2007) Chytridiomycosis widespread in anurans of northeastern United States. *Journal of Wildlife Management*, **71**, 435–444.

Longo AV, Burrowes PA (2010) Persistence with chytridiomycosis does not assure survival of direct-developing frogs. *EcoHealth*, **7**, 185–195.

Lowry DB (2010) Landscape evolutionary genomics. *Biology Letters*, **6**, 502–504.

Luikart G, Pilgrim K, Visty J, Ezenwa VO, Schwartz MK (2008) Candidate gene microsatellite variation is associated with parasitism in wild bighorn sheep. *Biology Letters*, **4**, 228–231.

May S, Zeisset I, Beebee TJC (2011) Larval fitness and immunogenetic diversity in chytrid-infected and uninfected natterjack toad (*Bufo calamita*) populations. *Conservation Genetics*, **12**, 805-811.

McCairns RJS, Bernatchez L (2008) Landscape genetic analyses reveal cryptic population structure and putative selection gradients in a large-scale estuarine environment. *Molecular Ecology*, **17**, 3901–3916.

Meagher S (1999) Genetic diversity and *Capillaria hepatica* (Nematoda) prevalence in Michigan deer mouse populations. *Evolution*, **53**, 1318–1324.

Murray KA, Skerratt LF, Speare R, McCallum H (2009) Impact and dynamics of disease in species threatened by the amphibian chytrid fungus, *Batrachochytrium dendrobatidis*. *Conservtion Biology*, **23**, 1242–1252.

Nagylaki T, Lucier B (1980) Numerical analysis of random drift in a cline. *Genetics*, **94**, 497–517.

Neher RA, Leitner T (2010) Recombination rate and selection strength in HIV intrapatient evolution. *PLoS Computational Biology*, **6**, e1000660.

Novembre J, Di Rienzo A (2009) Spatial patterns of variation due to natural selection in humans. *Nature Reviews Genetics*, **10**, 745–755.

Oláh-Hemmings V, Jaeger JR, Sredl MJ *et al.* (2010) Phylogeography of declining relict and lowland leopard frogs in the desert Southwest of North America. *Journal of Zoology*, **280**, 343–354.

Ortego J, Aparicio JM, Calabuig G, Cordero P (2007a) Risk of ectoparasitsm and genetic diversity in a wild lesser kestrel population. *Molecular Ecology*, **16**, 3712–

3720.

Ortego J, Cordero PJ, Aparicio JM, Calabuig G (2007b) No relationship between individual genetic diversity and prevalence of avian malaria in a migratory kestrel. *Molecular Ecology*, **16**, 4858–4866.

Osnas EE, Lively CM (2004) Parasite dose, prevalence of infection and local adaptation in a host-parasite system. *Parasitology*, **128**, 223–228.

Peakall R, Smouse PE (2006) GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, **6**, 288–295.

Pearman PB, Garner TWJ (2005) Susceptibility of Italian agile frog populations to an emerging strain of Ranavirus parallels population genetic diversity. *Ecology Letters*, **8**, 401–408.

Piotrowski JS, Annis SL, Longcore JE (2004) Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. *Mycologia*, **96**, 9–15.

Platz JE, Frost JS (1984) *Rana yavapaiensis*, a new species of leopard frog (*Rana pipiens* complex). *Copeia*, **1984**, 940–948.

Qihan C, Zhaoxue H, Haiyang J, Dacheng T, Sihai Y (2010) Strong positive selection drives rapid diversification of R-genes in *Arabidopsis* relatives. *Journal of Molecular Evolution*, **70**, 137–148.

Råberg L, Sim D, Read A (2007) Disentangling genetic variation for resistance and tolerance to infectious diseases in animals. *Science*, **318**, 812–814.

Rangel TF, Diniz-Filho JAF, Bini LM (2010) SAM: a comprehensive application for Spatial Analysis in Macroecology. *Ecography*, **33**, 46-50.

Reich DE, Cargill M, Bolk S *et al.* (2001) Linkage disequilibrium in the human genome. *Nature*, **411**, 199–204.

Richter-Boix A, Quintela M, Segelbacher G, Laurila A (2011) Genetic analysis of differentiation among breeding ponds reveals a candidate gene for local adaptation in

Rana arvalis. Molecular Ecology, **20**, 1582–1600.

Rohr JR, Halstead NT, Raffel TR (2011) Modelling the future distribution of the amphibian chytrid fungus: the influence of climate and human-associated factors. *Journal of Applied Ecology*, **48**, 174-176.

Rohr JR, Raffela TR, Romansica JM, McCallum H, and Hudson PJ (2008). Evaluating the links between climate, disease spread, and amphibian declines. *Proceedings: Biological Sciences*, **105**, 17436–17441.

Ron SR, Duellman WE, Coloma LA, Bustamante MR (2003) Population decline of the jambato toad *Atelopus ignescens* (Anura: Bufonidae) in the Andes of Ecuador. *Journal of Herpetology*, **37**, 116–126.

Rosenblum EB, Poorten TJ, Settles M *et al*. (2009) Genome-wide transcriptional response of *Silurana* (*Xenopus*) *tropicalis* to infection with the deadly chytrid fungus. *PLoS ONE*, **4**, e6494.

Savage AE, Jaeger JR (2009) Isolation and characterization of microsatellite markers in the lowland leopard frog (*Rana yavapaiensis*) and the relict leopard frog (*R. onca*), two declining frogs of the North American desert southwest. *Molecular Ecology Resources*, **9**, 199–202.

Savage AE, Zamudio KR (2011) MHC genotypes associate with resistance to a frog-killing fungus. *Proceedings: Biological Sciences,* 108: 16705-16710.

Savage AE, Sredl MJ, Zamudio KR (2011a) Disease dynamics vary spatially and temporally in a North American amphibian. *Biological Conservation,* **144**, 1910–1915.

Savage AE, Grismer LL, Anuar S *et al.* (2011b) First record of *Batrachochytrium dendrobatidis* infecting four frog families from Peninsular Malaysia. *EcoHealth*, **7**, 165–170.

Schork NJ (2002) Power calculations for genetic association studies using estimated probability distributions. *American Journal of Human Genetics*, **70**, 1480–1489.

Skerratt LF, Berger L, Speare R *et al.* (2007) Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth*, **4**, 125–134.

Slatkin M, Takahata N (1985) The Average Frequency of Private Alleles in a Partially Isolated Population. *Theoretical Population Biology*, **28**, 314-331.

Sredl MJ (2005) *Rana yavapaiensis* Platz and Frost, 1984, Lowland leopard frog. In: *Amphibian declines: the conservation status of United States species*, 596–599.

Schlaepfer MA, Sredl MJ, Rosen PC, Ryan MJ (2007) High prevalence of *Batrachochytrium dendrobatidis* in wild populations of lowland leopard frogs *Rana yavapaiensis* in Arizona. *EcoHealth*, **4**, 421–427.

Scott NJ (1993) Postmetamorphic death syndrome. *Froglog*, **7**, 1–2.

Tishkoff SA, Verrelli BC (2003) Patterns of human genetic diversity: Implications for Human Evolutionary History and Disease. *Annual Review of Genomics & Human Genetics*, **4**, 293–340.

Thrall PH, Jarosz AM (1994) Host–pathogen dynamics in experimental populations of Silene alba and Ustilago violacea. II. Experimental tests of theoretical models. *Journal of Ecology*, **82**, 561–569.

Tobler U, Schmidt BR (2010) Within- and among-population variation in chytridiomycosis-induced mortality in the toad *Alytes obstetricans. PloS ONE*, **5**, e10927.

Valdés AM, Slatkin M, Freimer NB (1993) Allele frequencies at microsatellite loci: the stepwise mutation model revisited. *Genetics*, **133**, 737–749.

van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, **4**, 535–538.

Vredenburg VT, Roland A, Tate S, Briggs CJ (2010) Dynamics of an emerging disease drive large-scale amphibian population extinctions. *Proceedings: Biological Sciences,*

107, 9689–9694.

Waldman B, van de Wolfshaar KE, Klena JD *et al.* (2001) Chytridiomycosis in New Zealand frogs. *Surveillance*, **28**, 9–11.

Wilson GA, Rannala B (2003) Bayesian inference of recent migration rates using multilocus genotypes. *Genetics*, **163**, 1177–1191.

Witte CL, Sredl MJ, Kane AS, Hungerford LL (2008) Epidemiologic analysis of factors associated with local disappearances of native ranid frogs in Arizona. *Conservation Biology*, **22**, 375–383.

Woodhams DC, Rollins-Smith LA, Carey C *et al.* (2006) Population trends associated with skin peptide defenses against chytridiomycosis in Australian frogs. *Oecologia*, **146**, 531–540.

Woodhams DC, Ardipradja K, Alford RA *et al.* (2007). Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defenses. *Animal Conservation*, **10**, 409–417.

Woodhams DC, Bosch J, Briggs CJ *et al*. (2011) Mitigating amphibian disease: strategies to maintain wild populations and control chytridiomycosis. *Frontiers in Zoology*, **8**, 8–31.

Yan G, Romero-Severson J, Walton M, Chadee DD, Severson DW (1999) Population genetics of the yellow fever mosquito in Trini- dad: comparisons of amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) markers. *Molecular Ecology*, **8**, 951–963.

Zhu Y, Chen H, Fan J *et al*. (2000) Genetic diversity and disease control in rice. *Nature*, **406**, 718–722.

CHAPTER 3:

MHC GENOTYPES ASSOCIATE WITH RESISTANCE TO A FROG-KILLING FUNGUS[†]

Anna E. Savage[1] and Kelly R. Zamudio[1]

[1]Department of Ecology and Evolutionary Biology, E150 Corson Hall, Cornell University, Ithaca, NY 14853 USA

Abstract

The emerging amphibian disease chytridiomycosis is caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*). Amphibian populations and species differ in susceptibility to *Bd*, yet we know surprisingly little about the genetic basis of this natural variation. Major Histocompatibility Complex (MHC) loci encode peptides that initiate acquired immunity in vertebrates, making them likely candidates for determining disease susceptibility. However, MHC genes have never been characterized in the context of chytridiomycosis. Here, we performed experimental *Bd* infections in lab-reared frogs collected from five populations that show natural variation in *Bd* susceptibility. We found that alleles of an expressed MHC class IIB locus associate with survival following *Bd* infection. Across populations, MHC heterozygosity was a significant predictor of survival. Within populations, MHC heterozygotes and individuals bearing MHC allele Q had a significantly reduced risk of death, and we detected a significant signal of positive selection along the evolutionary lineage leading to allele Q. Our findings demonstrate that immunogenetic variation affects chytridiomycosis survival under controlled

experimental conditions, confirming for the first time that host genetic polymorphisms contribute to chytridiomycosis resistance.

Introduction

Amphibians face a biodiversity crisis (Stuart *et al.* 2004) brought on in large part by the emerging infectious disease chytridiomycosis (Lips *et al.* 2006, Skerratt *et al.* 2007; Wake & Vredenburg 2008; Rohr *et al.* 2008). The fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) causes chytridiomycosis in amphibian species worldwide, but the extent of morbidity and mortality varies within and among populations (Lips 1998; Bradley *et al.* 2002), species (Lips 1998), and assemblages (Lips 1998; Crawford *et al.* 2010). Some of this variation is attributable to regional and local ecological factors such as climate (Pounds *et al.* 2006), elevation (Lips *et al.* 2006), host life history traits (Lips *et al.* 2003), and different *Bd* strains (Retallick & Miera 2007). However, host immunity likely also contributes to disease outcomes. In the model frog *Silurana tropicalis*, experimental *Bd* inoculations significantly change host gene expression profiles (Rosenblum *et al.* 2009), and innate (Ribas *et al.* 2009) and acquired (Ramsey *et al.* 2010) immune responses are activated at host-optimal temperatures. In addition, experimental infections of the toad *Alytes obstetricans* show that *Bd* survival varies among individuals and populations under constant environmental conditions (Tobler & Schmidt 2010), and upon reinfection, previously *Bd*-infected *Leiopelma archeyi* clear the pathogen significantly faster than control animals (Shaw *et al.* 2010), suggesting a secondary acquired immune response. Identifying heritable determinants of *Bd* resistance has the potential to enhance the success of amphibian conservation efforts (Woodhams *et al.* 2011). However, to date immunogenetic correlates of survival remain underexplored (Richmond *et al.* 2009).

Vertebrate Major Histocompatibility Complex (MHC) genes are good candidates

for influencing host *Bd* dynamics because they encode cell-surface glycoproteins that regulate the vertebrate acquired immune response and affect the development and progression of wildlife disease (Bernatchez & Landry 2003). To initiate acquired immunity, MHC proteins bind pathogen molecules on their peptide-binding regions (PBRs) and present them to T-cells (Jones *et al.* 2006). This central function in pathogen defense creates strong diversifying selection within host populations for numerous MHC proteins that can collectively bind a wide array of pathogens (Hughes & Yeager 1998). MHC genes are divided into three classes (I, II and III) that are genomically linked across tetrapods (Ohta *et al.* 2006). Class I loci are expressed on all nucleated somatic cells and present peptides derived from intracellular pathogens, whereas class II genes are expressed in epithelial cells in the thymus and antigen-presenting cells in the periphery, and primarily present peptides derived from extracellular pathogens (Kaufman *et al.* 1985; Braciale *et al.* 1987). The class III region encodes a group of structurally unrelated proteins that are not involved in antigen presentation (but have immune function; Kaufman *et al.* 1985). Class II MHC loci are the most likely candidate immunity genes for chytridiomycosis for two reasons: (i) they are the primary presenters of extracellular fungal pathogens (Braciale *et al.* 1987) and (ii) class II-expressing dendritic and Langerhans lymphocytes are present in amphibian skin (Carillo-Farga *et al.* 1990; Du Pasquier & Flajnik 1990), the primary location of *Bd* infections. In spite of these data, class II genes have only been described in a few amphibians (Kaufman *et al.* 1985; Sato *et al.* 1993; Sammut *et al.* 1999; Liu *et al.* 2002; Bos & DeWoody 2005; Hauswaldt *et al.* 2007; May *et al.* 2011) and never in the context of *Bd* susceptibility.

Associations between disease susceptibility and MHC polymorphisms are widespread in natural wildlife populations (Bernatchez & Landry 2003). Most studies focus on variation in the peptide-binding region (PBR) of class I and class IIB loci because PBR amino acid variability determines the repertoire of pathogen peptides to

which T-cells can respond (Jones *et al.* 2006). Across diverse vertebrate taxa, survival after disease exposure has been associated with particular PBR alleles (Langefors *et al.* 2001), with elevated PBR heterozygosity (Penn *et al.* 2002), and with intermediate levels of PBR diversity (Wegner *et al.* 2003). These multiple mechanisms of disease resistance indicate that MHC-associated immunity is context-dependent and varies with each combination of host, pathogen, and environment. Chytridiomycosis is caused by a single pathogen affecting numerous amphibian hosts that occupy diverse habitats, thereby providing a unique epidemiological combination for immunogenetic studies of independent hosts evolving under varied environmental regimes.

The lowland leopard frog (*Lithobates yavapaiensis*) is a North American amphibian that began experiencing mass chytridiomycosis die-offs in the early 1990s (Bradley *et al.* 2002). Currently, populations persist with *Bd* infections that vary in prevalence and intensity across space and time (Savage *et al.* 2011, Schlaepfer *et al.* 2007). In some populations, a proportion of individuals die from chytridiomycosis during winter months when temperatures are pathogen-optimal (Savage *et al.* 2011). In other populations, *Bd* infection prevalence and intensity increase during winter months, but host frogs never develop signs of disease (Savage *et al.* 2011). This pattern suggests two non-exclusive hypotheses: (i) genetic resistance to chytridiomycosis occurs in some populations, or (ii) environmental variation explains differences in disease dynamics among populations. However, the spatial and seasonal variation in pathogen and disease occurrence across populations precludes distinguishing genetic from environmental determinants of *Bd*-associated mortality in the field.

Here, we determined the role of host genetic variation in *Bd*-associated mortality by experimentally infecting lab-reared *L. yavapaiensis* collected from five geographically distinct populations that naturally differ in disease dynamics. We exposed individuals to *Bd* and induced chytridiomycosis under constant, pathogen-optimal environmental

conditions. We then tested the following four hypotheses: (i) lab survival varies across individuals and/or populations in patterns that correspond to observed chytridiomycosis mortality in the field; (ii) pathogen burdens are lower in surviving individuals compared to individuals that die; (iii) genetic variation at MHC loci associates with survival following infection; and (iv) MHC alleles associated with survival show a signature of positive selection. Combined, these hypotheses address the overarching question of whether heritable host immune factors explain differences in *Bd* susceptibility in natural populations.

Results

We characterized natural chytridiomycosis in five *L. yavapaiensis* populations during 2007–2010 (Figure 3.1A; Figure S3.1; Savage *et al.* 2011) to generate *a priori* expectations for survival patterns in our experimental *Bd* challenges. We collected egg masses in 2008 and reared them through metamorphosis in a light, temperature, and humidity controlled growth chamber. Prior to the experiment we tested for, but did not detect, native *Bd* infections among our experimental animals. Metamorphosed frogs were randomly assigned to two replicated treatments: (i) infected with Arizona *Bd* strain PsTr2004 via exposure to 1×10^5 zoospores in liquid media, or (ii) kept as uninfected controls exposed to liquid media only. Among the 99 frogs experimentally infected with *Bd*, 14 individuals survived the experiment (Figure 3.1B; Table S3.1); survivors were from Aravaipa Canyon (AC) and the Santa Maria River (SM). These two populations showed 41% and 27% survival in the laboratory, respectively, and are also the two populations with the lowest proportion of mortalities in the field (Figure 3.1A). No experimentally infected frogs from Cienega Creek (CIC), Muleshoe Ranch (MR), or Willow Creek (WC) survived, corroborating the hypothesis that lab survival varies across populations according to chytridiomycosis mortality in the field. Mean infection intensity,

defined as the number of *Bd* genome equivalents recovered per swab, was not significantly different from zero at one (P=0.74) or three (P=0.39) days post infection (DPI), but zoospore counts increased significantly in all populations at seven DPI (P=0.0005; Figure 3.1C-D). At 14 DPI, mean infection intensity was no longer significantly different from zero for individuals from AC and SM that survived (P=0.5; open symbols, Figure 3.1D) but remained significantly higher than zero for all individuals that eventually died (P<0.0001; closed symbols, Figure 3.1D). Thus, among *Bd*-infected individuals, those that died maintained *Bd* infection intensities higher than zero for the duration of the experiment, whereas surviving individuals cleared *Bd* infections by 14 DPI (Figure 3.1C-D).

Population genetic background can bias disease association analyses (Pritchard *et al.* 2000a), therefore we genotyped all 99 *Bd*-infected frogs at 14 unlinked microsatellite loci (Savage & Jaeger 2009) to characterize population structure for comparison with immunogenetic variation. We implemented a Bayesian assignment test (Pritchard *et al.* 2000b) that confirmed the independent evolutionary history and current genetic isolation of each sampled population: frogs were assigned to six genetic demes (lnL = –2984.1) corresponding to their five natal populations, with further differentiation between clutches from population CIC (Figure 3.2A). We next characterized the PBR of an expressed MHC class IIB gene (Kiemnec-Tyburczy *et al.* 2010) and sequenced PBR genotypes for all *Bd*-infected individuals to test for associations between allelic polymorphisms and responses to *Bd* infection. We recovered 33 unique PBR alleles (GenBank accession nos. JN638850–JN638882; Figure S3), of which only six occurred in multiple genetic demes. Ten of the 12 sampled clutches showed evidence of multiple paternity based on microsatellite and PBR genotypes (Figure S3.2; Table S3.1), thus the majority of clutches included half-sibships, rather than full sibships.

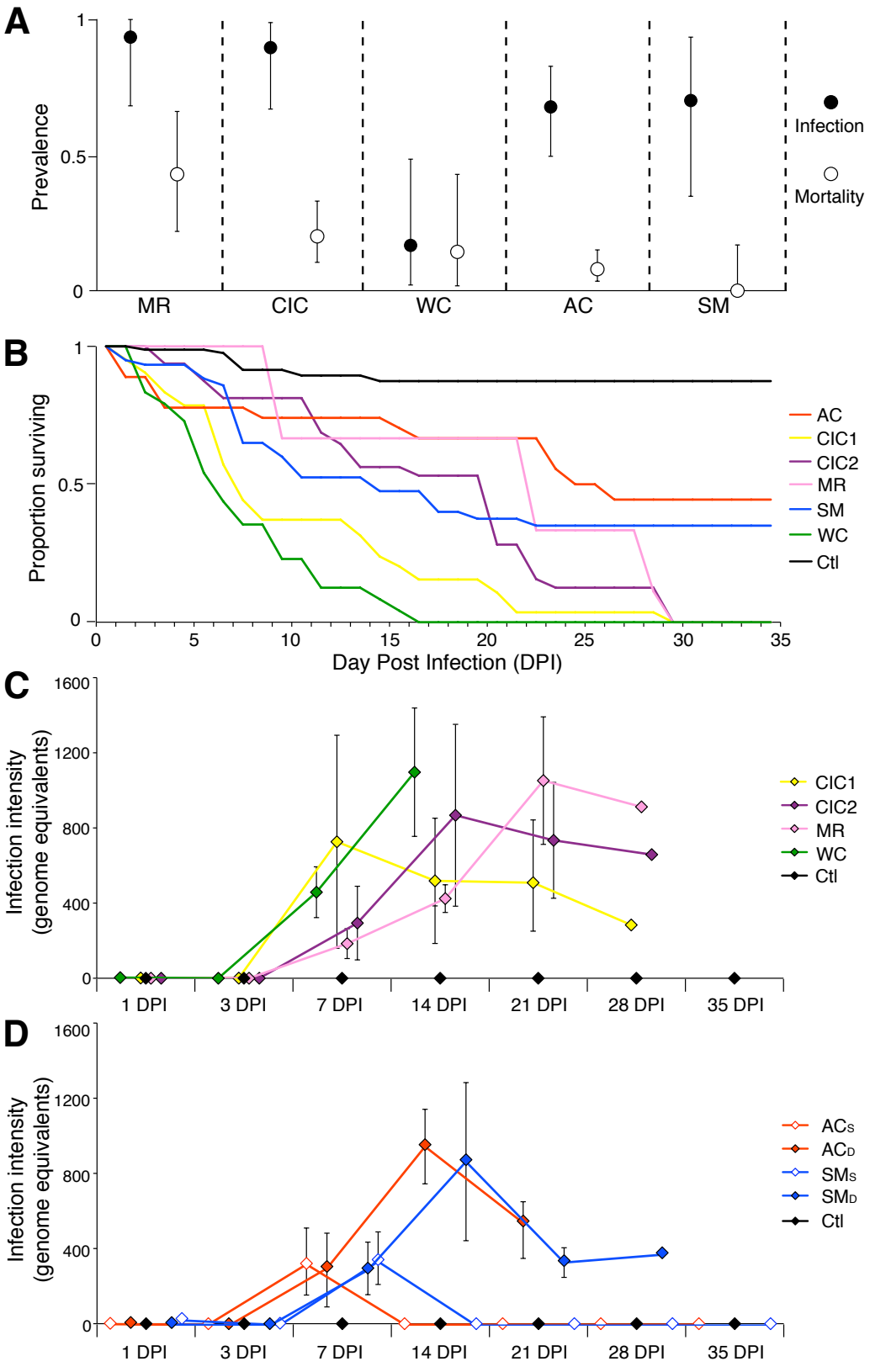
A
Prevalence
1
0.5
0
MR
CIC
WC
AC
SM
Infection
Mortality
B
Proportion surviving
1
0.5
0
0
5
10
15
20
25
30
35
Day Post Infection (DPI)
AC
CIC1
CIC2
MR
SM
WC
Ctl
C
Infection intensity
(genome equivalents)
1600
1200
800
400
0
1 DPI
3 DPI
7 DPI
14 DPI
21 DPI
28 DPI
35 DPI
CIC1
CIC2
MR
WC
Ctl
D
Infection intensity
(genome equivalents)
1600
1200
800
400
0
1 DPI
3 DPI
7 DPI
14 DPI
21 DPI
28 DPI
35 DPI
ACS
ACD
SMS
SMD
Ctl

Figure 3.1: *Bd* infection and mortality vary among populations of *Lithobates yavapaiensis* in nature and in the laboratory. (A) Field estimates of winter *Bd* infection prevalence (proportion of frogs testing positive for *Bd*; filled symbols) and mortality (proportion of *Bd*-positive frogs that died; open symbols) in five natural populations. AC = Aravaipa Canyon; CIC = Cienega Creek; MR = Muleshoe Ranch; SM = Santa Maria River; WC = Willow Creek; 95% Clopper-Pearson binomial confidence intervals are indicated (modified from 37). (B) Survival following *Bd* infection varies among individuals from the same five populations when reared in a growth chamber. Survival was tracked for 35 days post infection (DPI) with 1×10^5 zoospores of *Bd* (colored lines) or sham infection (black line). Population abbreviations follow (A); CIC1 and CIC2 denote genetically distinct clutches from population CIC. Numbers of individuals per population (*Bd*-infected/uninfected) are: AC, 17/11; CIC, 34/22; MR, 5/2; WC, 17/11; SM, 26/15; all, 99/59. (C) Mean *Bd* infection intensity (zoospore equivalents ± SD) measured at various days post infection (DPI) in populations with no survival (CIC, MR and WC). **(D)** Mean *Bd* infection intensity over time for survivors (open symbols; AC_S and SM_S) and non-survivors (filled symbols; AC_D and SM_D) from populations with survivors (AC and SM). Control frogs (black symbols) remained uninfected for the duration of the experiment.

