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RESEARCH ARTICLE

Population genetic structure and disease in montane boreal toads: more heterozygous individuals are more likely to be infected with amphibian chytrid

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Abstract Amphibians are more threatened than any other vertebrate group, with 41 % of species classified as threatened. The causes of most declines are not well understood, though many declines have been linked to disease. Additionally, amphibians are physiologically constrained to moist habitats and considered poor dispersers; thus, they may suffer genetic consequences of population isolation. To understand threats to the persistence of boreal toads (*Bufo boreas*) in Glacier National Park, USA, we genotyped 551 individuals at 11 microsatellite loci and used Bayesian clustering methods to describe population genetic structure and identify barriers to gene flow. We found evidence of two primary genetic groups that differed substantially in elevation and two secondary groups within the high elevation group. There was also evidence of further substructure within the southern high elevation group, suggesting mountain ridges are barriers to gene flow at local scales. Overall, genetic variation was high, but allelic richness declined with increasing elevation, reflecting greater isolation or smaller effective population sizes of high altitude populations. We tested for *Batrachochytrium dendrobatidis* (Bd), the fungal pathogen which causes chytridiomycosis, and we found that 35 of 199 toads were positive for Bd. Unexpectedly, more heterozygous individuals were more likely to be infected. This suggests that dispersal facilitates the spread of disease because heterozygosity may be highest where dispersal and gene flow are greatest.

Keywords Genetic variation · Mountains · Elevation · Chytridiomycosis

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Introduction

A central focus of conservation biology is determining why certain species are more at risk than others. Species traits such as poor dispersal ability, ecological specialization, and restricted geographic range, often lead to isolation of populations and can facilitate declines (Purvis et al. 2000; Harcourt et al. 2002). Geographically isolated populations are less likely to receive immigrants that help maintain positive population growth (Brown and Kodric-Brown 1977). Loss of genetic variation and inbreeding depression are also common in small, isolated populations (Frankham 2005). Both reduced genetic variation and inbreeding depression elevate extinction risk and weaken the ability of a population to cope with environmental change (Newman and Pilson 1997; Saccheri et al. 1998; Frankham 2005). Therefore, understanding the causes of population isolation and associated genetic effects is paramount for conservation and management, and many recent studies have identified landscape features (e.g., mountains, rivers) that lead to isolation by restricting movement and gene flow (e.g., McRae et al. 2005; Spear et al. 2005; Epps et al. 2005; Pérez-Espona et al. 2008).

Amphibians are more threatened than any other vertebrate class (Stuart et al. 2004). Forty-one percent of

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described amphibian species are classified as threatened, compared to 25 % of mammals, 13 % of birds, and 22 % of reptiles (Hoffmann et al. 2010). Amphibians may be more vulnerable to extinction than other vertebrates due to life history characteristics that lead to patchy distributions and geographically isolated populations. For example, because amphibians have highly permeable skin that is subject to evaporative water loss (Duellman and Trueb 1994), movements are often restricted to riparian corridors and forested areas where desiccation risks are low (Rothermel and Semlitsch 2002). Additionally, amphibians are generally considered poor dispersers and many species exhibit high breeding site fidelity (Tracy and Dole 1969; Daugherty and Sheldon 1982; Blaustein et al. 1994). Such physiological constraints and dispersal limitation can lead to limited connectivity among populations and related genetic and demographic effects of isolation (Lowe and Allendorf 2010).

An important emerging cause of amphibian declines is disease (Stuart et al. 2004; Kilpatrick et al. 2010). Chytridiomycosis, an infectious disease caused by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), has been linked to amphibian declines worldwide (Berger et al. 1998; Lips et al. 2006; Skerratt et al. 2007). The disease is transmitted via contact with infected individuals and by zoospores in water (Nichols et al. 2001). Chytridiomycosis has been reported on all six continents where amphibians occur (Fisher et al. 2009) and in a range of habitats, including lowland rainforests, cold mountaintops, and deserts (Ron 2005).