To identify parameters with significant effects on survival, we used the Cox proportional hazard model including treatment, genetic deme, clutch, replicate, number of frogs per replicate, initial mass, change in mass, PBR heterozygosity, maximum infection intensity (MII), and interactions between MII and all other parameters (complete model: ΔlnL=36.2, df=22, P<0.0001). As expected, significantly more uninfected individuals survived compared to *Bd*-infected individuals (P=0.001). Among infected individuals, PBR heterozygosity (P=0.0002), MII (P=0.005), and the interaction between

MII and PBR heterozygosity (P=0.0005) were significant explanatory variables predicting survival. In contrast, density-dependent dynamics within replicates did not influence survival, as the number of frogs per replicate and the interaction between number of frogs per replicate and MII were not significant.

We generated an unrooted genealogy of unique PBR sequences to examine the distribution of alleles in relation to genetic demes and disease outcomes (Figure 3.2B). PBR alleles did not cluster by genetic deme. Of the alleles recovered from multiple populations (A–E and Q), frogs with alleles B and Q showed a significantly reduced risk of mortality (Table 3.1). This pattern could arise because alleles B and Q occur only within AC and SM, the two genetic demes in which some individuals survived. Thus, we tested for allelic associations within each of those demes, and found a significant reduction in risk associated with allele Q in SM but not AC (Table 3.1). Similarly, SM (signed-rank test, P=0.03), but not AC (P=0.09), showed significantly different PBR allele frequency distributions between survivors and non-survivors (Figure 3.3A). Across populations, PBR heterozygotes had a significantly reduced risk of death (Table 3.1; Figure 3.3C). Within populations, risk of death remained significantly reduced for heterozygotes in demes AC and SM (Table 3.1, Figure 3.3B), demonstrating that heterozygote advantage was not an artifact of higher heterozygote frequency in demes with survivors.

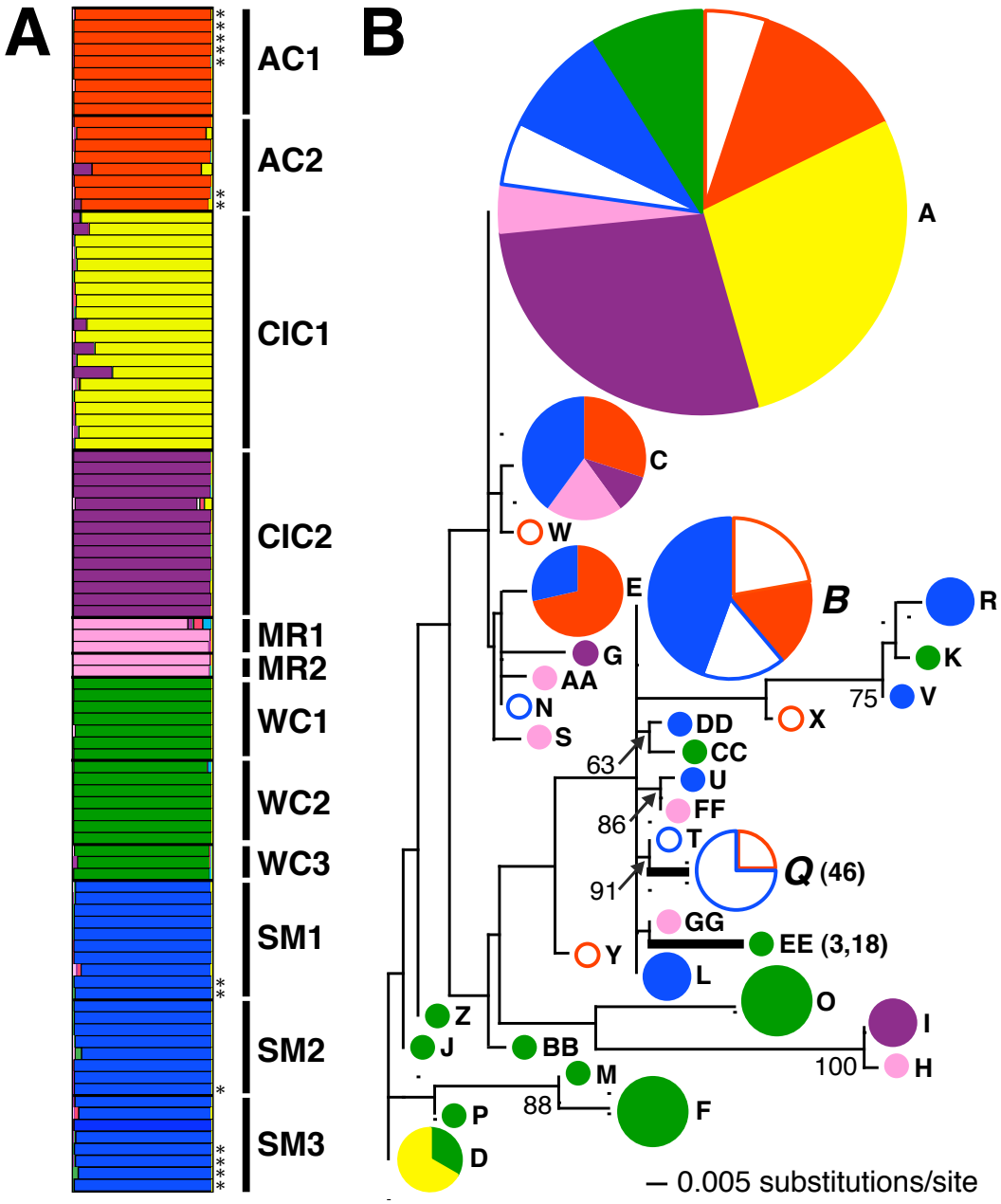

Figure 3.2: (A) Clutch genetic structure inferred by Bayesian assignment of 99 *Bd*-infected *L. yavapaiensis* individuals. Thin bars separate individuals, thick bars separate clutches, and asterisks indicate individuals that survived experimental infection. Horizontal bars show the coefficient of membership across genetic demes for each *Bd*-infected frog, with each color representing a unique genetic deme. (B) Maximum

likelihood genealogy of 33 MHC class IIB PBR alleles. Circle size is proportional to frequency, colors correspond to the genetic demes in (A), alleles recovered from survivors are shown with open symbols, and alleles recovered from non-survivors are shown with filled symbols. Bold denotes terminal branches with significantly elevated nonsynonymous substitution rates ($\omega > 1$) followed by the codon positions under selection in parentheses. Alleles showing significant associations with survival are enlarged and italicized. Bootstrap values >50% are indicated.

Table 3.1: Relative Risk (RR) for survival-associated MHC risk factors among *Bd*-infected frogs. 95% confidence intervals are in parentheses, followed by two-tailed Fisher exact test *P* values. Significant RR values after sequential Bonferroni correction are shown in bold.

	Risk Factor		
Genetic deme	*Heterozygote*	*Allele B*	*Allele Q*
AC (N=17)	***0.13 (0.13 – 0.43) P = 0.001***	0.46 (0.14 – 1.2) *P* = 0.16	0.0 (0.0 – 1.3) *P* = 0.41
SM (N=26)	***0.43 (0.32 – 0.77) P = 0.005***	0.64 (0.26 – 1.2) *P* = 0.31	***0.0 (0.0 – 0.71) P = 0.017***
All individuals (N=99)	***0.58 (0.54 – 0.78) P < 0.00001***	***0.47 (0.27 – 0.74) P = 0.001***	***0.0 (0.0 – 0.55) P < 0.00001***

To distinguish between selection and neutral evolution, we estimated ω, the ratio of nonsynonymous (amino acid-changing) to synonymous (silent) nucleotide substitutions. Values of ω equal to 1 indicate neutral evolution, whereas values greater than 1 indicate positive selection and values less than 1 indicate purifying selection (Kimura 1977). Along the 82 PBR codons, models including positive selection (allowing $\omega > 1$ as a rate class) fit the alignment significantly better than models excluding positive selection (Table 3.2). We detected significant positive selection in codons 3, 18 and 46, which correspond to three of 14 codon positions that determine peptide binding in the human class II PBR (Tong *et al.* 2006). These substitution rate analyses detect selection over the entire history of allelic lineages (Li *et al.* 1985) and do not distinguish among ancient versus recent selective events. Thus, to identify PBR alleles that may have recently fixed nonsynonymous substitutions in response to chytridiomycosis, we tested for positive selection ($\omega > 1$) along terminal branches of the genealogy, which represent the most recent molecular changes. We detected $\omega > 1$ in two terminal branches (Figure 3.2B, bold; Table S3.2). Codons 3 and 18 showed $\omega > 1$ along the branch leading to an allele present in an individual that died, thus we conclude this signal of selection is unrelated to *Bd*. In contrast, codon position 46 showed $\omega > 1$ along the branch leading to allele Q, the only allele recovered exclusively from surviving individuals (Figure 3.2B). Codon 46 may therefore be particularly important in determining *Bd* peptide binding, and allele Q is a candidate allele for recent immunogenetic adaptation in response to disease pressure.

Table 3.2: Evidence of positive selection among 82 amino acid residues of *Lithobates yavapaiensis* MHC class IIB PBR sequences. dN–dS = normalized difference between nonsynonymous substitutions per nonsynonymous sites (dN) and synonymous substitutions per synonymous sites (dS); ω = dN/dS ratio; PP = posterior probability.

Rate class method	**Likelihood ratio test (*P*-value)**	**Percentage of sites in ω>1 rate classes**	**Mean ω per ω>1 rate class**
PARRIS	9.16 (0.01)	15%	4.3
Evolutionary Fingerprinting	26.1 (0.006)	27%	4.4
Codon-specific method	**Codon 3 dN–dS (*P*-value/PP)**	**Codon 18 dN–dS (*P*-value/PP)**	**Codon 46 dN–dS (*P*-value/PP)**
SLAC	18.1 (0.09)		
FEL	1.1 (0.03)	0.93 (0.03)	0.74 (0.04)
REL	3.2 (0.99)	3.3 (0.99)	

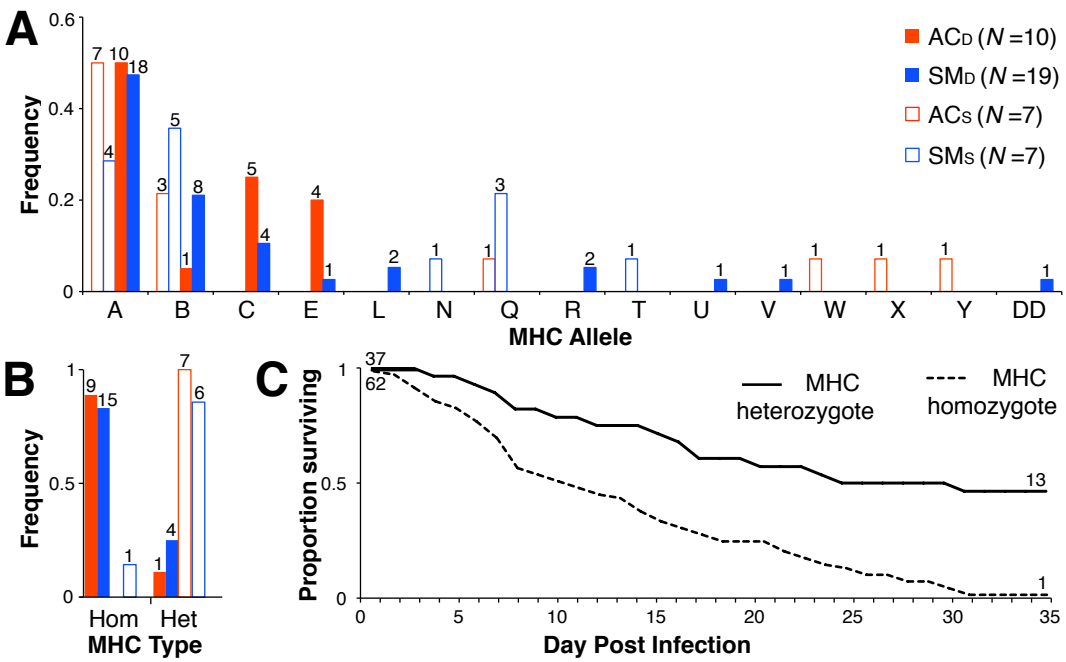

Figure 3.3: MHC class IIB PBR allele and heterozygote frequencies for *Bd*-infected survivors and non-survivors. PBR allele distribution (A) and PBR heterozygote and homozygote frequency (B) among survivors (open bars) and non-survivors (filled bars) in AC (red bars) and SM (blue bars), the two genetic demes with survivors. Allele counts and numbers of heterozygous and homozygous individuals are indicated above frequency bars. (C) Survival curves for PBR heterozygotes (solid line) and homozygotes (dotted line) across *Bd*-infected individuals from all five genetic demes.

Discussion

We have shown that MHC class IIB-associated genetic factors explain differences in *Bd* susceptibility in *L. yavapaiensis* by confirming our four hypotheses: (i) *Bd* survival was significantly different among individuals and populations, and was concordant with observed variation in field mortality (Figure 3.1A-B), (ii) maximum pathogen burdens were significantly lower in surviving individuals (Figure 3.1C-D), (iii)

MHC heterozygotes and MHC allele Q showed significant associations with survival (Table 3.1; Figure 3.2B); and (iv) allele Q had a signal of recent positive selection acting on codon 46 (Figure 3.2B). Thus, MHC class IIB genotypes are significantly associated with differences in *Bd* survival in *L. yavapaiensis* populations. Our results should be interpreted with the caveat that the MHC contains numerous immune-related loci that are in strong linkage disequilibrium (Ohta *et al.* 2006), and MHC loci experience frequent and diverse forms of selection for pathogen resistance, self and non-self discrimination, and kin selection (Bernatchez & Landry 2003; Apanius *et al.* 1997). These varied evolutionary processes present a challenge for identifying specific effects of individual MHC genes. Indeed, we cannot exclude the possibility that the class IIB locus examined here shows significant associations with survival due to linkage with another locus, because at least two class II genes are expressed in *L. yavapaiensis* (Kiemnec-Tyburczy *et al.* 2010). However, the signal of positive selection we detected along the PBR lineage leading to the only allele significantly associated with *Bd* survival challenges linkage as the sole explanation for our results. To the contrary, recent positive selection acting in the peptide-binding region of a resistance-associated allele found in natural populations provides compelling evidence that this locus encodes a molecule with specific involvement in the immunological response to *Bd*.

Our results underscore the evolutionary advantage of diverse genetic resistance pathways in an ever-changing environment. Lowland leopard frogs show spatial and temporal variation in demography and *Bd* exposure (Savage *et al.* 2011), which likely creates population-specific selective regimes for the evolution of chytridiomycosis resistance. Furthermore, host *Bd*-resistance alleles may carry some fitness cost with regard to other *Bd* strains or pathogens (Apanius *et al.* 1997; Zuk & Stoehr 2002). Due to the specificity of MHC-peptide binding, individual alleles often provide a fitness advantage against some pathogen epitopes while simultaneously incurring a fitness cost

against others (Gandon 2002; Bernatchez & Landry 2003). Thus, the strength and targets of selection on the MHC are likely to differ across populations, depending on the genetic background of the host and the full complement of pathogens at each site. In this context, our finding that PBR heterozygosity was a fitness advantage across all populations underscores that the broader peptide binding capabilities inherent to having two MHC alleles can be more advantageous than having one allele with particular efficacy against one pathogen epitope (Zuk & Stoehr 2002). Heterozygote advantage may thus be the general mechanism for enhancing chytridiomycosis resistance in natural populations. However, we also detected a resistance allele in population SM, highlighting that individual alleles can be selectively advantageous for specific genetic backgrounds and environmental regimes. Numerous studies of wildlife populations demonstrate that genetic disease resistance is a host, strain, and context-dependent process (Bernatchez & Landry 2003; Apanius *et al.* 1997), and our results confirm that lowland leopard frogs have reduced chytridiomycosis susceptibility via multiple evolutionary mechanisms.

The ability of host populations to evolve *Bd* resistance could be limited due to the recent timescale of chytridiomycosis emergence. Lowland leopard frogs have suffered from *Bd*-induced declines for only 20 generations (Bradley *et al*. 2002), and genetic theory predicts that the shorter generation time of pathogens should provide them an advantage in the co-evolutionary arms race (Gandon 2002). However, hosts can benefit from high standing genetic diversity, because the rate of pathogen evolution is negatively correlated with host genetic variability (Lipstich *et al*. 1995; Regoes *et al*. 2000; Zhan *et al*. 2002). Empirical studies also demonstrate that relative generation times of hosts and pathogens do not influence local adaptation (Morgan & Buckling 2006). In fact, when pathogen genetic variability is low, as is the case for *Bd* in the New World (Morgan *et al.* 2007), short generations increase the strength of selection for host

resistance and decrease the capacity of pathogens to adapt to an evolving host **(**Gandon & Michalakis 2002). Chytridiomycosis in *L. yavapaiensis* thus fits a scenario with strong potential for the evolution of heritable disease resistance in host populations. In our study, SM and AC were the only populations where individuals varied in *Bd* survival and the only populations with the PBR allele (Q) showing a signal of recent positive selection. Importantly, these populations also had the lowest proportions of *Bd* mortalities in our field surveys, indicating that our experimental results have biological relevance to *Bd* dynamics in natural populations. These populations are therefore the best candidates for future studies of the evolutionary dynamics of *Bd* resistance in natural populations.

Animal hosts evolve two mechanisms to combat infections: resistance, defined as the ability to limit pathogen burden, and tolerance, the ability to limit the damage caused by a given pathogen burden (Råberg *et al.* 2007). The distinction between tolerance and resistance is critical for understanding the evolutionary consequences of chytridiomycosis disease dynamics, because resistance has a negative effect on pathogen fitness and tolerance does not (Råberg *et al.* 2007). Under our experimental conditions, surviving frogs cleared *Bd* infections, demonstrating disease resistance. However, the same lowland leopard frog populations (AC and SM) show evidence of disease tolerance in the field, where individuals carry high pathogen burdens with no disease signs (Savage *et al.* 2011). These differences underscore the need to consider both experimental infection studies and disease dynamics in wild populations. Controlled experiments allow us to pinpoint the effects of immunogenetic factors in the absence of environmental variation. However, the critical next step will be studies of natural populations incorporating environmental factors that mediate host resistance and tolerance, pathogen virulence, and population MHC variability.

Given the scale of the amphibian biodiversity crisis (Wake & Vredenburg 2008), understanding host-pathogen chytridiomycosis dynamics is essential for amphibian conservation. Our study shows that genetically distinct lowland leopard frog populations experimentally infected with *Bd* have significant differences in survival that correlate with immunogenetic heterozygosity and polymorphism. This is the first experimental demonstration that host genetic variability determines *Bd* infection outcomes under controlled environmental conditions. To date, studies of natural amphibian populations have compared chytridiomycosis susceptibility across sympatric species (Lips *et al.* 2006; Pounds *et al.* 2006; Crawford *et al.* 2010), among intraspecific populations (Rachowicz *et al.* 2006), and between individuals (Briggs *et al.* 2005). Our findings emphasize the need for studies that quantify host population genetics and immunogenetics at these same hierarchical levels. For highly susceptible species, captive breeding is one of the few remaining strategies for maintaining natural populations (Mendelson *et al.* 2006). Species reintroductions with assisted selection could be a viable chytridiomycosis mitigation strategy, but will require a better understanding of amphibian immune responses and immunity genes targeted by natural selection (Woodhams *et al.* 2011). Although allele Q may be unique to *L. yavapaiensis*, we also detected significant heterozygote advantage, and selection for MHC heterozygosity is a strategy that could be implemented for any *Bd*-susceptible amphibian species. We identify for the first time genetic determinants of chytridiomycosis resistance in natural populations of a declining amphibian, a critical step in developing genetically-informed breeding programs for species recovery.

Methods

***Egg Mass Collection and Husbandry*.** We collected *L. yavapaiensis* egg masses from five populations in Arizona, USA in March 2008 (Figure S3.1). We detached partial

clutches and shipped them overnight to Cornell University. Frogs were housed in a 12h:12h light:dark regime at 30% humidity (±3%) and 21°C (±1°C) to mimic winter field conditions. Upon hatching, we equally divided larvae from each clutch into eight replicate 16 × 30 × 9 cm plastic cages filled with dechlorinated lab water. We fed larvae 90% spirulina algae and 10% fish flakes *ad libitum*, replacing water with fresh dechlorinated lab water three times per week. Variable survival resulted in unequal numbers of frogs per replicate, ranging from 1-5 per cage (Table S3.3). We transferred metamorphosed frogs to 16 × 30 × 9 cm plastic cages containing plastic perches and kept moist with a film of dechlorinated lab water. Frogs were fed 3 week old pathogen-free crickets *ad libitum*. Only metamorphs reaching the stage of complete tail resorbtion (post-Gosner stage 46) were used in experimental infections.

Bd *and Chytridiomycosis Field Surveys*. We surveyed populations for *Bd* during winter (January–February) of 2007-2010 (Savage *et al.* 2011). We categorized each dead individual testing positive for *Bd* as a chytridiomycosis mortality event. Additionally, we collected frogs with signs of chytridiomycosis (i.e., skin redness, lethargy, and loss of righting ability) for overnight observation, and categorized these as *Bd* mortalities if death occurred within 24h and the individuals were *Bd*-positive. We estimated *Bd* infection and mortality prevalence with 95% Clopper-Pearson binomial confidence intervals (Clopper & Pearson 1934).

Experimental* Bd *exposures. Frogs were infected with *Bd* strain PsTr2004, an Arizona strain isolated from a *Pseudacris triseriata* individual collected from Coconino County, AZ (UTMS: 446716E, 3871380N) in 2003. This locality is geographically distinct from our five populations, minimizing the potential that they were locally adapted to this *Bd* strain. Frogs from each replicate group were split into two cages; one cage was seeded with 1

X 10^5 PsTr2004 *Bd* zoospores in 1mL of TGhL broth and the other cage was sham-infected with 1mL of TGhL broth only. The film of water/TGhL/*Bd* or water/TGhL covering the floor of each cage was left undisturbed for 24h, and a new film of dechlorinated lab water was added to each cage daily. Cages were rotated daily to avoid the potential for microclimatic effects. Frogs were weighed immediately prior to infection, and at death or the end of the experiment, whichever came first.

Infection intensity over time. To quantify *Bd* infections, we used sterile swabs and followed standard amphibian swabbing protocols (Hyatt *et al.* 2007). *Bd* DNA was extracted using Prepman Ultra, and we used qPCR (Boyle *et al.* 2004) to determine *Bd* infection intensity. Before experiments began, we tested individuals for pre-existing *Bd* infection at all three developmental stages (eggs, larvae and metamorphs). One day prior to experimental *Bd* infections, we assayed all metamorphs for *Bd* infection. Frogs were handled with unused latex gloves to prevent *Bd* transmission. Following *Bd* exposures, we quantified infection intensity for each surviving replicate group on 1, 3, 7, 14, 21, 28 and 35 DPI.

Statistical Analysis of Survival. We analyzed survival data using the Cox proportional hazard model (Cox 1972), which assumes an underlying function describing how hazard changes over time and fits effect parameters using Cox's likelihood. Survival was censored, meaning that individuals may die after the end of the study period. To identify potential predictors, we first performed a univariate analysis for each variable and each interaction among variables. We performed log-rank tests of equality across strata for categorical variables (replicate, clutch, source population, number of cage mates, and MHC heterozygosity), and univariate Cox proportional hazard regression for continuous variables (initial mass, change in mass, and maximum infection intensity). Predictors

with a *P*-value of 0.1 or less in the univariate analyses were included as potential effect parameters in the final model. Significant *P*-values were determined using sequential Bonferroni correction (Rice 1989). We tested whether infection intensities were significantly different from zero for survivors and non-survivors using Wilcoxon signed-rank tests. Tests were performed in JMP version 9.0 (SAS Institute Inc.).

Screening of Microsatellite Loci. We genotyped all *Bd*-infected individuals at 14 previously characterized microsatellite loci (Savage & Jaeger 2009). DNA was extracted using a 5% Chelex 100 solution with 0.5μg proteinase K per sample. Chelex extractions were incubated at 55°C for 120 minutes, and 99°C for 10 minutes. PCR amplification was performed under the following conditions: 5 min initial denaturation at 94°C; 35 cycles of 1 min denaturing at 94°C, 1 min annealing at primer-specific annealing temperatures, 1 min extension at 72°C; and a final extension of 75°C for 5 min. Amplified products with different labels or non-overlapping size ranges were multiplexed and run on a 3730 Genetic Analyser (Applied Biosystems). Fragment sizes were determined by comparison with a LIZ-500 standard using GeneMapper version 3.5 (Applied Biosystems).

Population Structure. Genetic structuring among populations was inferred for all *Bd*-infected individuals. We used the program Structure version 2.3 (Pritchard *et al.* 2000b) to identify the most likely number of genetic clusters (*K*) present and the proportion of membership in those clusters for each *Bd*-infected clutch. This method uses Bayesian assignment techniques to identify clusters of genetically similar individuals from multilocus genotypes without prior knowledge of their population affinities. Twenty independent runs were performed for each value of *K* ranging from 1–12, with a burn-in of one million Markov chain Monte Carlo (MCMC) iterations and a data collection period

of three million MCMC iterations.

***MHC amplification, cloning and sequencing*.** We extracted genomic DNA from toe-clips of *Bd*-infected individuals using Qiagen DNeasy kits. We used a degenerate MHC class IIB forward primer (MHC-F; Kiemnec-Tyburczy *et al.* 2010) and an intron-specific reverse primer (B1intron2_R; Hauswaldt *et al.* 2007) to amplify 246 base pairs (bp) of exon 2 and 189 bp of adjacent 3' flanking intron. This primer pair was designed to amplify alleles from a single locus and ensure that only orthologues were compared, because at least two MHC class IIB loci are expressed in *L. yavapaiensis* (Kiemnec-Tyburczy *et al.* 2010). Subsequent analyses included the exon 2 coding region only. Amplifications were performed for 35 cycles at 95°C for 50 s; 60°C for 45 s; and 72°C for 1 min. We cloned PCR products into the Promega pGEM® T vector and transformed recombinant DNA into Invitrogen TOP-10 *E. coli*. Cells were grown on Luria agar plates for 18-22 hours at 37°C. We used blue/white screening to choose 8-32 clones from each transformation and amplified them using M13 primers with standard reaction conditions. PCR products were purified using an alkaline phosphatase-exonuclease reaction and sequenced on an ABI 3730 Sequencer using Big Dye v3.1 chemistry. We discarded MHC sequences recovered singly with ≤ 2 nucleotide polymorphisms to other sequences, attributing these small differences to PCR or cloning error. After discarding these sequences, no more than two alleles were recovered from any individual. We used translated amino acid queries in GenBank to confirm MHC class IIB homology and aligned sequences in Sequencher v. 4.10 (Gene Codes Corporation).

Genealogy reconstruction. We tested the alignment of MHC sequences for recombination using Genetic Algorithm for Recombination Detection (Kosakovsky Pond

et al. 2006), and detected no recombination, thus we performed an unrooted maximum likelihood (ML) analysis. Model parameters were determined using Akaike and Bayesian information criteria in jModeltest version 0.1.1 (Posada 2008). The best-fit model (HKY85) was used in a ML analysis in PAUP* (Sinauer Associates) using the heuristic search option, TBR branch swapping, MulTrees option in effect, and a single neighbor-joining tree as a starting topology. Node support was estimated from 1000 bootstrap replicates.

Tests of selection. We ran tests of selection using the HyPhy software package (Kosakovsky Pond *et al.* 2005) with the ML genealogy as our input tree. We used the PARRIS method to test for positive selection across the alignment, and the Evolutionary Fingerprinting method to infer positive selection rate classes and selection intensity. We tested for residue-specific selection in PBR lineages using three maximum likelihood methods: Single Likelihood Ancestral Counting (SLAC), Fixed Effects Likelihood (FEL), and Random Effects Likelihood (REL; Kosakovsky Pond *et al.* 2005).

***Analysis of risk factors*.** We calculated Relative Risk (RR) using equation (1):

(1) $RR = (p^{+} \times c^{-}) \div (p^{-} \times c^{+})$,

where p^{+} is the frequency of frogs with allele *x* that died, p^{-} is the frequency of frogs with allele *x* that did not die, c^{-} is the frequency of frogs without allele *x* that died, and c^{+} is the frequency of frogs without allele *x* that did not die. Significance of each RR value was assessed using Fisher's exact test and sequential Bonferroni correction (Rice 1989).

Acknowledgements: We thank S. Bogdanowicz, M. Lenker, K. Kiemnec, J.Q. Richmond, M. Schlaepfer, E. Davidson, V. Miera, R. Retallick, and E. Retallick for lab and field support, and A. Longo, G. Becker, and J. Cavatorta for constructive comments on the manuscript. This work was funded by Cornell University, a Population and Evolutionary Process NSF Grant (DEB-0815315) to K.Z., and a NSF-DDIG (DEB-0909013) to A.E.S.

REFERENCES

Apanius V, Penn D, Slev P, Ruff L (1997) The nature of selection on the major histocompatibility complex. *Critical Reviews in Immunology,* **17**, 179–224.

Bernatchez L, Landry C (2003) MHC studies in nonmodel vertabrates: what have we learned about natural selection in 15 years? *Journal of Evolutionary Biology*, **16**, 363–377.

Bos D, DeWoody JA (2005) Molecular characterization of major histocompatibility complex class II alleles in wild tiger salamanders (*Ambystoma tigrinum*). *Immunogenetics*, **57**, 775–781.

Boyle DG, Boyle DB, Olsen V, Morgan JAT, Hyatt AD (2004) Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Diseases of Aquatic Organisms*, **60**, 141–148.

Braciale TJ, Morrison LA, Sweetser MT, *et al.* (1987) Antigen presentation pathways to class I and class II MHC-restricted T lymphocytes. *Immunological Reviews*, **98**, 95–114.

Bradley G, Rosen PC, Sredl MJ, *et al* (2002) Chytridiomycosis in native Arizona frogs. *Journal of Wildlife Diseases*, **38**, 206–212.

Briggs CJ, Vredenburg VT, Knapp RA, Rachowicz LJ (2005) Investigating the population-level effects of chytridiomycosis, an emerging infectious disease of amphibians. *Ecology*, **86**, 3149–3159.

Carillo-Farga J, Castell A, Perez A, Rondan A (1990) Langerhans-like cells in amphibian epidermis. *Journal of Anatomy,* **172**, 39–45.

Clopper C, Pearson S (1934) The use of confidence or fiducial limits illustrated in the case of the binomial. *Biometrika*, **26**, 404–413.

Cox DR (1972) Regression models and life tables. *Journal of the Royal Statistical*

Society B, **34**,187–220.

Crawford AJ, Lips KR, Bermingham E (2010) Epidemic disease decimates amphibian abundance, species diversity, and evolutionary history in the highlands of central Panama. *Proceedings: Biological Sciences*, **107**, 13777–13782.

Du Pasquier L, Flajnik MF (1990) Expression of MHC class II antigens during *Xenopus* development. *Developmental Immunology,* **1**, 85–95.

Gandon S (2002) Local adaptation and the geometry of host–parasite coevolution. *Ecology Letters,* **5**, 246–256.

Gandon S, Michalakis Y (2002) Local adaptation, evolutionary potential and host-parasite coevolution: interactions between migration, mutation, population size and generation time. *Journal of Evolutionary Biology,* **15**, 451–462.

Hauswaldt JS, Stuckas H, Pfautsch S, Tiedemann R (2007) Molecular characterization of MHC class II in a non-model anuran species, the fire-bellied toad (*Bombina bombina*). *Immunogenetics*, **59**, 479–491.

Hughes A, Yeager M (1998) Natural selection at major histocompatibility complex loci of vertebrates. *Annual Review of Genetics,* **32**, 415–435.

Hyatt AD, Boyle DG, Olsen V *et al.* (2007) Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms*, **73**, 175–192.

Jones, EY, Fugger L, Strominger JL, Siebold C (2006) MHC class II proteins and disease: a structural perspective. *Nature Reviews* Immunology, **6***,* 271–282.

Kaufman JF, Flajnik MF, Du Pasquier L, Riegert P (1985) *Xenopus* MHC class II molecules I. Identification and structural characterization. *Journal of Immunology*, **134**, 3248– 3257.

Kiemnec-Tyburczy KM, Richmond JQ, Savage AE, Zamudio KR (2010) Selection, trans-species polymorphism and locus identification of major histocompatibility complex

class IIβ alleles of New World ranid frogs. *Immunogenetics*, **62**, 741–749.

Kimura M (1977) Preponderance of synonymous changes as evidence for the neutral theory of molecular evolution. *Nature*, **267**, 275–276.

Kosakovsky Pond SL, Frost SDW, Muse SV (2005) HyPhy: hypothesis testing using phylogenies. *Bioinformatics*, **21**, 676–679.

Kosakovsky Pond SL, Posada D, Gravenor MB, Woelk CH, Frost, SDW (2006) Automated phylogenetic detection of recombination using a genetic algorithm. *Molecular Biology and Evolution*, **23**, 1891–1901.

Langefors A, Lohm J, Grahn M, Andersen O, von Schantz T (2001) Association between major histocompatibility complex class IIB alleles and resistance to *Aeromonas salmonicida* in Atlantic salmon. *Proceedings: Royal Society B*, **268**, 479–85.

Li WH, Wu CI, Luo CC (1985) A new method for estimating synonymous and nonsynonymous rates of nucleotide substitution considering the relative likelihood of nucleotide and codon changes *Molecular Biology and Evolution*, **2**, 150–174.

Lips KR (1998) Decline of a tropical montane amphibian fauna. *Conservation Biol*ogy, **12**, 106–117.

Lips KR, Brem F, Brenes R *et al.* (2006) Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proceedings: Biological Sciences,* **103**, 3165–3170.

Lips KR, Reeve JD, Witters LR (2003) Ecological traits predicting amphibian population declines in Central America. *Conservation Biology,* **17**, 1078–1088.

Lipsitch M, Herre EA, Nowak MA (1995) Host population structure and the evolution of virulence – a law-of-diminishing-returns. *Evolution*, **49**, 743–748.

Liu Y, Kasahara M, Rumfelt LL, Flajnik MF (2002) *Xenopus* class II A genes: studies of genetics, polymorphism, and expression. *Developmental and Comparative Immunology*, **26**, 735–750.

May S, Zeisset I, Beebee TJC (2011) Larval fitness and immunogenetic diversity in chytrid-infected and uninfected natterjack toad (*Bufo calamita*) populations. *Conservation Genetics*, **12**, 805–811.

Mendelson JR 3rd, Lips KR, Gagliardo RW *et al*. (2006) Confronting amphibian declines and extinctions. *Science*, **313**, 48–51.

Morgan AD, Buckling A (2006) Relative number of generations of hosts and parasites does not influence parasite local adaptation in coevolving populations of bacteria and phages. *Journal of Evolutionary Biology*, **19**, 1956–1963.

Morgan JA, Vredenburg VT, Rachowicz LJ *et al*. (2007) Enigmatic amphibian declines and emerging infectious disease: population genetics of the frog killing fungus *Batrachochytrium dendrobatidis*. *Proceedings: Biological Sciences*, **104**,1385–1390.

Ohta Y, Goetz W, Hossain MZ, Nonaka M, Flajnik MF (2006) Ancestral organization of the MHC revealed in the amphibian *Xenopus*. *Journal of Immunology*, **176**, 3674–3685.

Penn DJ, Damjanovich K, Potts W (2002) MHC heterozygosity confers a selective advantage against multiple-strain infections. *Proceedings: Biological Sciences*, **99**, 11260–11264.

Posada D (2008) jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution*, **25**, 1253–1256.

Pounds AJ, Bustamante MR, Coloma L *et al*. (2006) Widespread amphibian extinctions from epidemic disease driven by global warming. *Nature*, **439**, 161–167.

Pritchard JK, Stephens M, Donnelly P (2000b) Inference of population structure using multilocus genotype data. *Genetics*, **155**, 945–959.

Pritchard JK, Stephens M, Rosenberg NA, Donnelly P (2000a) Association mapping in

structured populations. *American Journal of Human Genetics,* **67**, 170–181.

Råberg L, Sim D, Read A (2007) Disentangling genetic variation for resistance and tolerance to infectious diseases in animals. *Science*, **318**, 812–814.

Rachowicz LJ, Knapp RA, Morgan JAT *et al*. (2006) Emerging infectious disease as a proximate cause of amphibian mass mortality in *Rana muscosa* populations. *Ecology*, **87**, 1671–1683.

Ramsey J, Reinert L, Harper L, Woodhams D, Rollins-Smith L (2010) Immune defenses against *Batrachochytrium dendrobatidis*, a fungus linked to global amphibian declines, in the South African Clawed Frog, *Xenopus laevis. Infection and Immunity*, **78**, 3981–3992.

Regoes RR, Nowak MA, Bonhoeffer S (2000) Evolution of virulence in a heterogeneous host population. *Evolution*, **54**, 64–71.

Retallick RWR, Miera V (2007) Strain differences in the amphibian chytrid *Batrachochytrium dendrobatidis* and non-permanent, sub-lethal effects of infection. *Diseases of Aquatic Organisms,* **75**, 201–207.

Ribas L, Li M, Doddington BJ *et al*. (2009) Expression profiling the temperature-dependent amphibian response to Infection by *Batrachochytrium dendrobatidis. PLoS ONE*, **4**, e8408.

Rice WR (1989) Analyzing tables of statistical tests. *Evolution*, **43***,* 223–225*.*

Richmond JQ, Savage AE, Rosenblum EB, Zamudio KR (2009) Towards immunogenetic studies of amphibian chytridiomycosis: Linking innate and acquired immunity. *BioScience*, **59**, 311–320.

Rohr JR, Raffel TR, Romansic JM, McCallum H, Hudson PJ (2008) Evaluating the links between climate, disease spread, and amphibian declines. *Proceedings: Biological Sciences*, **105**, 17436–17441.

Rosenblum EB, Poorten TJ, Settles M *et al*. (2009) Genome-wide transcriptional response of *Silurana* (*Xenopus*) *tropicalis* to infection with the deadly chytrid fungus. *PLoS ONE*, **4**, e6494.

Sammut B, Du Pasquier L, Ducoroy P *et al.* (1999) Axolotl MHC architecture and polymorphism. *European Journal of Immunology,* **29**, 2897–2907.

Sato K, Flajnik MF, Du Pasquier L, Katagiri M, Kasahara M (1993) Evolution of the MHC: isolation of class II B-chain cDNA clones from the amphibian *Xenopus laevis. Journal of Immunology,* **150**, 2831–2843.

Savage AE, Jaeger JR (2009) Isolation and characterization of microsatellite markers in the lowland leopard frog (*Rana yavapaiensis*) and the relict leopard frog (*R. onca*), two declining frogs of the North American desert southwest. *Molecular Ecology Resources,* **9**, 199–202.

Savage AE, Sredl MJ, Zamudio KR (2011) Disease dynamics vary spatially and temporally in a North American amphibian. *Biological Conservation*, **144**, 1910–1915.

Schlaepfer MA, Sredl MJ, Rosen PC, Ryan MJ (2007) High prevalence of *Batrachochytrium dendrobatidis* in wild populations of Lowland Leopard Frogs *Rana yavapaiensis* in Arizona. *EcoHealth*, **4**, 421–427.

Shaw SD, Bishop PJ, Berger L *et al.* (2010) Experimental infection of self-cured *Leiopelma archeyi* with the amphibian chytrid *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms,* **92**, 159–163.

Skerratt LF, Berger L, Speare R *et al.* (2007) Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth*, **4**, 125–134.

Stuart SN, Chanson JS, Cox NA *et al.* (2004) Status and trends of amphibian declines and extinctions worldwide. *Science*, **306**, 1783–1786.

Tobler U, Schmidt BR (2010) Within- and among-population variation in chytridiomycosis-induced mortality in the toad *Alytes obstetricans. PloS ONE*, **5**,

e10927.

Tong J, Bramson J, Kanduc D *et al.* (2006) Modeling the bound conformation of *Pemphigus vulgaris*-associated peptides to MHC Class II DR and DQ alleles. *Immunome Research*, **2**, 1–10.

Wake DB, Vredenburg VT (2008) Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proceedings: Biological Sciences*,**105**, 11466–11473.

Wegner KM, Kalbe M, Kurtz J, Reusch TRB, Milinski M (2003) Parasite selection for immunogenetic optimality. *Science*, **301**, 1343–1343.

Woodhams DC, Bosch J, Briggs CJ *et al*. (2011) Mitigating amphibian disease: strategies to maintain wild populations and control chytridiomycosis. *Frontiers in Zoology*, **8**, 8–31.

Zhan J, Mundt CC, Hoffer ME, McDonald BA (2002) Local adaptation and effect of host genotype on the rate of pathogen evolution: an experimental test in a plant pathosystem. *Journal of Evolutionary Biology*, **15**, 634–647.

Zuk M, Stoehr AM (2002) Immune defense and life history. *American Naturalist,* **160**, S9–S22.

CHAPTER 4:

IMMUNOGENETIC ADAPTATION TO AN EMERGENT INFECTIOUS DISEASE IN NATURAL POPULATIONS OF A DECLINING AMPHIBIAN

Anna E. Savage[1] and Kelly R. Zamudio[1]

[1]Department of Ecology and Evolutionary Biology, E150 Corson Hall, Cornell University, Ithaca, NY 14853 USA

Abstract

For the last three decades, amphibians have been declining worldwide due to the emerging fungal disease chytridiomycosis, but we are just beginning to understand how immunogenetic variability contributes to disease susceptibility. We recently showed that lineages of an expressed Major Histocompatibility Complex (MHC) class II locus involved in acquired immunity associate with chytridiomycosis resistance and susceptibility among frog populations and individuals in controlled laboratory challenge assays. Here, we extended these findings to a survey of natural populations that vary in their exposure and response to the pathogen *Batrachochytrium dendrobatidis* (*Bd*). We found that MHC allelic lineages associated with disease resistance in the field show a molecular signal of positive selection, while those associated with susceptibility do not, confirming that heritable disease resistance may be rapidly evolving in the wild. We confirmed that natural selection has shaped MHC variability by comparing allele frequencies of MHC exons to neutral loci. In a population with complete *Bd* resistance in nature, we detected a significant signal of directional selection for the same chytridiomycosis resistance allele (allele Q) that showed a significant association to survival in our lab experiments. Our findings indicate that strong selective pressure for

disease resistance can drive rapid immunogenetic adaptation in natural populations, despite differences in environment, demography, and the many other factors that differ among natural populations. Our field-based survey of immunogenetic variation confirms that natural amphibian populations have the evolutionary potential to adapt to chytridiomycosis.

Introduction

Emerging infectious disease in natural populations is among the five leading threats to biodiversity, and mitigating its effects is a top conservation priority (Balmford *et al*. 2005). Amphibians are early indicators of environmental change (Blaustein & Wake 1990; Welsh & Ollivier 1998), thus the amphibian disease chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), raises particular concern for biodiversity and human health (Daszak *et al.* 2000). Chytridiomycosis has resulted in the decline or extinction of hundreds of species worldwide (Berger *et al.* 1998; Skerratt *et al.* 2007). Amphibian species demonstrate a wide range of responses to chytridiomycosis (Stuart *et al*. 2006) that are largely driven by environmental and ecological factors (Vredenburg *et al.* 2010; Becker & Zamudio 2011; Rohr *et al.* 2011; Searle *et al.* 2011). Host immunological responses also contribute to *Bd* susceptibility (Woodhams *et al.* 2007; Richmond *et al.* 2009; May *et al.* 2011; Rollins-Smoth *et al.* 2010; Savage & Zamudio 2011), but it has been difficult to identify differences in resistance among species or natural populations because of the confounding effects of environmental control of pathogen dynamics (Piotrowski *et al.* 2004; Rohr *et al.* 2008) and demographic factors contributing to disease. Thus, the potential evolution of hosts in response to this emergent disease remains underexplored in natural populations.

Amphibian immune systems are structurally and functionally similar to other vertebrate animals in possessing innate and acquired immune pathways with cellular

and humoral pathogen destruction mechanisms (Du Pasquier *et al.* 1989). A unique feature of the amphibian immune system is the production of diverse antimicrobial skin peptides, some of which are correlated with higher *Bd* resistance (Woodhams *et al.* 2007). Frog antibodies capable of binding to *Bd* have also been identified in infected individuals (Ramsey *et al.* 2010), suggesting humoral immune defenses are activated in response to *Bd* infection. In addition to these mechanisms, a likely immune system component contributing to host *Bd* responses is the Major Histocompatibility Complex (MHC), a family of immune-related genes that are conserved across vertebrate animals (Ohta *et al*. 2000). Class I and II MHC molecules must bind pathogen molecules on their peptide-binding regions (PBRs) and present them to T-cells to initiate an acquired (as opposed to innate) immune response (Jones *et al.* 2006). This central role in initiating and regulating immune responses creates strong selection on MHC loci for numerous polymorphisms and gene copies, thereby maximizing the array of pathogens that can be recognized (Bernatchez & Landry 2003; Shiina *et al.* 2004). Class II MHC genes are expressed on immune surveillance cells in amphibian skin (Du Pasquier *et al.* 1989; Carillo-Farga *et al.* 1990) and typically recognize bacterial and fungal pathogens, whereas class I molecules are involved primarily in viral immunity and self-discrimination (Bevan 1987). Class II loci are therefore ideal targets for immunogenetic studies of chytridiomycosis, a fungal disease that is restricted to amphibian epidermal cells (Berger *et al.* 1998).

Natural wildlife populations commonly show correlations between MHC polymorphism and disease susceptibility (Bernatchez & Landry 2003). Four non-exclusive evolutionary mechanisms may explain the response of MHC allele distributions after pathogen-imposed selection in populations. First, overdominance may arise if individuals that are heterozygous at MHC loci are able to bind a wider inventory of antigens (Hughes & Nei 1989; Hughes & Nei 1992), resulting in higher fitness compared

to homozygotes (Doherty & Zinkernagel 1975; Thursz *et al.* 1997; Buchbinder *et al.* 1999). Second, directional selection may occur if a specific allele (or lineage of alleles) increases in frequency over successive generations because it confers resistance to a common pathogen imposing strong selective pressure (Teacher *et al.* 2009; Vassilakos *et al.* 2009). Third, frequency-dependent selection may occur when rare MHC alleles have a selective advantage because pathogens are selected to be adapted to the most common host genotype. Once those rare MHC alleles increase in frequency as a consequence of their advantage against common alleles (Ladle 1992, Ebert & Hamilton 1996), pathogens in turn adapt to them, resulting in a balanced polymorphism of numerous alleles (Clarke & Kirby 1966; Takahata & Nei 1990; Meyer & Thomson 2001). Finally, diversifying selection may take place if spatially heterogeneous selection from multiple pathogens or pathogen strains selects for numerous resistance-conferring alleles within populations (Hill 1998), leading to balanced MHC polymorphism (Hedrick 2002; Bernatchez & Landry 2003) that is indistinguishable from frequency-dependent selection. Some or all of these mechanisms have likely shaped MHC diversity over the history of natural wildlife populations; thus, teasing apart the specific immunogenetic consequences of *Bd*-imposed selection will require multiple lines of evidence and the ability to distinguish historical versus recent selective events.

In amphibians, the MHC has only been extensively characterized in two model frog species, *Silurana* **(***Xenopus) laevis* (Kaufman *et al.* 1985; Flajnik & Du Pasquier 1990) and *S. tropicalis* (Ohta *et al.* 2006). *Silurana* has the ancestral tetrapod MHC gene organization (Nonaka *et al.* 1999), making it a good model for higher-level comparative studies of MHC evolution (Flajnik *et al.* 1990). However, *Silurana* diverged relatively early in the anuran phylogeny (Frost *et al.* 2006) making it a poor model for MHC evolution in the remaining 6000+ frog species. Recent experimental studies in *Silurana* find that under some conditions, *Bd* infection activates innate but not acquired immune

defenses (Ribas *et al.* 2009) or minimal immune responses of any kind (Rosenblum *et al*. 2009), while under other conditions, acquired immunity plays a role in host responses (Ramsey *et al.* 2010). However, *Silurana* appears to be resistant to chytridiomycosis in the wild, never showing clinical signs of disease despite *Bd* infections (Weldon *et al.* 2004), calling into question the applicability of these studies to amphibian species highly susceptible to chytridiomycosis. MHC genes have been examined in few natural populations of non-model amphibians (Sammut *et al.* 199; Bos & DeWoody 2005; Hauswaldt *et al.* 2007; Teacher *et al.* 2009; Kiemnec-Tyburczy *et al.* 2010). Only one study to date explores MHC dynamics in relation to chytridiomycosis: in larval populations of the natterjack toad (*Bufo calamita)*, MHC class II genotype frequencies varied in a pattern consistent with directional selection in response to pathogen prevalence among populations (May *et al.* 2011). Combined, these studies suggest a role for MHC genes in chytridiomycosis dynamics, but further studies of naturally susceptible species are necessary to elucidate the precise mechanisms underlying the evolution of immunogenetic resistance to *Bd*.

The lowland leopard frog (*Lithobates yavapaiensis*) is a North American amphibian that has been declining from seasonal chytridiomycosis outbreaks for at least twenty years (Bradley *et al.* 2002; Savage *et al*. 2011a). We recently reported on experimental *Bd* infections of lab-reared lowland leopard frogs originating from five populations, that showed specific MHC genotypes associated with survival, both within and among populations (Savage & Zamudio 2011). In particular, we found that PBR heterozygotes and individuals bearing PBR allele Q had significantly higher probabilities of surviving *Bd* infection (Savage & Zamudio 2011). Here, we test the generality of those findings in natural populations; we characterize the same PBR region of an expressed MHC class II gene (Kiemnec-Tyburczy *et al.* 2010) in field-sampled frogs from eight lowland leopard frog populations currently infected with *Bd* and interpret genetic

variation at this putative functional locus in light of multi-year field estimates of population and individual *Bd* susceptibilities. We also characterize two neutral genetic markers from these same populations to compare neutral and immunogenetic genotypes in the context of disease dynamics and identify significant signals of natural selection in response to chytridiomycosis. Our efforts to identify host immunogenetic factors that contribute to *Bd* susceptibility in natural populations complement our lab-based association studies by elucidating the mechanisms of evolutionary response to disease across a variable ecological and environmental landscape.

Results

Intra- and Interpopulation Variation in Field Bd Disease Dynamics

Lowland leopard frog populations are genetically and geographically distinct (Table S4.1; Table S4.2) and respond differently to *Bd* infection (Figure 4.1). In three populations (HR, SM and SS), *Bd* infected frogs persist without mortality, while in five populations (AC, CIC, MR, TV, and WC), a proportion of individuals develop chytridiomycosis and die (Figure 4.1A), but that proportion varies significantly across populations, with mortality being highest in population TV and lowest in AC. In a previous laboratory study, we performed *Bd* challenge assays on frogs collected from AC, CIC, MR, SM and WC (Savage & Zamudio 2011; hereafter referred to as our experimental infection study), and we found a strong association with *Bd* resistance and two immunogenetic factors: class II MHC heterozygosity, and the presence of class II allele Q.

In addition to differences in *Bd* infection prevalence, infection intensity (measured as the logarithm of the number of genome equivalents recovered per swab) also varies across natural populations (Figure 4.1B). Surprisingly, some populations with no mortality have higher mean infection intensities than other populations with mortality

(e.g., HR versus WC; Figure 4.1B), suggesting that infection intensities have functionally different consequences in different locations. However, within populations, frogs that died had significantly higher mean *Bd* infection intensities than frogs that survived (two-tailed paired Student's t-test, P=0.046; Figure 4.1C), confirming that an inability to limit pathogen burden is the proximate cause of *Bd*-associated mortality within populations that show susceptibility to *Bd*. At present, we are incapable of clearly distinguishing between *Bd* resistance (limiting pathogen burden) and tolerance (limiting the damage incurred by a given pathogen burden; Råberg *et al.* 2007), and how the two mechanisms differ within and among populations, thus for simplicity we hereafter refer to any process of surviving *Bd* infection as resistance, with the knowledge that tolerance is involved in at least some populations.

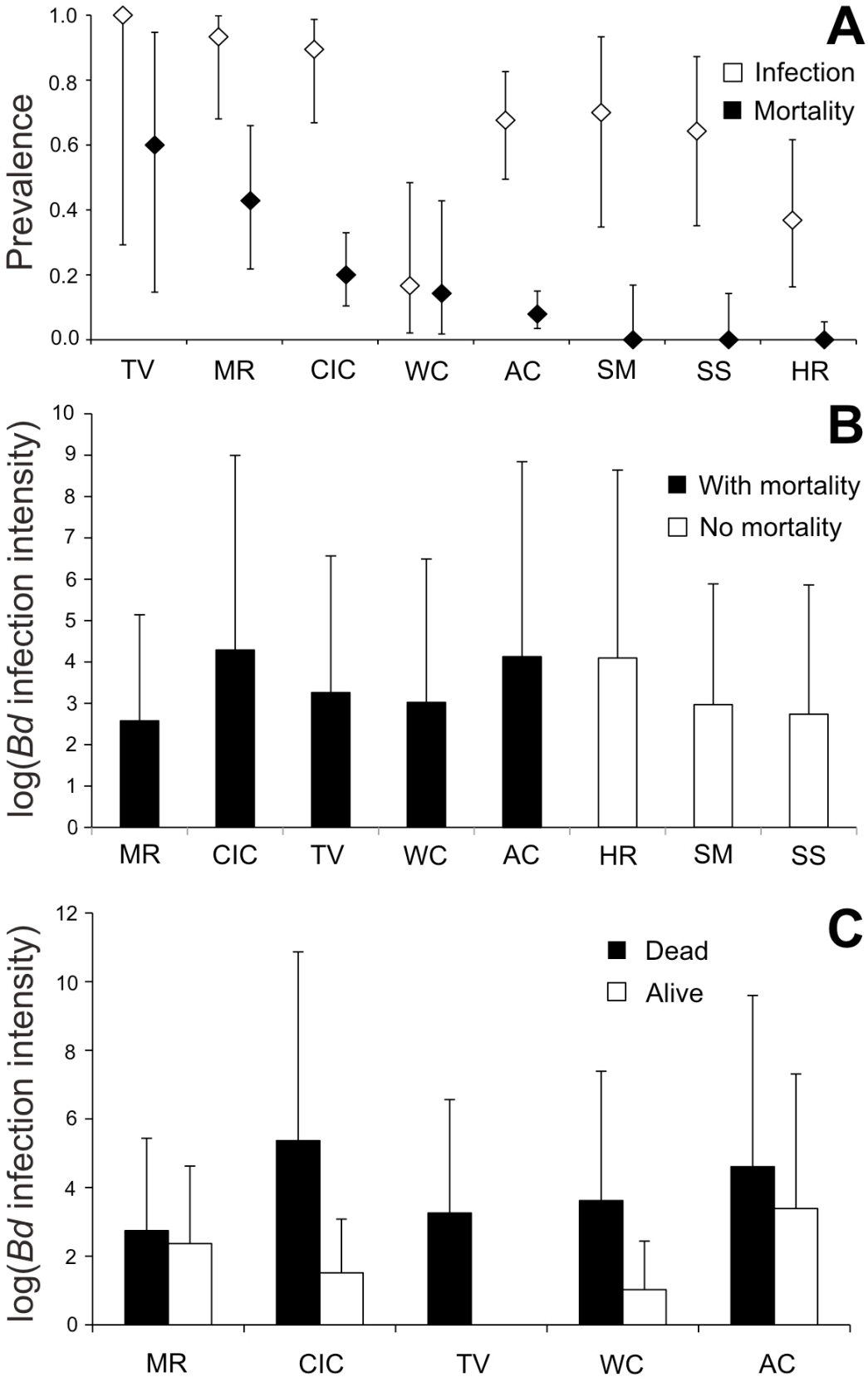
A
Prevalence
1.0
0.8
0.6
0.4
0.2
0.0
Infection
Mortality
TV
MR
CIC
WC
AC
SM
SS
HR
B
log(Bd infection intensity)
10
9
8
7
6
5
4
3
2
1
0
With mortality
No mortality
MR
CIC
TV
WC
AC
HR
SM
SS
C
log(Bd infection intensity)
12
10
8
6
4
2
0
Dead
Alive
MR
CIC
TV
WC
AC

Figure 4.1: *Batrachochytrium dendrobatidis* (*Bd*) infection and chytridiomycosis mortality vary among *Lithobates yavapaiensis* populations. (A) Prevalence of *Bd* infection (proportion of frogs testing positive for *Bd*; open symbols) and mortality (proportion of frogs that died; closed symbols) in populations with and without chytridiomycosis mortality. AC = Aravaipa Canyon; CIC = Cienega Creek; HR = Hassayampa River; MR= Muleshoe Ranch; SM = Santa Maria River; SS = Seven Springs; TV = Tanque Verde Canyon; WC = Willow Creek; error bars indicate 95% binomial confidence intervals. (B) Logarithm of the mean *Bd* infection intensity (genome equivalents +SD) among individuals from populations with (closed symbols) and without (open symbols) chytridiomycosis mortality (C) Logarithm of the mean *Bd* infection intensity (genome equivalents +SD) among surviving individuals (open symbols) and dying individuals (filled symbols) in populations with chytridiomycosis mortality.

MHC Alleles and Lineages Associate with Bd *Resistance*

We cloned and sequenced MHC class II PBR alleles from a single expressed locus (Kiemnec-Tyburczy *et al.* 2010) for 128 frogs from eight populations in Arizona, USA (Figure S3.1). We identified 84 unique PBR alleles, and we reconstructed a Bayesian genealogy to examine their distribution among populations with and without chytridiomycosis mortality (Figure 4.2). Three PBR alleles were recovered at high frequency (alleles A, N and Q); the same three alleles that were recovered in our earlier experimental *Bd* infection of lab-reared frogs (Savage & Zamudio 2011). Sixty-six of the 84 alleles were singleton private alleles (SPA), meaning the sequence was recovered once among all individuals sequenced from eight populations. An additional seven sequences were private alleles that were recovered more than once, but only from a single population. Due to the high proportion of singleton alleles, in addition to analyzing genetic association of the common alleles (A, N, Q) with *Bd* susceptibility, we also tested

for associations of clades with ≥99% support and ≥5 alleles recovered from dead individuals (Clades R1, R2 and S1, Figure 4.2), as groups of closely related alleles may bind the same pathogen epitopes and provide the same level of protective immunity (MacDonald *et al.* 2000).

To explore associations between PBR genotypes and *Bd* dynamics within populations, we examined the distribution of the three high-frequency alleles (A, N, and Q), the three well-supported clades (R1, R2 and S1), and PBR heterozygotes versus homozygotes among individual frogs found dead or alive in the field (Table 4.1). PBR allele A and clade S1 were both significantly associated with disease susceptibility; individuals with these alleles had more than a three-fold increased risk of death. Notably, allele A was also significantly associated with mortality in experimentally infected lowland leopard frogs (Savage & Zamudio 2011). In contrast, PBR allele Q and clade R1 were significantly associated with disease resistance; individuals with these sequences had a three- to four-fold reduced risk of death. Allele Q was the only allele that also showed a significant association with survival in experimentally infected lowland leopard frogs (Savage & Zamudio 2011). Although clade R2 did not show a significant association with survival due to its small sample size, this allele was only recovered from surviving individuals from a *Bd*-resistant population (SS). Surprisingly, PBR heterozygotes showed no reduced risk of mortality in the field, despite a strong survival advantage of heterozygosity in experimentally infected frogs (Savage & Zamudio 2011).

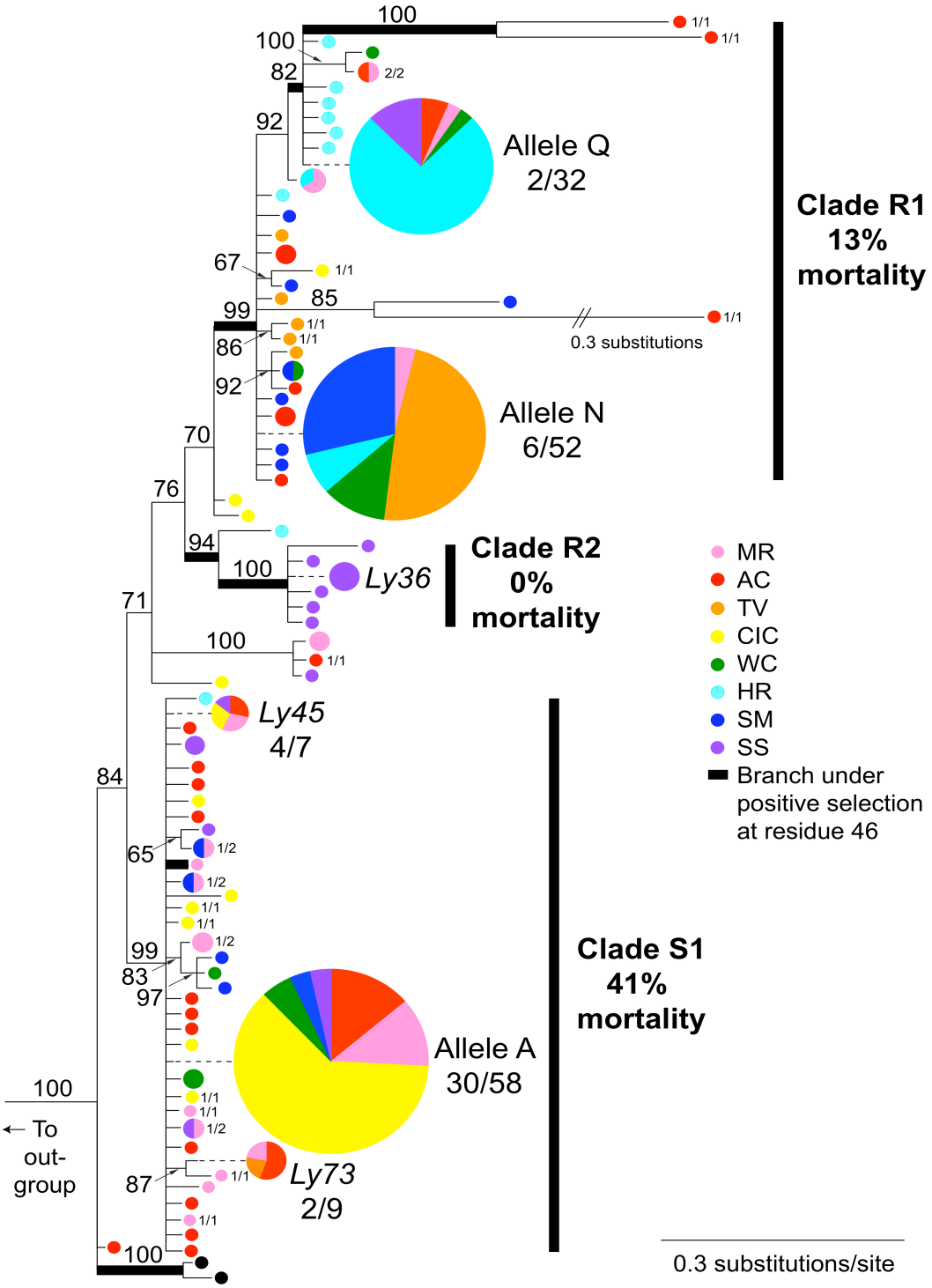
Allele Q
2/32
Allele N
6/52
Ly36
Ly45
4/7
Allele A
30/58
Ly73
2/9
0.3 substitutions
Clade R1
13%
mortality
Clade R2
0%
mortality
Clade S1
41%
mortality
MR
AC
TV
CIC
WC
HR
SM
SS
Branch under
positive selection
at residue 46
← To
out-
group
0.3 substitutions/site

Figure 4.2: MHC class II peptide-binding region (PBR) lineages experience positive selection and associate with chytridiomycosis mortality across frog populations and individuals. Maximum likelihood genealogy of 84 PBR sequences. Alleles from populations with mortality are in black and those from populations without mortality are in white, with circle size proportional to frequency. One high frequency allele associates with populations that do not experience chytridiomycosis mortality (I), while another associates with populations that do (II). Significantly elevated nonsynonymous substitution rates occur along three terminal branches leading to MHC alleles from four populations. Alleles from individuals in populations without mortality are in white, from dying individuals in populations with mortality are in black, and from surviving individuals in populations with mortality are in gray. The codon positions and amino acid substitutions occurring along lineages under positive selection are in boxes. Posterior Probabilities (PP) >70% are indicated. Population name abbreviations follow Figure 4.1. Dashed lines indicate spacing for presentation purposes and are not branches.

Table 4.1: MHC risk factors associated with *Bd* mortality.

MHC Risk Factor	Relative Risk*	*P*-value	95% CI
Heterozygote	0.68	0.22	0.35 – 1.30
Clade R1	**0.40**	**<0.0001**	**0.23 – 0.68**
Clade R2	0.00	0.12	0.00 – 1.41
Clade S1	***3.64***	***<0.0001***	***2.15 – 6.37***
Allele A	***3.20***	***<0.0001***	***2.10 – 4.79***
Allele N	0.51	0.091	0.20 – 1.14
Allele Q	**0.23**	**0.008**	**0.04 – 0.86**

*Significant values (after Bonferroni correction) are shown in bold for reduced risk of mortality, and bold italics for increased risk of mortality.

Codon 46 Is Positively Selected in Lineages Associated with Resistance

To distinguish selection at MHC loci from neutral evolution, we examined nucleotide substitution rate ratios within the 83 codons of our PBR alleles. A substitution rate ratio is the ratio of nonsynonymous nucleotide substitutions per nonsynonymous sites (dN) to synonymous substitutions per synonymous sites (dS), or ω, for each codon position in a sequence alignment. Values of ω that are significantly greater than 1 indicate positive or diversifying selection, whereas values of ω that are significantly less than 1 indicate negative or purifying selection. Evolutionary models that included positive selection ($\omega > 1$ for a proportion of codons) fit the alignment of PBR sequences significantly better than models excluding positive selection (Evolutionary Fingerprinting, log likelihood = -1951.54, $P < 0.001$). The best-fit model included three nucleotide substitution rate classes, and estimated that 65% of codons experienced negative selection ($\omega = 0.78$), 30% of codons experienced moderate positive selection ($\omega = 1.28$), and 5% of codons experienced strong positive selection ($\omega = 1.95$). Using the most conservative codon-specific test of selection, Single Likelihood Ancestral Counting (SLAC), we detected a significant signature of positive selection acting on codon 46 of the PBR alignment ($P = 0.05$, Normalized dN–dS = 4.08). Positive selection was detected at codon 46 along the branches leading to clades R1 and R2, but not clade S1 (Figure 4.2, highlighted branches). This residue is located at one of the 14 codon positions that determine peptide binding in the human MHC class II PBR (Tong *et al.* 2006), corroborating its function in binding pathogen peptides to initiate amphibian acquired immune responses. Further, this was the same residue where a significant signal of positive selection was detected in our experimental infection study (Savage & Zamudio 2011). This result corroborates the finding that codon 46 may be particularly important in determining *Bd* peptide binding, such that a single nonsynonymous

substitution has resulted in a chytridiomycosis resistance allele.

Neutral and PBR Loci Show Discordant Patterns of Population Differentiation

To disentangle the genetic signatures of selection and demography, we compared MHC class II PBR genotype frequencies to genotype frequencies of two putatively neutral markers: (1) the adjacent MHC intron, and (2) 14 microsatellite loci (Figure 4.3). Concordant patterns among neutral and MHC loci would indicate that demography dictates population differentiation. In contrast, directional or balancing selection for the same MHC allele(s) across populations should produce lower differentiation at MHC compared to neutral loci, whereas if selection on the MHC is distinct in each locality, populations should be more differentiated at MHC compared to neutral loci. For all eight populations, indices of population genetic differentiation (F_{ST}) estimated from MHC versus neutral markers are incongruent, demonstrating that selection and not demography has shaped MHC differentiation among populations. In some population comparisons, neutral markers show higher differentiation compared to MHC (Figure 4.3; values below the dashed line), a pattern expected if balancing selection is homogenizing MHC allele frequencies and reducing between-population differentiation. In the other population comparisons, MHC differentiation is higher than neutral markers (Figure 4.3; values above the dashed line), consistent with directional selection for different MHC alleles among those population pairs.

The MHC intron is in tight linkage with the PBR exon under selection, whereas microsatellite loci are distributed throughout the genome, thus only very recent demographic processes would differentiate results from these two loci. We detected the same pattern of differentiation using microsatellites and intronic sequences; however, the incongruence between PBR and neutral pairwise F_{ST} measures was higher when comparing PBR genotypes to microsatellite genotypes. HR was the only population with

all pairwise population comparisons falling above the dashed line (Figure 4.3B), indicating directional selection for a single PBR allele, while in all other populations, some pairwise comparisons indicated higher MHC differentiation and others indicated lower MHC differentiation. Combined, indices of population differentiation demonstrate that selection is shaping PBR evolution, but the mode(s) of selection are distinct in each population, perhaps reflective of the different pathogen epidemiologies across localities (Figure 4.1B).

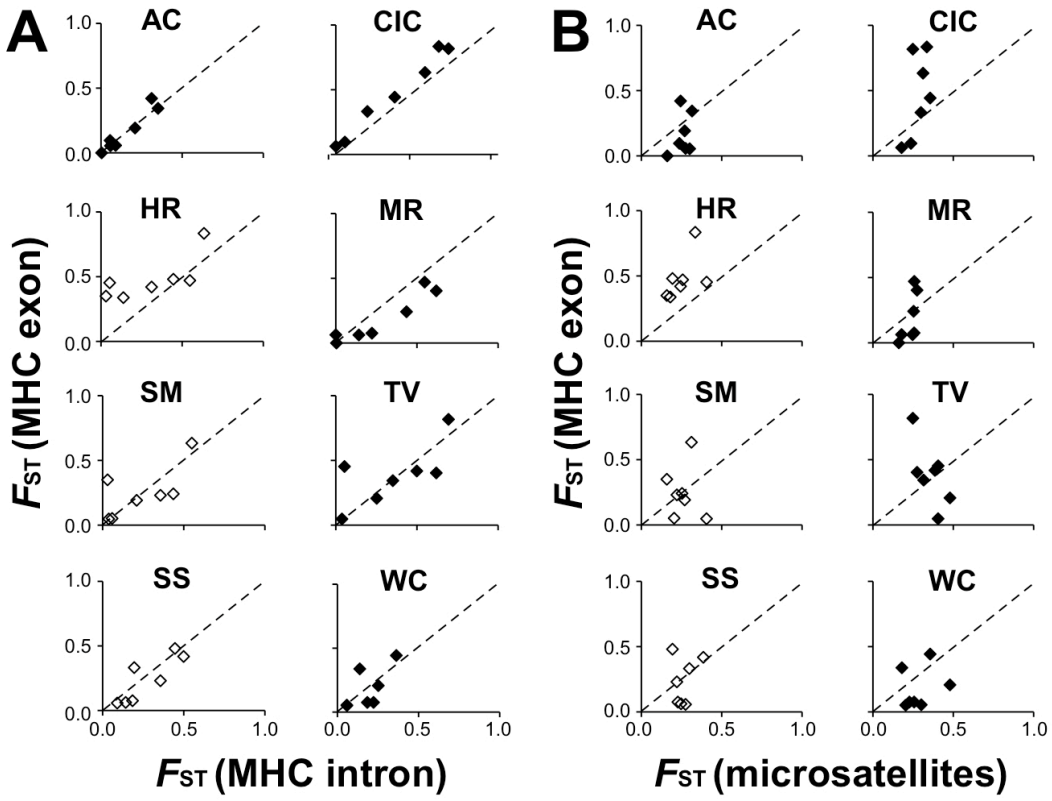

Figure 4.3: MHC class II PBR alleles show signatures of selection in *Lithobates yavapaiensis* populations. (A) Pairwise population differentiation indices (F_{ST}) calculated from MHC class II PBR exon and MHC class II intron genotypes. (B) Pairwise population differentiation indices (F_{ST}) calculated from MHC class II PBR exon and 14-locus

microsatellite genotypes. The dashed line represents perfect concordance among F_{ST} values measured from both genetic marker types; values above the line indicate higher differentiation at PBR genotypes, and values below the line indicate higher differentiation at non-coding genotypes. Filled symbols represent populations with mortality and open symbols represent populations without mortality.

Neutral and PBR Loci Detect Population Histories of Directional Selection and Demographic Expansion

We inferred the mode of selection acting within each population by examining neutral and MHC genotypes. Genetic diversity was significantly higher for PBR exons compared to introns when measured either as nucleotide diversity (π; two-tailed paired Student's t-test, P=0.014) or the number of segregating sites (θ; two-tailed paired Student's t-test, P=0.0097; Table 4.2). We conducted Ewens-Watterson, Tajima's D, and Fu's F_S tests of selective neutrality on MHC class II PBR exon alleles to infer signatures of directional or balancing selection, and also performed these tests on MHC intron and microsatellite alleles (where possible) as a control for the effects of demography (Nielsen *et al.* 2009). For the PBR exon, at least one of the three tests produced a significant signal of directional selection for populations AC, CIC, HR, SM, and TV; in contrast, we detected no signal of directional selection for populations MR, SS and WC, and no significant signal of diversifying selection in any population (Table 4.2, Figure 4.4).

Table 4.2: Population genetic variability and signatures of selection.

Pop.	Genetic region	E-W test *P*-value	Tajima's *D* (*P* -value)	Fu's F_S (*P* -value)	π	θ
Populations with mortality						
CIC† (*N*=24)	MHC exon	***1.00****	***-2.1 (0.004)***	*-1.8 (0.25)*	2.97	7.89
	MHC intron	***0.99***	*-1.5 (0.06)*	***-3.7 (0.04)***	1.70	3.15
	Microsatellites	*0.73*	*NA*	*NA*	*NA*	*NA*
MR† (*N*=15)	MHC exon	0.65	-0.43 (0.40)	1.27 (0.74)	18.5	9.76
	MHC intron	0.85	-0.21 (0.47)	-1.2 (0.24)	2.21	2.39
	Microsatellites	0.92	*NA*	*NA*	*NA*	*NA*
TV (*N*=15)	MHC exon	***0.99***	**-1.9 (0.01)**	*0.66 (0.67)*	2.72	5.96
	MHC intron	***0.99***	**-1.9 (0.01)**	***-4.3(0.004)***	1.52	3.48
	Microsatellites	*0.87*	*NA*	*NA*	*NA*	*NA*
WC† (*N*=8)	MHC exon	*0.84*	1.36 (0.93)	2.41 (0.86)	10.4	7.84
	MHC intron	***1.00***	0.80 (0.81)	-1.2 (0.24)	3.79	3.14
	Microsatellites	0.73	*NA*	*NA*	*NA*	*NA*
AC‡ (*N*=20)	MHC exon	**0.99**	-1.4 (0.063)	*-1.0 (0.37)*	20.9	27.5
	MHC intron	**0.98**	-0.67 (0.31)	***-7.6 (0.001)***	2.63	3.33
	Microsatellites	**0.99**	*NA*	*NA*	*NA*	*NA*
Populations without mortality						
HR (*N*=19)	MHC exon	**1.00**	**-2.1 (0.004)**	-3.4 (0.09)	3.46	8.81
	MHC intron	**0.99**	**-1.6 (0.04)**	-3.3 (0.05)	2.10	4.05
	Microsatellites	**0.99**	*NA*	*NA*	*NA*	*NA*
SM‡ (*N*=14)	MHC exon	**1.00**	-1.27 (0.10)	0.26 (0.55)	8.69	13.1
	MHC intron	**0.97**	-0.024 (0.5)	-1.4 (0.22)	2.55	2.57
	Microsatellites	**0.99**	*NA*	*NA*	*NA*	*NA*
SS (*N*=11)	MHC exon	0.76	-0.20 (0.49)	0.78 (0.66)	17.3	14.5
	MHC intron	0.50	0.48 (0.75)	-1.1 (0.31)	5.27	4.66
	Microsatellites	0.92	*NA*	*NA*	*NA*	*NA*

* Bold values indicate a significant signature of directional selection ($P > 0.95$ for Ewens-Watterson tests and $P < 0.05$ for Tajima's D and Fu's F_S tests), and italicized values indicate discordance among test results for different genetic markers.

† Experimentally *Bd*-infected population with 100% mortality in the lab

‡ Experimentally *Bd*-infected population with surviving individuals in the lab

The three populations without *Bd* mortality showed distinct selective and demographic patterns. A significant signal of directional selection in population HR was congruent among the MHC exon and intron for Ewens-Watterson and Tajima's *D* tests, but neither locus showed a significant pattern for Fu's F_S, demonstrating that selection and not demographic expansion produced the observed patterns. In contrast, population SM had congruent significant tests from PBR exon, intron, and microsatellite alleles, indicating that demographic processes dominate. Finally, population SS showed no significant patterns from any loci. However, genealogical analysis found SS to be the only population with a unique clade of alleles, with a strong signal of positive selection acting on that allelic lineage (Figure 4.2), suggesting the clade may have recently diversified, possibly due to enhanced *Bd* resistance conferred by those alleles.

The five populations with *Bd* mortality did not show evidence of selection acting on PBR alleles; four of them showed significant patterns of historical demographic events and one showed no significant deviations from neutrality. Populations AC, CIC and TV had significant signals of directional selection from PBR alleles, but Fu's F_S tests, the most powerful tests for detecting population growth (Ramos-Onsins & Rozas 2002), were only significant for the PBR intron, indicating that the high frequency alleles in these populations reflect low MHC diversity following population expansion after initial *Bd*-induced bottlenecks, rather than directional selection. Population WC only had a significant Ewens-Watterson test for the MHC intron, also indicating demographic rather than selective processes. Consistent with demographic rather than selective explanations for patterns of MHC diversity, the high frequency alleles in these four populations are allele A, which associates with mortality, and allele N, which shows no *Bd*-associated pattern (Table 4.1). Finally, population MR showed no selective or demographic patterns from any genetic loci, likely due to the constant source of juvenile recruits from a nearby thermal spring that is environmentally sheltered from *Bd* and

experiences no selection for resistance (Savage *et al.* 2011a).

In summary, two of the populations without *Bd* mortality (HR and SS) show evidence of selection for PBR alleles associated with resistance, while in populations with *Bd* mortality, demographic processes (AC, CIC, TV, and WC) or neutral evolution (MR) explain neutral and MHC genetic patterns.

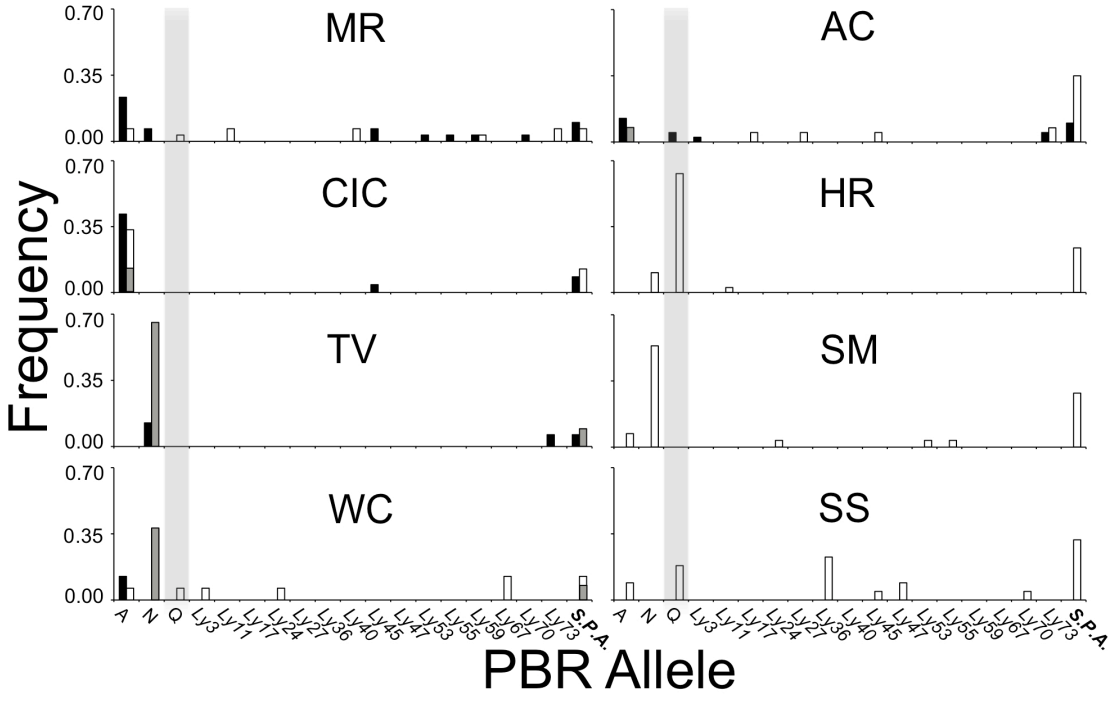

Figure 4.4: Class II PBR allele distributions of within populations. Filled and open bars represent alleles recovered from dead and live frogs, respectively. Grey bars represent individuals sampled in summer with unknown disease outcomes. Shaded bars denote the allele (Q) associated with *Bd* survival in controlled experimental infections (Savage & Zamudio 2011). Singleton private alleles (S.P.A.) are grouped together as a total proportion of the alleles recovered from each population.

Discussion

Lowland leopard frogs have been declining in recent decades due to chytriodiomycosis outbreaks, habitat loss, and invasive species (Witte *et al.* 2008; Bradley *et al.* 2002). The seasonal selective pressure imposed by *Bd* (Savage *et al.* 2011a) means that populations either must adapt, persist, or they become extirpated. Our results indicate that some populations are adapting to *Bd* via increased immunogenetic resistance, while other populations lack resistance-conferring MHC alleles and face ongoing chytridiomycosis declines. Our genealogical analyses revealed that MHC allelic lineages are not evenly distributed across populations, with alleles recovered from *Bd* resistant populations clustered in clades R1 and R2 and alleles recovered from *Bd* susceptible populations clustered in clade S1 (Figure 4.2). Additionally, positive selection acting on a pathogen-binding residue of the PBR only occurred along lineages leading to resistance-associated clades and alleles (Figure 4.2; Table 4.1). Thus, at the time of initial chytridiomycosis outbreaks, populations with standing MHC variation that included resistance alleles, or with large enough population sizes to generate novel *Bd*-resistance alleles for natural selection to act upon, have evolved partial or complete *Bd* resistance. In contrast, populations without standing immunogenetic variation for *Bd* resistance that were decimated by initial chytridiomycosis outbreaks now have small populations sizes, limited genetic diversity for selection to act upon, and struggle to persist in the face of repeated bouts of *Bd* mortality (Savage *et al.* 2011a).

Demographic processes can produce population genetic patterns that mimic the signatures of natural selection (Nielson *et al*. 2009). For example, population bottlenecks and population expansions can produce an excess or deficiency of heterozygotes, respectively (Watterson *et al.* 1986). Thus, our comparison of MHC genotypes to neutral

genetic loci further elucidates the evolutionary mechanisms enabling or inhibiting population adaptation to emergent diseases. Because the PBR exon and intron sequences are adjacent and therefore in tight linkage disequilibrium, a shift in the frequency of a PBR exon should produce the equivalent shift in intron allele frequencies. Differences between PBR exons and introns therefore reflect very recent and/or strong selection, whereas differences between PBR alleles and multi-locus microsatellites can reflect selective shifts over longer periods of time (Barton 1986; Charlesworth 2009). (Patterns of population differentiation reflect this difference; pairwise differentiation measured from PBR exons and introns produce similar values, whereas PBR exons and microsatellite markers produce discordant measures of differentiation (Figure 4.3). However, this pattern is not consistent; some pairwise F_{ST} comparisons reflect higher PBR differentiation and others reflect higher neutral differentiation, underscoring that some populations show similar PBR allele frequency distributions, producing excessively low PBR differentiation measures, and others do not, resulting in disproportionately high levels of PBR differentiation (Figure 4.4). A notable exception to this pattern is population HR, which shows consistently high measures of PBR differentiation compared to both PBR intron and microsatellites, indicative of recent directional selection for a PBR allele in this, but no other, population.

Both directional (Harf & Sommer 2005; Cutrera & Lacey 2007) and diversifying (Paterson *et al.* 1998; Bonneaud *et al.* 2005) selection on MHC alleles are commonly observed evolutionary responses to emergent pathogens in natural populations. Given that *Bd* currently imposes strong selective pressure in susceptible lowland leopard frog populations, we might expect directional selection for one or a subset of MHC alleles to predominate in our system. Indeed, significant Ewens-Watterson and Tajima's *D* tests, high PBR differentiation compared to neutral markers, and an allele frequency of 0.63 for allele Q indicate that HR, a population with *Bd* infection but no mortality in the last five

years (Figure 4.1; Savage *et al.* 2011a), experiences directional selection for allele Q, the *Bd* resistance allele previously identified in the laboratory (Savage & Zamudio 2011). However, in SS and SM, the other two populations that harbor *Bd* but show no mortality, the mechanisms underlying resistance are less clear. Population genetics at neutral and PBR loci in SM are consistent with demographic expansion alone, and PBR alleles recovered from this population show no associations with resistance, thus environmental factors, pathogen variation, or other uncharacterized genetic loci may be predominantly responsible for the observed lack of disease. In population SS, the absence of a population genetic signal of selection is likely due to the recent emergence of a group of closely related resistance alleles (Figure 4.2, clade R2); if this population continues expanding in future generations, further sampling may reveal a significant pattern of directional or diversifying selection for one or all of these alleles. Finally, the lack of any signals of selection acting on PBR genotypes in the five populations with *Bd* mortality is consistent with their ongoing *Bd* susceptibility; if any of these populations develop *Bd* resistance in future generations, we would expect a concomitant increase in the frequency of one or a few PBR alleles. Indeed, continued population monitoring in future generations may provide direct evidence for the benefits of particular PBR alleles if the expected correlated changes in allele frequency and disease susceptibility occur. Among our sampled populations, AC has the highest likelihood of evolving future disease resistance, as this population currently harbors allele Q at low frequency.

Collectively, our analyses show that selection for chytridiomycosis resistance has acted on a class II MHC locus in the declining lowland leopard frog. In population HR, *Bd*-induced selection has generated a high frequency of allele Q, consistent with adaptation arising from standing genetic variation (Feder *et al.* 2003; Colosimo *et al.* 2005). Notably, allele Q occurred in all but one of the surviving individuals in experimental *Bd* infections (Savage & Zamudio 2011). Thus, allele Q has a confirmed

role in providing enhanced *Bd* resistance, and is likely responsible for the absence of *Bd* mortality in population HR. However, allele Q also occurred at low frequency in four other populations (AC, MR, SM and WC) with no signal of directional selection, suggesting that selection for allele Q may not be strong enough to overwhelm neutral and demographic processes in some of the smaller lowland leopard frog populations that continue to decline from disease. Alternately, the benefit of allele Q may vary across populations due to eco-immunological differences in the host or the pathogen under distinct environmental regimes (Rollins-Smith *et al.* 2011).

In addition to selection acting on standing genetic variants, novel PBR alleles providing *Bd* resistance equal to or better than allele Q may have arisen in some populations. We found evidence of this in population SS, which has a low frequency of allele Q, no observed *Bd* mortality, and a clade of private alleles (clade R2) with a significant signal of positive selection acting on the same amino acid residue detected for allele Q. Although these sequences were not recovered at high enough frequency to measure a significantly reduced relative risk (Table 4.1), the observed pattern suggests that adaptation to *Bd* may have occurred rapidly in population SS from *de novo* genetic mutations, a less frequently documented phenomenon (Linnen *et al.* 2009).

Global declines caused by chytridiomycosis have had catastrophic consequences for amphibian diversity (Skerratt *et al.* 2007) and *Bd* continues to spread to new regions and hosts (Cheng *et al.* 2011; Savage *et al.* 2011b). Our study highlights the importance of examining fine-scale demographic, epidemiological, and genetic patterns if we are to elucidate the key processes underlying *Bd* disease dynamics and evolution of resistance in free-living amphibians. Identifying immunogenetic correlates of chytridiomycosis outcomes provides a mechanism to explain variable host susceptibility among individuals, populations, and perhaps species. Analyses of *Bd* dynamics within and across amphibian species, pathogen strains, and geographic regions, which have

been important in elucidating environmental and ecological predictors of disease dynamics (Briggs *et al*. 2010; Rohr *et al.* 2011), may also benefit from incorporation of host genetic contributions. Immunogenetic markers for *Bd* resistance also provide a potential tool for proactive conservation via marker-assisted selection (Woodhams *et al.* 2011), a potentially powerful tool in mitigating the impact of this global pandemic on amphibian biodiversity. Finally, identifying immunogenetic hallmarks of *Bd* resistance in natural populations is a critical step towards species recovery, as the global spread and persistence of *Bd* means that wild populations must ultimately evolve disease resistance to achieve long-term species survival.

Methods

***Field Surveys*.** We surveyed eight *L. yavapaiensis* populations for *Bd* and chytridiomycosis during winter months (January–February) of 2007-2011 (Savage *et al*. 2011a) and pooled samples across the five survey years. Using a standardized protocol (Hyatt *et al.* 2007) we swabbed the epidermis of live, sick and dead *L. yavapaiensis* individuals with sterile fine-tip swabs (Medical Wire & Equipment Co. MW113). We categorized each dead individual that tested positive for *Bd* infection as a chytridiomycosis mortality event. Additionally, we collected any individual with signs of chytridiomycosis (i.e., skin redness, lethargy, failure to seek shelter, and loss of righting ability) for overnight observation, and also categorized these as chytridiomycosis-induced mortality events if death occurred within 24h and the individual tested positive for *Bd*. We estimated *Bd* and chytridiomycosis mortality prevalence and 95% Clopper-Pearson binomial confidence intervals (Clopper & Pearson 1934) for each *L. yavapaiensis* population sample.

Microsatellite genotyping. We genotyped 282 *L. yavapaiensis* individuals at 14

previously characterized microsatellite loci (Savage & Jaeger 2008). Template DNA for microsatellite genotyping was extracted using a 5% Chelex 100 solution (Bio-Rad Laboratories) with 0.5μg proteinase K per sample. Chelex extractions were incubated at 55°C for 120 minutes, and 99°C for 10 minutes. The supernatant from these extractions was used directly as template for PCR amplification, performed under the following conditions: 5 min initial denaturation at 94°C; 35 cycles of 1 min denaturing at 94°C, 1 min annealing at primer-specific annealing temperatures, 1 min extension at 72°C; and a final extension of 75°C for 5 min. Amplified products with different labels or non-overlapping size ranges were multiplexed and run on a 3730 Genetic Analyzer (Applied Biosystems). Fragment sizes were determined by comparison with a LIZ-500 standard using GeneMapper version 3.5 (Applied Biosystems). We used GENEPOP version 3.4 (Raymond & Rousset 1995) to calculate observed and expected heterozygosities and test for deviations from Hardy-Weinberg equilibrium (HWE) at each locus and population locality using a Monte Carlo chain method (1000 dememorizations, 100 batches, 1000 iterations; Guo & Thompson 1992) and a Bonferroni correction for multiple tests (adjusted $P = 0.00022$).

MHC amplification, cloning and sequencing. We extracted genomic DNA from toe clips (preserved in ethanol) from 128 *L. yavapaiensis* individuals using Qiagen DNeasy kits (Qiagen, Valencia, CA). The majority (108) of the individuals were sampled in winter, but for populations with prohibitively small winter sample sizes (CIC, TV and WC), we sampled 20 additional frogs collected in summers of 2006 and 2007. We used a degenerate frog class II MHC β1 forward primer (MHC-F; Hauswaldt *et al.* 2009) and a ranid frog MHC β1 intron-specific reverse primer (B1intron2_R; Kiemnec-Tyburczy *et al.* 2010) to amplify 249 base pairs (bp) of exon 2 and 189 bp of adjacent 3' flanking intron. Amplifications were performed on genomic DNA for 35 cycles (95°C for 50 s; 60°C for 45

s; and 72°C for 1 min) with standard reaction conditions. We cloned the PCR products from all reactions into pGEM® T vectors (Promega, Madison, WI) and transformed recombinant DNA into TOP-10 *E. coli* cells (Invitrogen). Cells were grown on Luria agar plates for 18 hours at 37°C. We used blue/white screening to randomly choose 6-24 positive clones from each transformation and amplified them using M13 primers for 30 cycles (95°C for 1 min; 55°C for 30 s; and 72°C for 30 s) with standard reaction conditions. PCR products were visualized on a 1% agarose gel and products in the appropriate size range were purified using an alkaline phosphatase-exonuclease reaction. The products were sequenced on an automated 3730 DNA analyzer using Big Dye v3.1 chemistry (Applied Biosystems). We screened each MHC sequence and only included those obtained from at least two clones. For each individual, sequences recovered only once with ≤ 2 nucleotide differences to other cloned sequences were attributed to PCR/cloning errors and discarded. After excluding these sequences, no more than two unique MHC alleles were recovered from any individual. We used translated amino acid queries in GenBank (tBLASTx) to confirm MHC class II homology and then aligned the sequences using Sequencher v. 4.10 (Gene Codes Corporation, Ann Arbor, MI).

Genealogy reconstruction. We first tested the alignment of MHC sequences for evidence of recombination using the Single Breakpoint (SBP) method (Pond *et al.* 2006). We found no evidence of recombination, thus we performed a Bayesian analysis to reconstruct genealogical relationships among MHC alleles. We used MHC class II β1 exon 2 sequences from the frogs *Silurana laevis* and *S. tropicalis* (GenBank accession numbers NM_001114771 and NM_001045794) as outgroups. Model parameters were determined using the Akaike information criteria (AIC) in jModeltest (Posada 2008). We used the best-fit model (GTR+I+G) to estimate a 95% credible set of rooted MHC

genealogies in the software MrBayes 3.1 (Ronquist & Huelsenbeck 2003). We ran two separate analyses in MrBayes for $1x10^7$ generations and sampled every 500^{th} generation of the Markov chain. We used Tracer v1.4 to assess the stationarity of model parameters, convergence of model parameters between runs, the number of burn-in samples, and the effective sample sizes for each parameter.

Tests of selection. We ran tests of selection using the HyPhy software package (Kosakovsky Pond *et al*. 2005). We used the Bayesian genealogy as our input tree, excluding the outgroup sequences. We first used the PARRIS method to test for positive selection in the entire alignment (Scheffler *et al.* 2006). This method expands on other maximum likelihood methods for detecting positive selection by allowing substitution rates to vary across sites, while accounting for recombination by detecting breakpoints when measuring nonsynonymous and synonymous substitution rates. Second, we used the Evolutionary Fingerprinting method to infer the number of positive selection rate classes and the intensity of selection in each rate class (Kosakovsky Pond *et al.* 2010). Third, we tested for residue-specific positive selection across evolutionary lineages of MHC class IIB exon 2 sequences using the most conservative maximum likelihood method, Single Likelihood Ancestral Counting (SLAC). This method infers ancestral codon states and calculates normalized expected and observed nonsynonymous (dN) and synonymous (dS) substitutions at each site (Kosakovsky Pond & Frost 2005).

Selection and genetic differentiation among populations

We estimated F_{ST} across all population pairs for (i) 14-locus microsatellite genotypes, (ii) PBR exon genotypes, and (ii) PBR intron genotypes using Arlequin version 3.5 (Excoffier & Lischer 2010). For each population, we calculated observed and expected heterozygosity, nucleotide diversity (π), theta(θ), and performed Ewens-Watterson tests

separately for PBR exon, intron and microsatellite genotypes using Arlequin version 3.5 (Excoffier & Lischer 2010). We also performed Tajima's *D* and Fu's F_S tests on PBR exon and intron genotypes.

Statistical Analyses

Differences in *Bd* infection intensity and nucleotide diversity across populations were assessed using Student's t-tests implemented in JMP software, version 9.0 (SAS). We calculated Relative Risk (RR; Sistrom & Garvan 2004) using equation (1):

(1) $RR = (p^+ \times c^-) \div (p^- \times c^+)$,

where p^+ is the frequency of frogs with MHC allele *x* that died from chytridiomycosis, p^- is the frequency of frogs with MHC allele *x* that did not die from chytridiomycosis, c^- is the frequency of frogs without MHC allele *x* that died from chytridiomycosis, and c^+ is the frequency of frogs without MHC allele *x* that did not die from chytridiomycosis. Significance of each RR value was assessed using Fisher's exact test, with sequential Bonferroni correction for multiple comparisons (Rice 1989).

Acknowledgements: We thank J.Q. Richmond, K. Kiemnec-Tyburczy, and M. Lenker for help in the laboratory; D. Caldwell, M. Sredl, M. Haberstitch, R. Rogers and M. Lawrence for field support; and members of the Zamudio lab for discussions and comments. This research was supported by an NSF Doctoral Dissertation Improvement Grant (DEB-0909013) to A.E.S. and a Population and Evolutionary Process NSF Grant (DEB-0815315) to K.R.Z.

REFERENCES

Balmford A, Bennun L, Brink B *et al*. (2005) The convention on biological diversity's 2010 target. *Science,* **307**, 212–213.

Barton NH (1986) The effects of linkage and density-dependant regulation on gene flow. *Heredity,* **57**, 415–426.

Becker C, Zamudio KR (2011) Tropical amphibian populations experience higher disease risk in natural habitats. *Proceedings: Biological Sciences,* **108**, 9893–9898.

Berger L, Speare R, Daszak P *et al.* (1998) Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings: Biological Sciences,* **95**, 9031–9036.

Bernatchez L, Landry C (2003) MHC studies in nonmodel vertebrates: what have we learned about natural selection in 15 years? *Journal of Evolutionary Biology,* **16**, 363–377.

Bevan MJ (1987) Class discrimination in the world of immunology. *Nature*, **325**, 192–194.

Blaustein AR, Wake DB (1990) Declining amphibian populations: a global phenomenon. *Trends in Ecology and Evolution*, **5**, 203–204.

Bonneaud C, Richard M, Faivre B, Westerdahl H, Sorci G (2005) An *Mhc* class I allele associated to the expression of T-dependent immune response in the house sparrow. *Immunogenetics*, **57**, 782–789.

Bos DH, DeWoody JA (2005) Molecular characterization of major histocompatibility complex class II alleles in wild tiger salamanders (*Ambystoma tigrinum*). *Immunogenetics*, **57**, 775–781.

Bradley G, Rosen P, Sredl M, Jones T, Longcore J (2002) Chytridiomycosis in native Arizona frogs. *Journal of Wildlife Diseases*, **38**, 206–212.

Briggs CJ, Knapp RA, Vredenburg VT (2010) Enzootic and epizootic dynamics of the

chytrid fungal pathogen of amphibians. *Proceedings: Biological Sciences, **107**,* 9695–9700.

Buchbinder S, Hoots K, O'Brien S (1999) HLA and HIV-1: heterozygote advantage and B*35-Cw*04 disadvantage. *Science*, **283**, 1748-1752.

Carillo-Farga J, Castell A, Perez A, Rondan A (1990) Langerhans-like cells in amphibian epidermis. *Journal of Anatomy*, **172**, 39–45.

Carrington M, Nelson G, Martin M *et al.* (1997) Heterozygote advantage for HLA class-II type in hepatitis B virus infection. *Nature Genetics*, **17**, 11-12.

Charlesworth B (2009) Fundamental concepts in genetics: effective population size and patterns of molecular evolution and variation. *Nature Reviews Genetics*, **10**, 195–205.

Cheng TL, Rovito SM, Wake DB, Vredenburg VT (2011) Coincident mass extirpation of neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis*. *Proceedings: Biological Sciences,* **108**, 9502–9507

Clarke B, Kirby D (1966) Maintenance of histocompatibility polymorphisms. *Nature*, **211**, 999–1000.

Clopper C, Pearson S (1934) The use of confidence or fiducial limits illustrated in the case of the binomial. *Biometrika*, **26**, 404–413.

Colosimo PF, Hosemann KE, Balabhadra S *et al.* (2005) Widespread parallel evolution in sticklebacks by repeated fixation of ectodysplasin alleles. *Science*, **307**, 1928–1933.

Cutrera AP, Lacey EA (2007) Trans-species polymorphism and evidence of selection on class II MHC loci in tuco-tucos (Rodentia: Ctenomyidae). *Immunogenetics*, **59**, 937–948.

Daszak P, Cunningham AA, Hyatt AD (2000) Emerging infectious diseases of wildlife--threats to biodiversity and human health. *Science*, **287**, 443–449.

Doherty P, Zinkernagel R (1975) Enhanced immunological surveillance in mice

heterozygous at the H-2 gene complex. Nature 256: 50–52.

Du Pasquier, L, Schwager J, Flajnik MF (1989) The immune system of *Xenopus*. *Annual Review of Immunology*, **7**, 251–275.

Du Pasquier, L, Schwager J, Flajnik MF (1989) The immune system of *Xenopus*. *Annual Review of Immunology*, **7**, 251–275.

Ebert D, Hamilton W (1996) Sex against virulence: the coevolution of parasitic diseases. *Trends in Ecology and Evolution*, **11**, 79–82.

Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, **10**, 564–567.

Feder JL, Berlocher SH, Roethele JB *et al*. (2003) Allopatric genetic origins for sympatric host-plant shifts and race formation in Rhagoletis. *Proceedings: Biological Sciences,* **100**, 10314–10319.

Flajnik MF, Du Pasquier L (1990) The major histocompatibility complex of frogs. *Immunological Reviews*, **113**, 47–63.

Flajnik MF, Ohta Y, Namikawa-Yamada C, Nonaka M (1999) Insight into the primordial MHC from studies in ectothermic vertebrates. *Immunological Reviews*, **167**, 59–67.

Frost DR, Grant T, Faivovich J *et al.* (2006) The amphibian tree of life. *Bulletin of the American Museum of Natural History*, **297**, 1-–370.

Guo SW, Thompson EA (1992) Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics,* **48**, 361–372.

Harf R, Sommer S (2005) Association between MHC Class II DRB alleles and parasite load in the hairy-footed gerbil, *Gerbillurus paeba*, in the Southern Kalahari. *Molecular Ecology*, **14**, 85–91.

Hauswaldt JS, Stuckas H, Pfautsch S, Tiedemann R (2007) Molecular characterization of MHC class II in a nonmodel anuran species, the fire-bellied toad *Bombina*

bombina. *Immunogenetics*, **59**, 479–491.

Hedrick P (2002) Pathogen resistance and genetic variation at MHC loci. *Evolution*, **56**, 1902–1908.

Hill AVS (1998) The immunogenetics of human infectious diseases. *Annual Review of Immunology*, **16**, 593–617.

Hughes A, Nei M (1992) Maintenance of MHC polymorphism. *Nature*, **355**, 402–403.

Hughes AL, Nei M (1989) Nucleotide substitution at major histocompatibility complex class II loci: Evidence for overdominant selection. *Proceedings: Biological Sciences,* **86**, 948–962.

Hyatt AD, Boyle DG, Olsen V *et al.* (2007) Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms*, **73**, 175–192.

Jones EY, Fugger L, Strominger JL, Siebold C (2006) MHC class II proteins and disease: a structural perspective. *Nature Reviews Immunology*, **6***,* 271–282.

Kaufman JF, Flajnik MF, Du Pasquier L, Riegert P (1985) *Xenopus* MHC class II molecules. I. Identification and structural characterization. *Journal of Immunology*, **134**, 3248–3257.

Kiemnec-Tyburczy KM, Richmond JQ, Savage AE, Zamudio KR (2010) Selection, trans-species polymorphism and locus identification of major histocompatibility complex class IIβ alleles of New World ranid frogs. *Immunogenetics*, **62**, 741–751.

Kosakovsky Pond SL, Frost SDW, Muse SV (2005) HyPhy: Hypothesis testing using phylogenies. *Bioinformatics*, **21**, 6760–679.

Kosakovsky Pond SL, Frost, SDW (2005) Not so different after all: comparison of various methods for detecting amino-acid sites under selection. *Molecular Biology and Evolution*, **22**, 1208–1222.

Kosakovsky Pond SL, Posada D, Gravenort MB, Woelk CH, Frost SDW (2006)

Automated phylogenetic detection of recombination using a genetic algorithm. *Molecular Biology and Evolution*, **23**, 1891–1901.

Kosakovsky Pond SL, Scheffler K, Gravenor MB, Poon AFY, Frost SDW (2010) Evolutionary fingerprinting of genes. *Molecular Biology and Evolution*, **27**, 520–536.

Ladle R (1992) Parasites and sex: catching the Red Queen. *Trends in Ecology and Evolution*, **7**, 405–408.

Linnen CR, Kingsley EP, Jensen JD, Hoekstra HE (2009) On the origin and spread of an adaptive allele in deer mice. *Science*, **325**, 1095–1098.

MacDonald KS, Fowke KR, Kimani J *et al*. (2000) Influence of HLA supertypes on susceptibility and resistance to Human Immunodeficiency Virus Type 1 infection. *Journal of Infectious Diseases,* **181**, 1581–1589.

May S, Zeisset I, Beebee TJC (2011) Larval fitness and immunogenetic diversity in chytrid-infected and uninfected natterjack toad (*Bufo calamita*) populations. *Conservation Genetics*, **12**, 805–811.

Meyer D, Thomson G (2001) How selection shapes variation of the human major histocompatibility complex: a review. *Annals of Human Genetics*, **65**, 1–26.

Nielsen R, Hubisz MJ, Torgerson D *et al.* (2009) Darwinian and demographic forces affecting human protein coding genes. *Genome Research*, **19**, 838–849.

Nonaka M, Namikawa C, Kato Y *et al.* (1997) Major histocompatibility complex gene mapping in the amphibian *Xenopus* implies a primordial organization. *Proceedings: Biological Sciences,* **94**, 5789–5791.

Ohta Y, Goetz W, Hossain MZ, Nonaka M, Flajnik MF (2006) Ancestral organization of the MHC revealed in the amphibian *Xenopus*. *Journal of Immunology*, **176**, 3674–3685.

Ohta Y, Okamura K, McKinney E *et al.* (2000) Primitive synteny of vertebrate major histocompatibility complex class I and class II genes. *Proceedings: Biological*

Sciences, **97**, 4712–4717.

Paterson S, Wilson K, Pemberton J (1998) Major histocompatibility complex variation associated with juvenile survival and parasite resistance in a large unmanaged ungulate population (*Ovis aries L.*). *Evolution,* **95**, 3714–3719.

Piotrowski JS, Annis SL, Longcore JE (2004) Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. *Mycologia*, **96**, 9–15.

Posada D. 2008. jModelTest: Phylogenetic Model Averaging. *Molecular Biology and Evolution*, **25**, 1253-1256.

Råberg L, Sim D, Read A (2007) Disentangling genetic variation for resistance and tolerance to infectious diseases in animals. *Science*, **318**, 812–814.

Ramos-Onsins S, Rozas J (2002) Statistical properties of new neutrality tests against population growth. *Molecular Biology and Evolution*, **19**, 2092–2100.

Ramsey J, Reinert L, Harper L, Woodhams D, Rollins-Smith L (2010) Immune defenses against *Batrachochytrium dendrobatidis*, a fungus linked to global amphibian declines, in the South African Clawed Frog, *Xenopus laevis. Infection and Immunity*, **78**, 3981–3992.

Raymond M, Rousset F (1995) GENEPOP: population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Ribas L, Li M, Doddington BJ *et al*. (2009) Expression profiling the temperature-dependent amphibian response to infection by *Batrachochytrium dendrobatidis*. *PLoS ONE*, **4**, e8408.

Rice WR (1989) Analyzing tables of statistical tests. *Evolution*, **43**, 223–225.

Richmond JQ, Savage AE, Rosenblum EB, Zamudio KR (2009) Towards immunogenetic studies of amphibian chytridiomycosis: Linking innate and acquired immunity. *BioScience*, **59**, 311–320.

Rohr JR, Halstead NT, Raffel TR (2011) Modelling the future distribution of the

amphibian chytrid fungus: the influence of climate and human-associated factors. *Journal of Applied Ecology*, **48**, 174–176.

Rohr JR, Raffela TR, Romansica JM, McCallum H, and Hudson PJ (2008). Evaluating the links between climate, disease spread, and amphibian declines. *Proceedings: Biological Sciences*, **105**, 17436–17441.

Rollins-Smith LA, Ramsey JP, Pask JD, Woodhams DC (2011) Amphibian immune defenses against chytridiomycosis: Impacts of changing environments. *Integrative and Comparative Biology*, **51**, 552–562.

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19**, 1572–1574.

Rosenblum EB, Poorten TJ, Settles M *et al*. (2009) Genome-wide transcriptional response of *Silurana* (*Xenopus*) *tropicalis* to infection with the deadly chytrid fungus. *PLoS ONE*, **4**, e6494.

Sammut B, Du Pasquier L, Ducoroy P *et al.* (1999) Axolotl MHC architecture and polymorphism. *European Journal of Immunology,* **29**, 2897–2907.

Savage AE, Jaeger JR (2009) Isolation and characterization of microsatellite markers in the lowland leopard frog (*Rana yavapaiensis*) and the relict leopard frog (*R. onca*), two declining frogs of the North American desert southwest. *Molecular Ecology Resources,* **9**, 199–202.

Savage AE, Sredl MJ, Zamudio KR (2011a) Disease dynamics vary spatially and temporally in a North American amphibian. *Biological Conservation*, **144**, 1910–1915.

Savage AE, Grismer LL, Anuar S *et al.* (2011b) First record of *Batrachochytrium dendrobatidis* infecting four frog families from Peninsular Malaysia. *EcoHealth*, **7**, 165–170.

Savage AE, Zamudio KR (2011) MHC genotypes associate with resistance to a frog-killing fungus. *Proceedings: Biological Sciences,* **108**, 16705–16710.

Scheffler K, Martin DP, Seoighe C (2006) Robust inference of positive selection from recombining coding sequences. *Bioinformatics*, **22**, 2493–2499.

Searle CL, Biga LM, Spatafora JW, Blaustein AR (2011). A dilution effect in the emerging amphibian pathogen *Batrachochytrium dendrobatidis*. *Proceedings: Biological Sciences,* **108**, 16322–16326.

Shiina T, Inoko H, Kulski JK (2004) An update of the HLA genomic region, locus information and disease associations: 2004. *Tissue Antigens*, **64**, 631–649.

Sistrom, CL, Garvan CW (2004) Proportions, odds, and risk. *Radiology*, **230**, 12–19.

Skerratt LF, Berger L, Speare R *et al.* (2007) Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth*, **4**, 125–134.

Stuart SN, Chanson JS, Cox NA *et al.* (2004) Status and trends of amphibian declines and extinctions worldwide. *Science*, **306**, 1783–1786.

Takahata N, Nei M (1990) Allelic genealogy under overdominant and frequency-dependent selection and polymorphism of major histocompatibility complex loci. *Genetics*, **124**, 967–978.

Teacher AGF, Garner TWJ, Nichols RA (2009) Evidence for directional selection at a novel major histocompatibility class I marker in wild common frogs (Rana temporaria) exposed to a viral pathogen (Ranavirus). *PLoS ONE*, **4**, e4616.

Vassilakos, D, Natoli A, Dahlheim M, Hoelzel AR (2009) Balancing and directional selection at exon-2 of the MHC DQB1 locus among populations of odontocete cetaceans. *Molecular Biology and Evolution*, **26**, 681–689.

Vredenburg VT, Briggs CJ, Tunstall TS, Knapp RA (2010) Dynamics of an emerging disease drive large-scale amphibian population extinctions. *Proceedings: Biological Sciences,* **107**, 9689–9694.

Watterson GA (1986) The homozygosity test after a change in population size. *Genetics*, **112**, 899–907.

Weldon C, du Preez LH, Hyatt AD *et al.* (2004) Origin of the amphibian chytrid fungus. *Emerging Infectious Diseases*, **10**, 2100–2105.

Welsh HHJ, Ollivier LM (1998) Stream amphibians as indicators of ecosystem stress: A case study from California's redwoods. *Ecological Applications,* **8**, 1118–1132.

Witte CL, Sredl MJ, Kane AS, Hungerford LL (2008) Epidemiologic analysis of factors associated with local disappearances of native ranid frogs in Arizona. *Conservation Biology*, **22**, 375–383.

Woodhams DC, Ardipradja K, Alford RA *et al.* (2007) Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defenses. *Animal Conservation,* **10**, 409–417.

Woodhams DC, Bosch J, Briggs CJ *et al*. (2011) Mitigating amphibian disease: strategies to maintain wild populations and control chytridiomycosis. *Frontiers in Zoology*, **8**, 8–31.

APPENDIX 1:

SUPPLEMENTAL FIGURES

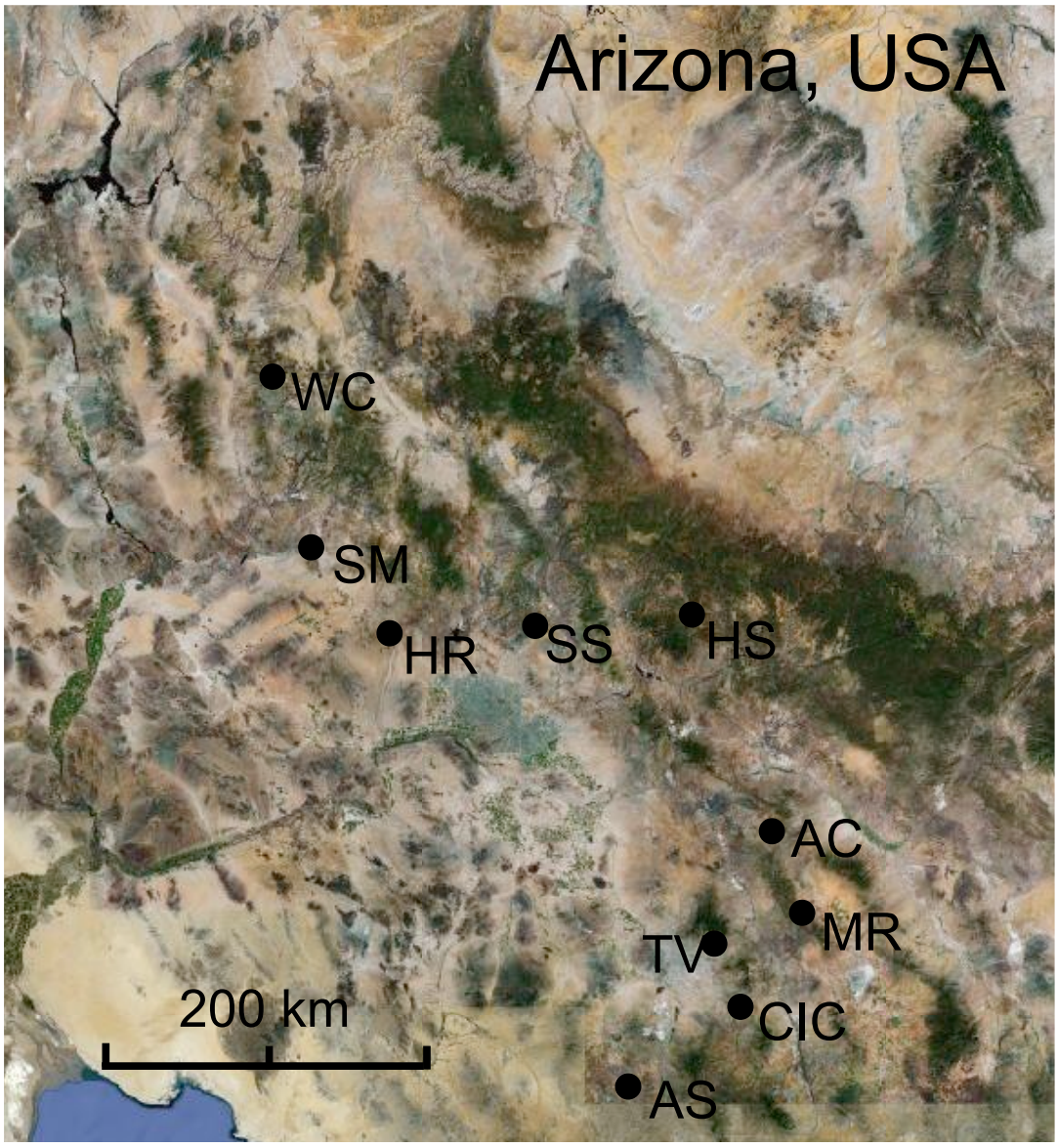

Figure S1.1: Map of *Lithobates yavapaiensis* population localities in Arizona, USA that were sampled in summers and winters of 2006 – 2010.

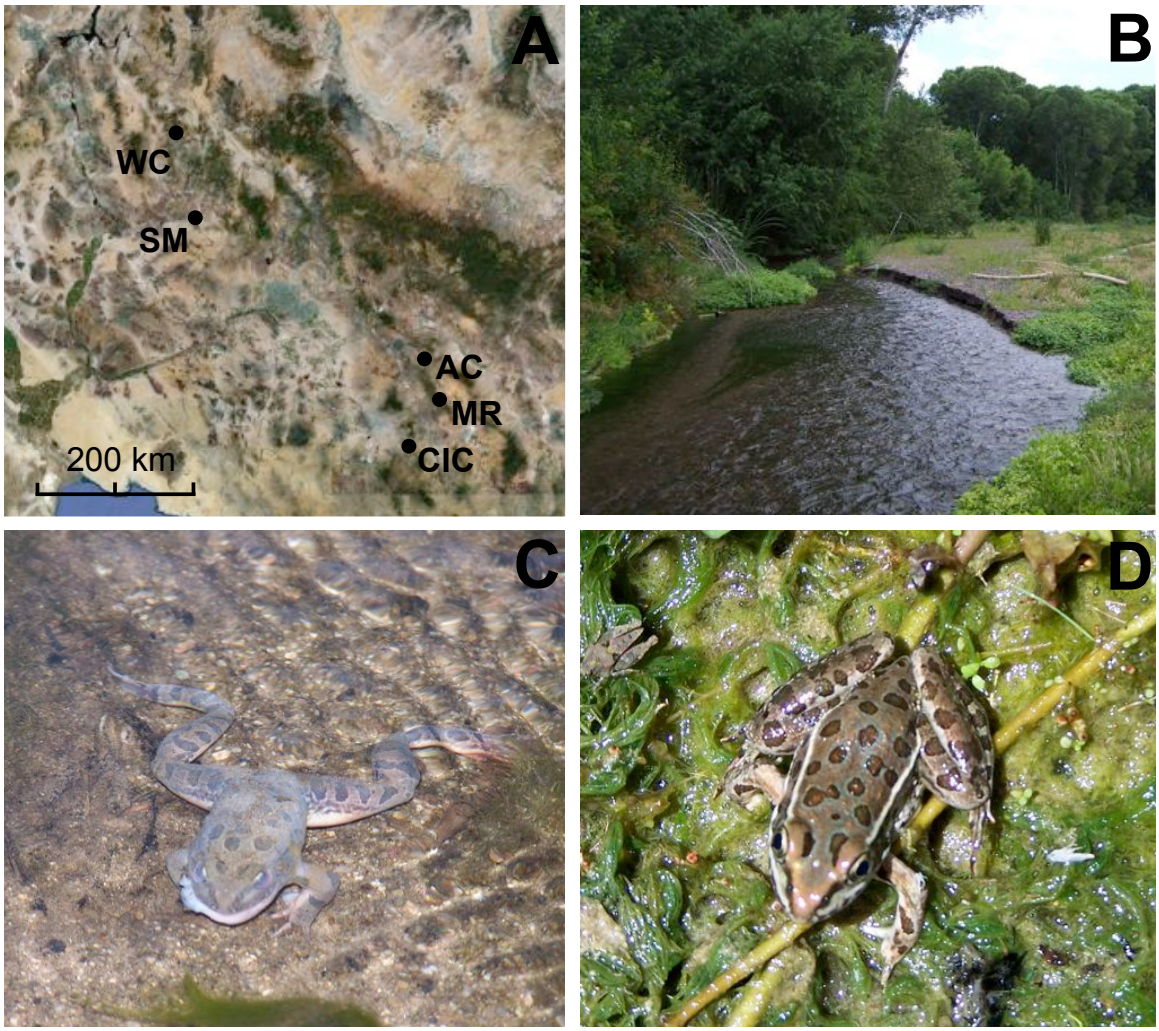

Figure S3.1: (A) Egg mass collection localities in Arizona, USA, for the five *Lithobates yavapaiensis* populations used in experimental *Bd*-infections. Site abbreviations follow Figure 1. (B) Photo of Aravaipa Canyon, showing typical *L. yavapaiensis* habitat. (C) Image of a *Bd*-infected frog found dead with signs of chytridiomycosis at Cienega Creek. (D) Image of a healthy frog from Cienega Creek.

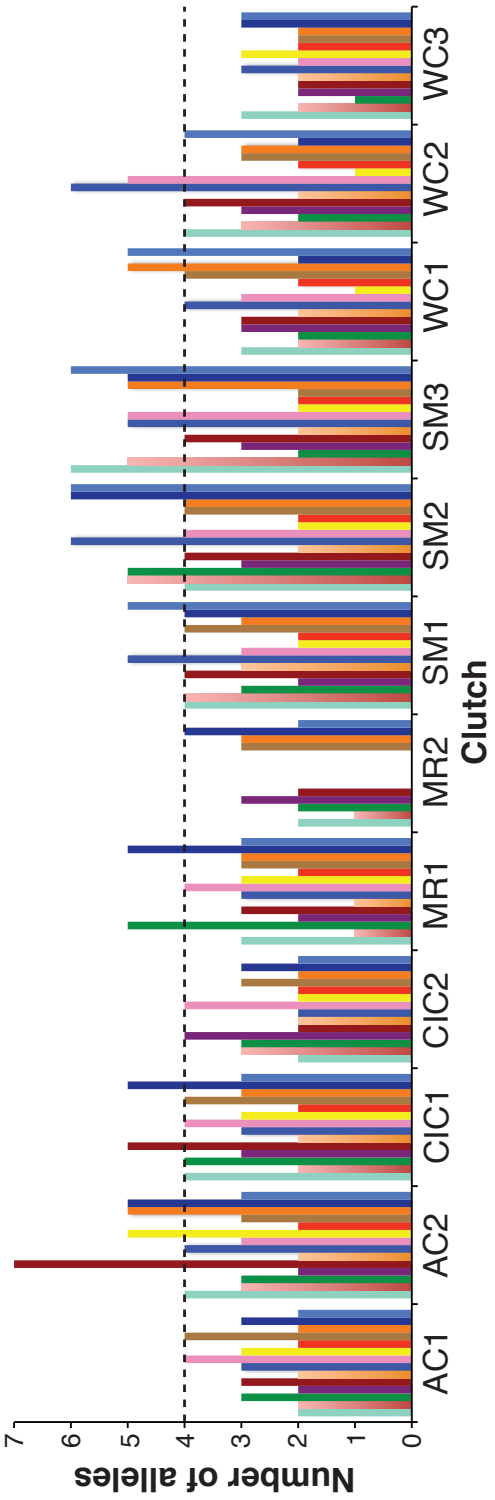

Figure S3.2: Number of microsatellite alleles recovered for each *Bd*-infected *Lithobates yavapaiensis* clutch. Colors represent each of the 14 genotyped microsatellite loci. The dotted line shows the threshold above which multiple paternity was inferred.

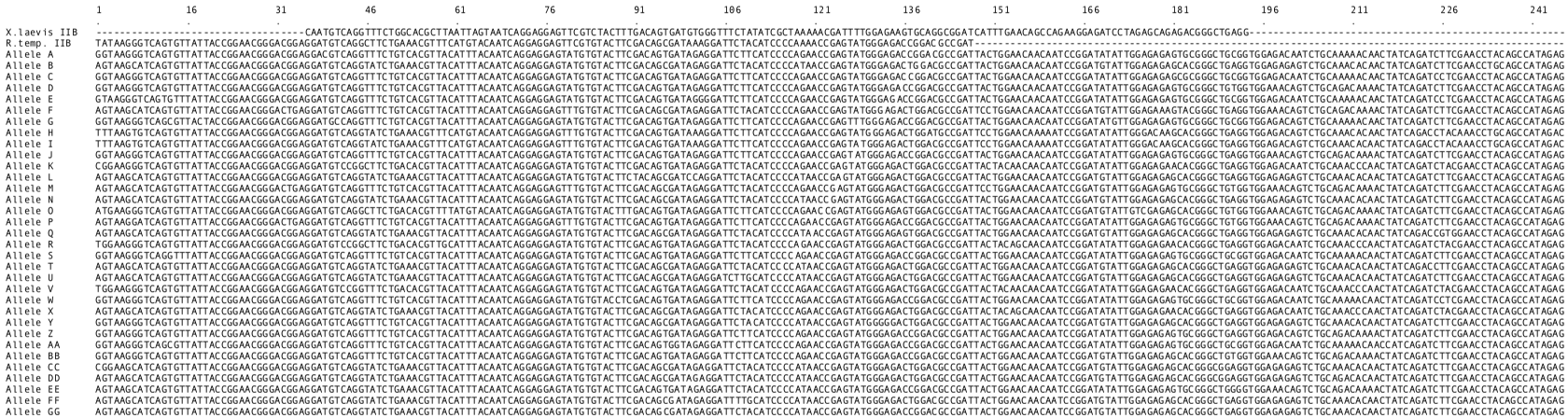
1 16 31 46 61 76 91 106 121 136 151 166 181 196 211 226 241
X.laevis IIB
R.temp. IIB
Allele A
Allele B
Allele C
Allele D
Allele E
Allele F
Allele G
Allele H
Allele I
Allele J
Allele K
Allele L
Allele M
Allele N
Allele O
Allele P
Allele Q
Allele R
Allele S
Allele T
Allele U
Allele V
Allele W
Allele X
Allele Y
Allele Z
Allele AA
Allele BB
Allele CC
Allele DD
Allele EE
Allele FF
Allele GG

Figure S3.3: Alignment of 33 MHC class IIB alleles recovered from 99 *Bd*-infected *Lithobates yavapaiensis* individuals. Alleles were aligned to *Silurana (Xenopus) laevis* and *Lithobates (Rana) temporaria* partial MHC class IIB sequences from GenBank (Accession numbers EF210752.1 and FJ876299.1, respectively).

APPENDIX 2:

SUPPLEMENTAL TABLES

Table S1.1: Summary of *L. yavapaiensis* sampling and die-offs.

Table S1. Summary of *Lithobates yavapaiensis* sampling localities, previously documented die-offs, and for field surveys spanning July 2006 through July 2010, seasonal sample size, seasonal *Bd* infection, and winter chytridiomycosis mortality. The number of recaptures are shown in parentheses.

Locality	Abbr.	UTMs (Zone 12; NAD27)	Elevation (m)	Documented die-offs	N_{SUMMER}	N_{WINTER}	N_{TOTAL}	Summer *Bd* infect. (95%CI)	Winter *Bd* infect. (95%CI)	Winter *Bd* mort. (95%CI)
Willow Creek	WC	276653E, 3897524N	1590		30	12	42 (1)	0 (0-0.22)	0.17 (0.02-0.48)	2/14 (0.02-0.43)
Santa Maria River	SM	299035E, 3805275N	970		107	10	117	0.001 (0.0002-0.05)	0.70 (0.35-0.93)	0/20 (0-0.17)
Hassayampa River	HR	343507E, 3755767N	630	LTM, 1986[a]	54	19	73	0 (0-0.09)	0.37 (0.16-0.62)	0/65 (0-0.06)
Seven Springs	SS	421596E, 3758299N	1270		69	14	83	0.01 (0.0004-0.08)	0.64 (0.35-0.87)	0/24 (0-0.14)
House Spring	HS	519897E, 3756427N	1520		17	7	24	0 (0-0.25)	0 (0-0.41)	0/9 (0-0.34)
Aravaipa Canyon	AC	556918E, 3637764N	990	LTM, 1986[a]	48	34	82	0.03 (0.0008-0.16)	0.68 (0.49-0.83)	8/101 (0.03-0.15)
Muleshoe Ranch Hot Spring	MR_{HS}	571674E, 3578056N	1260	LTM, 1988[a]	64	3	67	0.15 (0.04-0.35)	0.33 (0.01-0.91)	0/68 (0-0.05)
Muleshoe Ranch Secret Spring	MR_{SS}	571192E, 3578378N	1250	*Bd* positive 1985[c]	61	4	65 (1)	0 (0-0.14)	0.25 (0.01-0.81)	0/81 (0-0.03)
Muleshoe Ranch Bass Canyon	MR_{BC}	570330E, 3588337N	1270		23	15	38	0.17 (0.05-0.39)	0.90 (0.56-0.99)	9/21 (0.22-0.66)
Tanque Verde Canyon	TV	532664E, 3568974N	1000	Winter 1998-99[b]	34	5	39	0 (0-0.10)	1 (0.29-1)	3/5 (0.15-0.95)
Cienega Creek	CIC	538111E, 3540254N	1080	Winter 1998-99[b]	26	19	45	0 (0-0.13)	0.90 (0.67-0.99)	11/55 (0.10-0.33)
Aliso Spring	AS	490458E, 3494128N	1210		12	5	17 (2)	0 (0-0.26)	0 (0-0.52)	0/6 (0-0.46)
Total	---	---	---	---	*545*	*147*	*692*	*0.016* *(0.006-0.035)*	*0.73* *(0.62-0.83)*	*0.08* *(0.05-0.10)*

[a] Nature Conservancy property with long-term monitoring (LTM) beginning in the indicated year
[b] Bradley *et al.* 2002
[c] Rosen and Schwalbe 2002

Table S2.2: GENEPOP tests of deviations from Hardy-Weinberg Equilibrium for each locus and population. *P*-values are indicated for all significant deviations (adjusted *P*=0.00022).

Locus	CC	AC	TV	AS	CIC	MR_{SS}	MR_{HS}	MR_{BC}	SM	WC	HR	SS	HS
D120			0.0002		0.0001								
D122			0		0			0					
D124		0							0				
D125			0										
C110								0	0				
C4		0	0										
C9	0												
DI-13												0	
TET-e					0			0	0		0		
D102												0	
Ry2													
DI-2r					0				0			0	
DI-7			0	0								0	
TET-f		0							0				

Table S2.3: (A) Recent migration among populations estimated using BAYESASS, populations 1-7.

Into	*Migration from* CC	AC	TV	AS	CIC	MR_{SS}	MR_{HS}
CC	**0.990 (0.969-1)**	0.001 (0-0.007)	0.001 (0-0.006)	0.001 (0-0.008)	0.001 (0-0.005)	0.001 (0-0.007)	0.001 (0-0.005)
AC	0.001 (0-0.006)	**0.982 (0.962-1)**	0.004 (0-0.015)	0.001 (0-0.006)	0.001 (0-0.004)	0.001 (0-0.006)	0.004 (0-0.014)
TV	0.001 (0-0.004)	0.001 (0-0.004)	**0.992 (0.974-1)**	0.001 (0-0.004)	0.001 (0-0.006)	0.001 (0-0.006)	0.001 (0-0.005)
AS	0.002 (0-0.015)	0.002 (0-0.013)	0.002 (0-0.014)	**0.977 (0.936-1)**	0.002 (0-0.014)	0.002 (0-0.014)	0.002 (0-0.013)
CIC	0.003 (0-0.013)	0.003 (0-0.013)	0.002 (0-0.012)	0.052 (0-0.101)	**0.950 (0.931-1)**	0.003 (0-0.014)	0.003 (0-0.013)
MR_{SS}	0.003 (0-0.017)	0.003 (0-0.017)	0.004 (0-0.017)	0.004 (0-0.017)	0.01 (0-0.038)	**0.679 (0.650-0.710)**	**0.265 (0.200-0.330)**
MR_{HS}	0.001 (0-0.007)	0.001 (0-0.006)	0.001 (0-0.008)	0.001 (0-0.008)	0.001 (0-0.008)	0.001 (0-0.007)	**0.988 (0.964-1)**
MR_{BC}	0.004 (0-0.017)	0.004 (0-0.017)	0.022 (0-0.057)	0.004 (0-0.006)	0.006 (0-0.026)	0.007 (0-0.027)	**0.213 (0.140-0.290)**
SM	0 (0-0.003)	0.001 (0-0.004)	0 (0-0.003)	0.001 (0-0.004)	0 (0-0.002)	0 (0-0.003)	0 (0-0.003)
WC	0.002 (0-0.012)	0.002 (0-0.013)	0.002 (0-0.012)	0.002 (0-0.014)	0.002 (0-0.015)	0.002 (0-0.013)	0.002 (0-0.011)
HR	0.001 (0-0.006)	0.001 (0-0.005)	0.001 (0-0.006)	0.001 (0-0.005)	0.001 (0-0.006)	0.001 (0-0.005)	0.001 (0-0.006)
SS	0.001 (0-0.005)	0.001 (0-0.005)	0.001 (0-0.004)	0.001 (0-0.005)	0.001 (0-0.004)	0.001 (0-0.004)	0.001 (0-0.005)
HS	0.002 (0-0.011)	0.002 (0-0.012)	0.002 (0-0.014)	0.002 (0-0.011)	0.002 (0-0.013)	0.002 (0-0.013)	0.002 (0-0.011)

Table S2.3: (B) Recent migration among populations estimated using BAYESASS, populations 8-13.

	Migration from					
Into	MR_{BC}	SM	WC	HRP	SS	HS
CC	0.001 (0-0.007)	0.001 (0-0.006)	0.001 (0-0.006)	0.001 (0-0.006)	0.001 (0-0.006)	0.001 (0-0.007)
AC	0.001 (0-0.005)	0.001 (0-0.004)	0.001 (0-0.005)	0.001 (0-0.005)	0.001 (0-0.006)	0.001 (0-0.005)
TV	0.001 (0-0.005)	0.001 (0-0.005)	0.001 (0-0.006)	0.001 (0-0.004)	0.001 (0-0.006)	0.001 (0-0.005)
AS	0.002 (0-0.015)	0.002 (0-0.014)	0.001 (0-0.012)	0.002 (0-0.012)	0.002 (0-0.011)	0.002 (0-0.013)
CIC	0.003 (0-0.017)	0.003 (0-0.015)	0.002 (0-0.013)	0.003 (0-0.015)	0.003 (0-0.013)	0.004 (0-0.020)
MR_{SS}	0.004 (0-0.019)	0.004 (0-0.017)	0.004 (0-0.019)	0.004 (0-0.018)	0.004 (0-0.017)	0.007 (0-0.030)
MR_{HS}	0.001 (0-0.006)	0.001 (0-0.009)	0.001 (0-0.007)	0.001 (0-0.008)	0.001 (0-0.007)	0.001 (0-0.008)
MR_{BC}	**0.681 (0.650-0.710)**	0.004 (0-0.019)	0.035 (0-0.077)	0.004 (0-0.016)	0.003 (0-0.017)	0.008 (0-0.032)
SM	0.001 (0-0.004)	**0.994 (0.982-1)**	0 (0-0.003)	0 (0-0.003)	0.001 (0-0.005)	0 (0-0.003)
WC	0.002 (0-0.012)	0.001 (0-0.011)	**0.980 (0.943-1)**	0.001 (0-0.01)	0.001 (0-0.01)	0.002 (0-0.011)
HR	0.001 (0-0.006)	0.002 (0-0.009)	0.001 (0-0.007)	**0.987 (0.967-1)**	0.001 (0-0.006)	0.001 (0-0.006)
SS	0.001 (0-0.005)	0.001 (0-0.006)	0.001 (0-0.005)	0.001 (0-0.005)	**0.991 (0.974-1)**	0.001 (0-0.006)
HS	0.002 (0-0.013)	0.002 (0-0.013)	0.001 (0-0.011)	0.001 (0-0.012)	0.002 (0-0.014)	**0.979 (0.940-1)**

Table S2.4: Model selection for genetic and environmental factors influencing mortality prevalence, *Bd* infection prevalence, and *Bd* infection intensity of *L. yavapaiensis* in Arizona. We compared all possible models using Akaike Information Criterion (AICc); Interactions among variables were disregarded; five best models are reported for each dataset.

Bd Mortality Prevalence

Model	AICc	nVars
H_O, BIO2, BIO12	41.89	3
Nº PRIVATE ALLELES, BIO2, BIO12	43.31	3
H_O, BIO2	47.58	2
Nº PRIVATE ALLELES, C110- H_O, BIO2	47.89	3
H_O, BIO2, BIO8	48.04	3

Bd Infection Prevalence

Model	AICc	nVars
H_O, Nº PRIVATE ALLELES, BIO6	62.50	3
H_O, Nº PRIVATE ALLELES, BIO6, BIO11	67.13	4
Nº PRIVATE ALLELES, BIO6	68.34	2
H_O, Nº PRIVATE ALLELES, BIO11	68.35	3
BIO6, BIO11	69.21	2

Bd Infection Intensity

Model	AICc	nVars
BIO7, BIO13, BIO17	25.16	3
BIO7, BIO13	25.56	2
BIO7, BIO16	28.61	2
BIO7, BIO13, BIO15	28.85	3
BIO7, BIO15, BIO17	29.93	3

Best predictors are as follows: for neutral loci, observed heterozygosity (H_O) and number of private alleles (Nº PRIVATE ALLELES); for the outlier locus, observed heterozygosity (C110-H_O); for environmental variables: mean diurnal temperature range [mean of monthly max temp - min temp] (BIO2), minimum temperature of coldest month (BIO6), temperature annual range [max temperature of warmest month – min temperature of coldest month] (BIO7), mean temperature of wettest quarter (BIO8), mean temperature of coldest quarter (BIO11), annual precipitation (BIO12), precipitation of wettest month (BIO13), precipitation seasonality [coefficient of variation] (BIO15), precipitation of wettest quarter (BIO16), and precipitation of driest quarter (BIO17).

Table S3.1: List of lab-reared, *Bd*-infected *Lithbates yavapaiensis* individuals, housing conditions, change in mass over the course of the experiment, maximum *Bd* infection intensity (in genome equivalents), and MHC class IIB peptide-binding region genotypes. Surviving individuals are shown in bold.

Frog	Pop	Rep-licate	Clutch	Days sur-vived	Sur-vived ?	No./ cage	0 DPI mass (g)	death /35 DPI mass (g)	change in mass	PBR het or hom	Max. inf. Inten -sity	PBR Allele 1	PBR Allele 2
CIC 1.1.A1 TUFI-1	CIC	1	1	7	no	2	1.36	0.91	-0.45	hom	515.7	A	A
CIC 1.1.A1 TUFI-2	CIC	1	1	3	no	2	1.21	1.41	0.2	hom	515.7	A	A
CIC 1.1.A2 TUFI-1	CIC	2	1	16	no	3	1.12	0.97	-0.15	hom	625.6	A	A
CIC 1.1.A2 TUFI-2	CIC	2	1	20	no	3	0.81	1.2	0.39	het	625.6	A	D
CIC 1.1.A2 TUFI-3	CIC	2	1	14	no	3	1.38	1.15	-0.23	hom	625.6	A	A
CIC 1.1.B1 TUFI-1	CIC	3	1	2	no	3	1.96	1.9	-0.06	hom	NA	A	A
CIC 1.1.B1 TUFI-3	CIC	3	1	4	no	3	1.88	1.98	0.1	hom	NA	A	A
CIC 1.1.B2 TUFI-1	CIC	4	1	5	no	1	1.45	1.65	0.2	het	NA	A	D
CIC 1.2.A1 TUFI-1	CIC	5	1	6	no	1	1.11	1.14	0.03	hom	1777.7	A	A
CIC 1.2.A2 TUFI-1	CIC	6	1	15	no	4	1.07	1.28	0.21	hom	948.9	A	A
CIC 1.2.A2 TUFI-2	CIC	6	1	21	no	4	1.38	1.6	0.22	hom	784.89	A	A
CIC 1.2.A2 TUFI-3	CIC	6	1	21	no	4	1.51	1.52	0.01	hom	784.89	A	A
CIC 1.2.A2 TUFI-4	CIC	6	1	29	no	4	1.8	1.62	-0.18	hom	784.89	A	A
CIC 1.2.B1 TUFI-1	CIC	7	1	13	no	5	2.82	3.2	0.38	hom	144.58	A	A
CIC 1.2.B1 TUFI-2	CIC	7	1	13	no	5	1.51	1.32	-0.19	hom	144.58	A	A
CIC 1.2.B1 TUFI-3	CIC	7	1	7	no	5	0.98	0.97	-0.01	hom	144.58	A	A
CIC 1.2.B1 TUFI-4	CIC	7	1	7	no	5	1.1	1.29	0.19	hom	144.58	A	A
CIC 1.2.B1 TUFI-5	CIC	7	1	14	no	5	1.24	1.43	0.19	hom	144.58	A	A
CIC 1.2.B2 TUFI-1	CIC	8	1	8	no	2	2.37	2.15	-0.22	hom	227.4	A	A
CIC 1.2.B2 TUFI-2	CIC	8	1	6	no	2	1.85	1.5	-0.35	hom	227.4	A	A
CIC 2.1.A1 TUFI-1	CIC	9	1	20	no	1	1.17	1.22	0.05	hom	1669.24	A	A
CIC 2.1.A2 TUFI-1	CIC	10	2	16	no	4	1.16	1.04	-0.12	het	981.96	A	C
CIC 2.1.A2 TUFI-2	CIC	10	2	23	no	4	1.46	1.32	-0.14	hom	981.96	A	A

Frog	Pop	Rep-licate	Clutch	Days sur-vived	Sur-vived ?	No./ cage	0 DPI mass (g)	death /35 DPI mass (g)	change in mass	PBR het or hom	Max. inf. Inten-sity	PBR Allele 1	PBR Allele 2
CIC 2.1.A2 TUFI-3	CIC	10	2	3	no	4	1.27	1.49	0.22	het	981.96	A	G
CIC 2.1.A2 TUFI-4	CIC	10	2	3	no	4	1.14	1.22	0.08	hom	981.96	A	A
CIC 2.1.B1 TUFI-1	CIC	11	2	29	no	1	1.48	1.11	-0.37	hom	441.87	A	A
CIC 2.1.B2 TUFI-1	CIC	12	2	22	no	1	0.97	1.13	0.16	hom	860.38	A	A
CIC 2.2.A1 TUFI-1	CIC	13	2	12	no	3	1.1	1.4	0.3	het	301.87	A	I
CIC 2.2.A1 TUFI-2	CIC	13	2	13	no	3	1.01	1.05	0.04	hom	301.87	A	A
CIC 2.2.A1 TUFI-3	CIC	13	2	13	no	3	1.33	0.95	-0.38	hom	301.87	A	A
CIC 2.2.A2 TUFI-1	CIC	14	2	5	no	2	1.46	1.99	0.53	hom	358.9	A	A
CIC 2.2.A2 TUFI-2	CIC	14	2	6	no	2	1.6	2.01	0.41	hom	358.9	A	A
CIC 2.2.B1 TUFI-1	CIC	15	2	20	no	1	1.67	1.51	-0.16	hom	948.9	A	A
CIC 2.2.B2 TUFI-1	CIC	16	2	11	no	1	1.95	2.1	0.15	hom	146.9	A	A
AC 1.1.A1 TUFI-1	AC	17	3	8	no	3	1.04	0.7	-0.34	hom	766.3	E	E
AC 1.1.A1 TUFI-2	AC	17	3	15	no	3	1.08	1.04	-0.04	hom	766.3	A	A
AC 1.1.A1 TUFI-3	AC	17	3	16	no	3	1.16	1.47	0.31	hom	766.3	A	A
AC 1.1.A2 TUFI-1	**AC**	**18**	**3**	**35**	**yes**	**3**	**3.08**	**2.34**	**-0.74**	**het**	**625.5**	**A**	**Q**
AC 1.1.A2 TUFI-2	**AC**	**18**	**3**	**35**	**yes**	**3**	**2.88**	**2.47**	**-0.41**	**het**	**625.5**	**A**	**B**
AC 1.1.A2 TUFI-3	**AC**	**18**	**3**	**35**	**yes**	**3**	**2.4**	**2.09**	**-0.31**	**het**	**625.5**	**A**	**B**
AC 1.1.B1 TUFI-1	**AC**	**19**	**3**	**35**	**yes**	**1**	**1.96**	**1.59**	**-0.37**	**het**	**214.3**	**A**	**B**
AC 1.2.A2 TUFI-1	**AC**	**20**	**3**	**35**	**yes**	**1**	**1.43**	**0.89**	**-0.54**	**het**	**114.1**	**A**	**X**
AC 1.2.B2 TUFI-1	AC	21	3	23	no	1	1.41	1.2	-0.21	hom	931.4	E	E
AC 2.1.A1 TUFI-1	AC	22	4	1	no	1	0.98	0.8	-0.18	hom	NA	A	A
AC 2.2.A1 TUFI-1	AC	23	4	3	no	1	1.31	1.04	-0.27	hom	NA	C	C
AC 2.2.A2 TUFI-1	**AC**	**24**	**4**	**35**	**yes**	**2**	**0.97**	**0.93**	**-0.04**	**het**	**328.8**	**A**	**Y**
AC 2.2.A2 TUFI-2	**AC**	**24**	**4**	**35**	**yes**	**2**	**1.26**	**1.27**	**0.01**	**het**	**328.8**	**A**	**W**
AC 2.2.B1 TUFI-1	AC	25	4	26	no	4	1.49	1.41	-0.08	het	1161.3	C	B
AC 2.2.B1 TUFI-2	AC	25	4	**26**	no	**4**	**1.05**	1.3	0.25	hom	1161.3	A	A
AC 2.2.B1 TUFI-3	AC	25	4	24	no	4	1.19	1.33	0.14	hom	1161.3	A	A
AC 2.2.B1 TUFI-4	AC	25	4	24	no	4	1.2	1.31	0.11	hom	1161.3	C	C

Frog	Pop	Rep-licate	Clutch	Days sur-vived	Sur-vived ?	No./ cage	0 DPI mass (g)	death /35 DPI mass (g)	change in mass	PBR het or hom	Max. inf. Inten-sity	PBR Allele 1	PBR Allele 2
MR 1.2.A2 TUFI-1	MR	26	5	22	no	1	0.77	1.07	0.3	het	1291.91	AA	A
MR 1.2.B2 TUFI-1	MR	27	5	28	no	3	1.3	1.51	0.21	het	811.98	H	A
MR 1.2.B2 TUFI-2	MR	27	5	28	no	3	1.54	1.63	0.09	het	811.98	C	S
MR 2.2.B2 TUFI-3	MR	27	5	29	no	3	0.94	1.28	0.34	hom	811.98	C	GG
MR 2.1.B2 TUFI-1	MR	28	5	9	no	1	1.24	1.32	0.08	het	137.6	A	FF
WC 2.2.A1 TUFI-1	WC	29	6	16	no	3	1.67	2.89	1.22	het	1338.32	D	O
WC 2.2.A1 TUFI-2	WC	29	6	15	no	3	1.14	1.31	0.17	het	1338.32	O	BB
WC 2.2.A1 TUFI-3	WC	29	6	11	no	3	0.87	0.6	-0.27	het	1338.32	A	K
WC 2.2.A2 TUFI-1	WC	30	6	3	no	3	0.99	1.43	0.44	het	855.11	O	EE
WC 2.2.A2 TUFI-2	WC	30	6	2	no	3	1.17	1.08	-0.09	het	855.11	A	J
WC 2.2.A2 TUFI-3	WC	30	6	14	no	3	0.99	1.17	0.18	het	855.11	P	A
WC 2.2.B1 TUFI-1	WC	31	6	11	no	2	0.95	1.01	0.06	het	266.1	O	F
WC 2.2.B1 TUFI-2	WC	31	6	6	no	2	1.2	1.02	-0.18	het	266.1	O	N
WC 1.1.A1 TUFI-1	WC	32	6	2	no	1	1.28	1.01	-0.27	hom	NA	F	F
WC 1.1.A2 TUFI-1	WC	33	6	7	no	4	1.5	1.03	-0.47	hom	526.4	F	F
WC 1.1.A2 TUFI-2	WC	33	6	6	no	4	1.08	1.05	-0.03	het	526.4	Z	F
WC 1.1.A2 TUFI-3	WC	33	6	7	no	4	1.04	1.12	0.08	hom	526.4	A	A
WC 1.1.A2 TUFI-4	WC	33	6	5	no	4	0.82	0.73	-0.09	hom	526.4	A	A
WC 1.1.B2 TUFI-1	WC	34	6	9	no	1	1.07	0.51	-0.56	het	400.1	A	CC
WC 3.1.B TUFI-1	WC	35	7	5	no	1	1.51	1.13	-0.38	hom	NA	A	A
WC 3.1.C TUFI-1	WC	36	7	4	no	2	2.21	1.91	-0.3	hom	NA	A	A
WC 3.1.C TUFI-2	WC	36	7	5	no	2	1.63	1.94	0.31	hom	NA	A	A
SM 1.1.A1 TUFI-1	SM	37	8	7	no	3	0.8	0.9	0.1	hom	133.6	A	A
SM 1.1.A1 TUFI-2	SM	37	8	7	no	3	0.81	0.96	0.15	hom	133.6	A	A
SM 1.1.A1 TUFI-3	SM	37	8	2	no	3	0.82	0.72	-0.1	hom	133.6	L	L
SM 1.1.A2 TUFI-1	**SM**	**38**	**8**	**35**	**yes**	**1**	**1.18**	**1.49**	**0.31**	**het**	**264.7**	**A**	**Q**
SM 1.1.B1 TUFI-1	SM	39	8	7	no	1	0.94	0.97	0.03	hom	611.7	A	A
SM 1.2.B1 TUFI-1	SM	40	8	1	no	2	1.2	1.2	0	hom	332.30	A	A

Frog	Pop	Rep-licate	Clutch	Days sur-vived	Sur-vived ?	No./ cage	0 DPI mass (g)	death /35 DPI mass (g)	change in mass	PBR het or hom	Max. inf. Inten-sity	PBR Allele 1	PBR Allele 2
SM 1.2.B1 TUFI-2	SM	41	8	17	no	2	1.24	1.04	-0.2	hom	332.30	A	A
SM 1.2.B2 TUFI-1	SM	42	8	1	no	2	1.27	1.33	0.06	het	1302.09	A	U
SM 1.2.B2 TUFI-2	SM	42	8	22	no	2	1.36	1.17	-0.19	hom	1302.09	A	A
SM 1.2.A1 TUFI-1	SM	43	9	10	no	1	2	2.1	0.1	hom	549.22	A	A
SM 2.1.A1 TUFI-1	**SM**	**44**	**9**	**35**	**yes**	**1**	**1.41**	**0.93**	**-0.48**	**hom**	**125.6**	**B**	**B**
SM 2.1.A2 TUFI-1	SM	45	9	10	no	2	1.22	1.01	-0.21	hom	886.98	B	B
SM 2.1.A2 TUFI-2	SM	45	9	19	no	2	1.28	1.31	0.03	hom	886.98	B	B
SM 2.1.B1 TUFI-1	SM	46	9	6	no	2	0.96	1.02	0.06	hom	359.6	B	B
SM 2.1.B1 TUFI-2	SM	46	9	7	no	2	1.42	0.58	-0.84	hom	359.6	R	R
SM 2.1.B2 TUFI-1	SM	47	9	7	no	1	0.95	0.65	-0.3	het	371.3	B	E
SM 2.2.B2 TUFI-1	SM	48	9	9	no	1	2.65	1.45	-1.2	het	422.3	B	DD
SM 2.2.B1 TUFI-1	**SM**	**49**	**9**	**35**	**yes**	**1**	**0.96**	**0.92**	**-0.04**	**het**	**321.8**	**B**	**Q**
SM 2.3.B2 TUFI-1	**SM**	**50**	**8**	**35**	**yes**	**1**	**1.09**	**0.9**	**-0.19**	**het**	**593.6**	**B**	**N**
SM 3.1.A1 TUFI-1	**SM**	**51**	**10**	**35**	**yes**	**1**	**1.15**	**0.82**	**-0.33**	**het**	**408.4**	**A**	**B**
SM 3.1.A2 TUFI-1	**SM**	**52**	**10**	**35**	**yes**	**1**	**1.56**	**1.57**	**0.01**	**het**	**523.2**	**A**	**T**
SM 3.1.B1 TUFI-1	**SM**	**53**	**10**	**35**	**yes**	**1**	**1.26**	**1.36**	**0.1**	**het**	**149.4**	**A**	**Q**
SM 3.2.A1 TUFI-1	SM	54	10	7	no	1	0.74	0.82	0.08	hom	288.4	A	A
SM 3.2.B2 TUFI-1	SM	55	10	5	no	1	0.8	0.8	0	hom	NA	C	C
SM 3.2.B1 TUFI-1	SM	56	10	17	no	1	1.29	1.1	-0.19	hom	1411.24	C	C
SM 3.2.A2 TUFI-1	SM	57	10	14	no	1	0.99	1.01	0.02	het	754.32	A	V

Table S3.2: Terminal branch evolution among 33 PBR sequences from five *L. yavapaiensis* populations experimentally infected with *Bd*. A Maximum Likelihood (ML) phylogeny of the 82-codon PBR alignment was used as the input tree for selection on particular codons within branches of the phylogeny. We used three different codon-based methods, all implemented with the HyPhy statistical software (66). The most conservative method, SLAC, infers ancestral codon state and then calculates normalized expected and observed nonsynonymous (dN) and synonymous (dS) substitutions at each site (66). The FEL method directly estimates rates of nonsynonymous and synonymous substitutions at each site, while the REL approach fits a distribution of substitution rates across sites and then infers the rate at which each site evolves (66). When the normalized difference between non-synonymous and synonymous substitutions (dN-dS) equals 0, the site is neutrally evolving; when dN-dS >0, the site is positively selected; and when dN-dS <0, the site is negatively selected.

Single Likelihood Ancestral Counting (SLAC)				
Terminal branch of ML phylogeny	*Codon site under positive selection*	*Normalized dN-dS*	*P-value*	*Amino acid change*
Leading to allele EE	3	18.1	0.09	Gly → His
Fixed-Effects Likelihood (FEL)				
Terminal branch of ML phylogeny	*Codon site under positive selection*	*Normalized dN-dS*	*P-value*	*Amino acid change*
Leading to allele EE	3	1.1	0.03	Gly → His
Leading to allele EE	18	0.93	0.03	Ser → Lys
Leading to allele Q	46	0.74	0.04	Leu → Val
Random-Effects Likelihood (REL)				
Terminal branch of ML phylogeny	*Codon site under positive selection*	*Normalized dN-dS*	*Posterior Probability*	*Amino acid change*
Leading to allele EE	3	3.21	0.99	Gly → His
Leading to allele EE	18	3.32	0.99	Ser → Lys

Table S3.3: Survival from hatching through start of experimental infections for lab-reared *Lithobates yavapaiensis*.

Clutch	Number of larvae hatched (%)	Number of larvae reaching Gosner stage 45 (%)	Number of frogs surviving metamorphosis (post-Gosner stage 46; %)
CIC1	143 (100%)	118 (83%)	32 (27%)
CIC2	134 (100%)	123 (92%)	24 (20%)
AC1	188 (100%)	91 (48%)	14 (15%)
AC2	205 (100%)	119 (58%)	14 (12%)
MR1	195 (100%)	11 (6%)	5 (57%)
MR2	118 (100%)	2 (2%)	2 (2%)
MR3	0 (0%)	–	–
WC1	96 (20%)	54 (56%)	7 (13%)
WC2	255 (100%)	167 (65%)	16 (10%)
WC3	117 (100%)	41 (35%)	5 (12%)
SM1	247 (100%)	159 (64%)	17 (11%)
SM2	155 (100%)	98 (63%)	13 (13%)
SM3	253 (100%)	115 (45%)	9 (8%)
Total	*1810*	*1044 (58%)*	*158 (15%)*
Mean	*144 (85%)*	*87.5 (55%)*	*13.2 (16%)*

Table S4.1: Pairwise estimates of F_{ST} (upper) and D (lower) among *Lithobates yavapaiensis* study populations. All values are significant: for pairwise F_{ST} values, adjusted P = 0.000549; for pairwise D, 95% confidence intervals did not include zero.

	AC	TV	CIC	MR	SM	WC	HR	SS
AC	–	0.3154	0.2365	0.1611	0.2694	0.3005	0.2439	0.275
TV	0.5412	–	0.2483	0.2745	0.4054	0.4793	0.4059	0.3857
CIC	0.5257	0.3373	–	0.1781	0.3116	0.3559	0.3352	0.2986
MR	0.4265	0.5021	0.4465	–	0.2531	0.2561	0.2572	0.2468
SM	0.7345	0.8592	0.7644	0.6991	–	0.2039	0.1585	0.2209
WC	0.7723	0.8849	0.8158	0.6336	0.4916	–	0.18	0.227
HR	0.5974	0.8044	0.8248	0.6836	0.4144	0.5005	–	0.1935
SS	0.7191	0.7840	0.7289	0.6738	0.6099	0.6239	0.5706	–

Table S4.2: Pairwise Euclidean distances among *Lithobates yavapaiensis* study populations in kilometers.

	AC	TV	CIC	MR	SS	SM	WC	HR
AC	–							
TV	73	–						
CIC	100	29	–					
MR	65	40.5	51.5	–				
SM	298	334	358	356	–			
WC	374	416	443	433	95	–		
HR	235	264	289	290	66	157	–	
SS	172	220	245	233	132	202	79	–

APPENDIX 3:

Isolation and characterization of microsatellite markers in the lowland leopard frog (*Rana yavapaiensis*) and the relict leopard frog (*R. onca*), two declining frogs of the North American desert southwest

Anna E. Savage* and Jef R. Jaeger†

**Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY 14853, USA*

† *School of Life Sciences and Public Lands Institute, University of Nevada, 4505 Maryland Parkway, Las Vegas, NV 89154-4004*

Abstract

We characterized 15 microsatellite loci for the lowland leopard frog (*Rana yavapaiensis*) and the relict leopard frog (*R. onca*) for future studies of population genetic structure and relatedness. Analysis of 20 individuals from single populations of each species showed that all markers were polymorphic in at least one species. Observed and expected heterozygosities ranged from 0 to 0.94 and 0.11 to 0.85, respectively, and there were 3 to 11 alleles per locus. No loci were in linkage disequilibrium, but six loci deviated significantly from Hardy-Weinberg equilibrium, and the presence of a null allele was detected in two of these loci.

Manuscript

The lowland leopard frog (*Rana yavapaiensis*) and the relict leopard frog (*R. onca*) are sister species within the *R. pipiens* complex (*Pantherana*) (Hillis and Wilcox

2005). *Rana onca* was once considered extinct (Jennings 1988), but recently, several extant populations were confirmed as *R. onca* based on mitochondrial haplotypes, assessment of randomly amplified polymorphic DNA (RAPD markers), and morphology (Jaeger *et al.* 2001). Taxonomic designations may be uncertain, however, because these two species are almost identical at mitochondrial DNA markers (approximately 98.8% similarity at the 12 S and 16 S genes), a level of divergence comparable to that among other ranid subspecies (Hillis and Wilcox 2005).

Both *R. yavapaiensis* and *R. onca* are species of conservation concern. *Rana onca* was known from only 24 locations in Utah, Nevada and Arizona prior to the 1970s, but by 2001 its range dwindled to 5 localities in Nevada (Bradford *et al.* 2004). In contrast, *R. yavapaiensis* is still patchily distributed throughout much of Arizona and Sonora (Hillis 1988), although the species has also experienced considerable declines and extirpations since the 1970s (Sredl *et al.* 1997), at least some of which were caused by the fungal disease chytridiomycosis (Bradley *et al.* 2002). Characterization of fine-scale levels of genetic variability within and among these species is therefore critical to establish conservation priorities and to inform questions of species designation.

To accomplish this objective, 15 microsatellite loci were isolated and characterized from two enriched partial genomic libraries (Hamilton *et al.* 1999), one prepared with tissue from an adult *R. yavapaiensis* individual from Maricopa County, Arizona, and one prepared with tissue from an adult *R. onca* individual from Clark County, Nevada. For the *R. yavapaiensis* library, genomic DNA was extracted using a Qiagen DNeasy tissue kit, followed by digestion with *AluI* and *HaeIII* (New England Biolabs). The linked genomic fragments were enriched for microsatellites with

biotinylated dimer, trimer and tetramer probes bound to streptavidin-coated magnetic beads (Dynabeads, Dynal Biotech). Subsequently, the microsatellite-containing DNA fragments were magnetically captured. These fragments were amplified by polymerase chain reaction (PCR) using linker-specific primers, followed by digested with *NheI* (New England Biolabs). Resulting fragments were cloned into pUC19 vector and transformed with DH5α competent cells (Invitrogen). Colonies were grown on X-Gal/IPTG-coated agar and transferred to Magna Lift nylon membranes (Osmonics Inc.) that were probed with the same di-, tri- and tetramer radiolabeled repeats. Positive clones were transferred to 30μl of de-ionized water, boiled at 99°C for 5 min, and 1μl of the resulting solution was used as template for PCR amplification with vector-specific primers (M13 F and R). Amplification products >300 base pairs were sequenced in one direction with the M13 F primer using dGTP BigDye terminator cycle sequencing components on a 3100 Genetic Analyzer (Applied Biosystems). For the *R. onca* library, a private company (Genetic Identification Services) was employed to isolate, clone and sequence microsatellite-containing DNA fragments.

PCR primers were designed in the flanking regions of 22 microsatellites from the *R. yavapaiensis* library using SEQMAN version 5.05 (DNAStar Inc.) and 46 microsatellites from the *R. onca* library using the program DESIGNERPCR version 1.03 (Research Genetics, Inc.). Eight *R. yavapaiensis* from 4 sites and 8 *R. onca* from 4 sites, representing a broad area of the current range for each species, were screened for amplification reliability and microsatellite polymorphism. Of the evaluated primers, 15 polymorphic loci were identified. These loci were used for a broader assessment of population and species differentiation.

Genotyping was conducted for 20 *R. yavapaiensis* individuals from a single

location on the Santa Maria River (Yavapai County, Arizona) and 20 *R. onca* individuals from Bighorn Sheep Spring along the Colorado River (Clark County, Nevada). DNA was extracted from toe clips in 150µl of a 5% Chelex-100 solution (Bio-Rad) with 1µl Proteinase K by incubation at 55 ºC for 180 min and 99 ºC for 10 min; supernatants were used directly as PCR templates. Each PCR had a total volume of 10µl, consisting of 1µl of each template, 0.1µl *Taq* DNA polymerase (5U/µl) (Roche), 1.5µl 10X PCR buffer (100mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl), 0.25µl dNTPs (40mM), and 0.5µl of each primer (10µM). Forward primers were 5'-labeled with a fluorescent dye (Applied Biosystems). Amplification was performed in a Hybaid PCR thermal cycler under the following conditions: 5 min initial denaturation at 94 ºC; 35 cycles of 1 min denaturing at 94 ºC, 1 min annealing at primer specific annealing temperatures (Table 1), 1 min extension at 72 ºC; and a final extension of 75 ºC for 5 min. Amplified products with different labels or nonoverlapping size ranges were multiplexed and electrophoresed on a 5% polyacrylamide gel on a 3100 Genetic Analyzer (Applied Biosystems). Fragment sizes were determined with the LIZ-500 standard using GENEMAPPER version 3.5 (Applied Biosystems). Tests of significant deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD), and calculations of the number of alleles (*A*), observed (H_O) and expected (H_E) heterozygosities were performed using GENEPOP version 3.4 (Raymond and Rousset 1995).

The majority of the microsatellite loci identified were tetramers or complex repeats mostly containing tetramers, while only two loci were pure dimers (Table 1). All 15 loci were polymorphic among *R. yavapaiensis* individuals, while only 10 loci were polymorphic among the *R. onca* sampled. Lower genetic diversity was expected in *R. onca* because the current remnant populations comprise a very small

geographic range (Bradford et al. 2004). The total number of alleles per locus across both species ranged from 3 to 11 (mean 6.7), with 2 to 9 alleles per locus in *R. yavapaiensis* (mean 5.1) and 1 to 5 alleles per locus in *R. onca* (mean 2.6). For 9 of the loci, 1 to 3 alleles were shared among *R. yavapaiensis* and *R. onca* individuals. Across all samples, observed and expected heterozygosities ranged from 0 to 0.94 and 0.11 to 0.85, respectively.

No significant LD was detected among any pair of loci; however, six loci showed significant deviations from HWE. Loci *Ro*C4, *Ro*D125, and *Ro*D122 showed significant heterozygote deficiency for *R. yavapaiensis* only, loci *Ro*D120 and *Ro*D124 showed significant heterozygote deficiency in population samples from both species, and locus *Ry*TET-e showed significant heterozygote excess for *R. onca* only. The program MICRO-CHECKER (van Oosterhout *et al.* 2004) was used to further evaluate these loci, and the presence of a null allele was detected at loci D120 and D122 for the *R. yavapaiensis* population sample. The 15 loci characterized here will be used in range-wide studies of population structure within and among *R. yavapaiensis* and *R. onca* populations, providing a comprehensive understanding of relatedness and conservation priorities for these threatened species.

Acknowledgements: This project was funded by a NSF Graduate Research Fellowship and a National Geographic Society Young Explorers Grant to A.E.S. and the Clark County Desert Conservation Program funded by Southern Nevada Public Lands Management Act (project # 2005-UNLV-575-P) to J.R.J. We thank S. Bogdanowicz and the Evolutionary Genetics Core Facility for help with microsatellite development, M.C. Duryea for help with data analysis, and David Bradford and Brett Riddle for their efforts in support of this project.

REFERENCES

Biek, R, Drummond AJ, Poss M (2006) A virus reveals population structure and recent demographic history of its carnivore host. *Science*, **311**, 538–540.

Bradford DF, Jaeger JR, Jennings RD (2004) Population status and distribution of a decimated amphibian, the relict leopard frog (*Rana onca*). *Southwestern Naturalist,* **49**, 218-228.

Bradley GA, Rosen PC, Sredl MJ, Jones TR, Longcore JE (2002) Chytridiomycosis in native Arizona frogs. *Journal of Wildlife Diseases,* **38**, 206-212.

Hamilton MB, PIncus EL, Di Fiore A, Fleischer RC (1999) Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. *Biotechniques,* **27**, 500-507.

Hillis DM, Wilcox TP (2005) Phylogeny of the New World true frogs (*Rana*). *Molecular Phylogenetics and Evolution,* **34**, 299-314.

Hillis DM (1988) Systematics of the *Rana pipiens* complex: puzzle and paradigm. *Annual Review of Ecology and Systematics,* **19**, 39-63.

Jaeger JR, Riddle BR, Jennings RD, Bradford DF (2001) Rediscovering *Rana onca*: Evidence for phylogenetically distinct leopard frogs from the border region of Nevada, Utah, and Arizona. *Copeia,* **2001**, 339-354.

Jennings MR (1988) *Rana onca* Cope, relict leopard frog. *Catalogue of American Amphibians and Reptiles,* **417**, 1-2.

Raymond M, Rousset F (1995) GENEPOP: population genetics software for exact tests and ecumenicism. *Journal of Heredity,* **86**, 248-249.

Sredl MJ, Howland JM, Wallace JE, Saylor LS (1997) Status and distribution of Arizona's native ranid frogs. In *Ranid Frog Conservation and Management. Non-game and Endangered Wildlife Program, Technical Report 121*. M. J. Sredl, ed.

Arizona Game and Fish Department, Phoenix, AZ.

Van Oosterhaut C, Hutchinson WF, Wills DPM, Shipley P (2004). MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes,* **4**, 535-538.

Table 1 Primer sequences, repeat motif, annealing temperatures (T_a), size range, and variability measures for 15 microsatellite loci from 20 individuals each of *Rana yavapaiensis* (*Ry*) and *R. onca* (*Ro*). Sequences of original clones have been accessioned in GenBank (Accession nos EU853423–EU853437). *A*, no. of alleles; *N*, no. of successfully genotyped individuals; H_E, expected heterozygosity; H_O, observed heterozygosity. *P* values represent significance values for exact tests of deviation from Hardy–Weinberg equilibrium (significant deviations are listed in bold); NA, not applicable

Locus	Repeat motif	Primer sequence (5′–3′)	T_a (°C)	Size range	*N*	A_{Ry}	A_{Ro}	A_{tot}	A_{shared}	*Ry* H_E/H_O	*Ry P* value	*Ro* H_E/H_O	*Ro P* value
RoC4	$(ATAG)_{19}(ATAC)_{12}$	F: (6-FAM)TTACCAAGGTAGCACTCTTTG R: ACTGCAAACCGGAAATGT	55	195–243	37	5	3	7	1	0.84/0.65	**0.02**	0.55/0.60	1.00
RoC123	$(TGTA)_{10}$	F: (PET)GGCTTACTTCTTGCCTTTAGC R: CATCCATTTTTCCCTTGTTC	51	124–156	37	3	3	5	1	0.57/0.78	0.08	0.34/0.46	1.00
RoD102	$(TATC)_{19}$	F: (NED)ATGGAGATTTAAGTGCAAGAGT R: CTTCCCAATATGGCATAGATAT	60	195–251	38	9	4	11	2	0.85/0.82	0.12	0.70/0.50	0.07
RoD125	$(AGAT)_{15}$	F: (6-FAM)TCAATGGTGGTGTGTCAC R: GCTCTGAAGTCAACTGGTC	60	282–298	37	5	3	5	3	0.79/0.71	**0.01**	0.44/0.58	0.46
RoC9	$(AT)_5(AGAT)_{10}(ACAT)_8$	F: (NED)CCAGCTCTAAACACATTAGCTC R: CAGCATAGGTTGGAATACAAAT	59	184–239	37	8	3	9	2	0.80/0.82	0.58	0.23/0.20	0.24
RoD124	$(TCTA)_9(TCTCTA)_6(TC)_3$	F: (VIC)ACCCCTCCAAGTCAAAATC R: AAAGGTGGGCAAACTCAA	55	232–292	37	7	4	11	0	0.83/0.63	**0.03**	0.71/0.53	**0.00**
RoD120	$(ATCT)_{11}$	F: (NED)TATCCGAGGCTTAAAATCCTTC R: ATATCGGTGCAACCCTAATACA	60	100–124	38	6	2	6	2	0.75/0.47	**0.00**	0.11/0	**0.03**
RoD122	$(TATC)_{19}(TA)_1(TATC)_2$	F: (NED)CTCTGAGTCTGTCTGTCTGTCT R: TAGTGGCTTAGTCCATTCTATG	51	190–260	39	5	1	6	0	0.75/0.33	**0.00**	NA	NA
RoC110	$(ATAC)_7(AC)_6$	F: (VIC)GGACCTGTCATACACAATGTC R: AAATGCTCTCAAACCAAGTAAC	60	124–148	37	4	1	5	0	0.53/0.47	0.26	NA	NA
RyDI-7	$(GT)_{14}$	F: (VIC)AATACTCTGTTCATCTTTGTCATCTGTTCTG R: AAATCCTTAGCACTCCTTCTGGTCACT	65	232–280	40	4	3	7	0	0.50/0.40	0.41	0.59/0.65	0.05
RyTET-G	$(ATCT)_{13}$	F:(PET)GGTGGTGTACAGAGCCAAAAGGATTAGAATTGTGTTGAT R: AACCCCTAGACGCGCACGAGGTGAAGAAG	55	124–224	40	7	1	8	0	0.75/0.74	0.05	NA	NA
RyTET-f	$(CTAT)_{16}$	F: (6-FAM)CTTTACCCTTAAGTTGTTTAGTGGATAA R: ACTTACAAACACAATAATATAACAGGACCGAGTA	61	219–289	39	2	5	7	0	0.41/0.25	0.11	0.51/0.61	0.06
RyTET-e	$(CAAA)_6$	F: (PET)ATAGTTCAGCAATTTTGTATATTTTTGTGCAAGATGTATG R: ACTTCTCCGAGGGGGTCAGCAGATGT	55	219–271	37	4	3	6	1	0.53/0.40	0.27	0.54/0.94	**0.01**
RyDI-2r	$(AC)_{11}$	F: (NED)TTCAACGTCCCATTAAGAGGAAACT R: GACTATTTGGGCGATATCAGAAAA	50	182–194	40	3	1	3	1	0.57/0.45	0.08	NA	NA
Ry2	$(CA)_6CC(AC)_4ACC(AC)_6$	F: (PET)GTGTGCGGCAGAGCCATGTGC R: GGCATATCCATTTTGATGGG	62	166–186	40	4	2	5	1	0.69/0.60	0.05	0.48/0.55	0.65