Amphibian populations differ in susceptibility to Bd (Crawford et al. 2010), and Savage and Zamudio (2011) showed major histocompatibility complex (MHC) heterozygosity was positively associated with survival in Bd-infected populations of the lowland leopard frog (*Rana* [*Lithobates*] *yavapaiensis*). More broadly, resistance to disease and parasites increases with heterozygosity at adaptive or neutral loci in many taxa (e.g. Meagher 1999; Coltman et al. 1999; Luikart et al. 2008). For example, Luikart et al. (2008) showed that bighorn sheep (*Ovis canadensis*) heterozygous at loci located within genes related to parasite susceptibility had lower lungworm burdens. Coltman et al. (1999) demonstrated a similar pattern at putatively neutral microsatellite loci, where inbred (less heterozygous) Soay sheep (*Ovis aries*) were more susceptible to parasitism by gastrointestinal nematodes. Such relationships highlight the need to monitor and maintain genetic diversity to ameliorate the effects of disease. Specifically, further efforts to detect a genetic basis for natural variation in host resistance to chytridiomycosis could be important for predicting local extinction risks and prioritizing conservation efforts for amphibians worldwide.

Boreal toads (*Bufo* [*Anaxyrus*] *boreas*) are widely distributed across western North America and occupy a variety of habitats, from desert springs to mountain wetlands (Hammerson et al. 2004). Boreal toads are of conservation concern because they are declining in portions of their range, causing the species to be listed as near-threatened by the IUCN (Hammerson et al. 2004). Declines of boreal toads in Colorado and Montana have been linked to chytridiomycosis (Muths et al. 2003; Pilliod et al. 2010). Factors that influence disease susceptibility, population isolation, and patterns of genetic variation are likely important for predicting long-term persistence of this species, particularly for populations in the southern Rocky Mountains where aridity and extreme geographic isolation make immigration an unlikely source of demographic and genetic rescue (Switzer et al. 2009).

Mountain ridges are barriers to gene flow in many amphibian species (Lougheed et al. 1999; Funk et al. 2005; Giordano et al. 2007), including boreal toads (Murphy et al. 2010). Additionally, gene flow is often restricted between low and high elevation amphibian populations (Funk et al. 2005; Giordano et al. 2007). Boreal toads are found at a range of elevations, from sea level to 3640 m (Hammerson et al. 2004), so it is reasonable to expect that elevation itself or environmental conditions correlated with elevation (e.g., temperature, snow accumulation, and vegetation) also influence patterns of genetic variation. Glacier National Park (GNP) in Montana provides a good opportunity to examine the effects of mountains and elevation on gene flow because convergence of two mountain ranges in the middle of the park with relatively flat land adjacent to either side creates diverse toad breeding habitat across a wide range of elevations.

Chytridiomycosis is common in boreal toads in GNP (Hossack et al. 2013a), providing an opportunity to identify factors that lead to variation in disease prevalence. Based on previous population genetic studies on disease resistance in wild populations, it might be expected that infected individuals will have lower mean heterozygosity than uninfected individuals. Alternatively, ecological and demographic processes (i.e., dispersal) that influence patterns of genetic variation may create an opposite pattern of disease prevalence—where more heterozygous individuals are more likely to be infected. For example, individuals in populations experiencing high gene flow, with resulting high heterozygosity, might experience higher exposure to Bd-infected immigrants and have a higher prevalence of Bd infection than individuals from isolated, low-heterozygosity populations. Distinguishing between these two alternatives is important because they have different management implications (i.e., promoting vs. preventing dispersal and gene flow).

Overall, this study had three major goals. The first goal was to describe the population genetic structure of boreal toads in GNP. Second, we identified landscape factors that are barriers to gene flow in boreal toads based on population genetic structure. Finally, we tested for a relationship between chytrid prevalence and boreal toad population genetic structure.

Methods

Study site and DNA sampling protocol

GNP is characterized by steep topography due to extensive glaciation during the Pleistocene. Boreal toads breed at a range of elevations, from 960 to >2190 m, and in a variety of habitats, including beaver ponds, forest ponds, and small cirque lakes. Historical processes operating in toad populations within GNP are unlikely to substantially influence patterns of genetic variation. Boreal toads in GNP are included in a clade that consists of toads from eastern Washington, Idaho, and northwestern Wyoming (Goebel et al. 2009). This clade has low mitochondrial genetic diversity despite its fairly broad spatial distribution, which may be explained by range expansions following retreating glaciers during the Pleistocene. Because GNP was glaciated during the Pleistocene, there were likely no refugia for boreal toads that could have allowed for divergence within the boundaries of the park.

We collected DNA samples from 551 toads >1 year old in GNP during the summers of 2008–2011 (Fig. 1). Twenty-two toads were sampled in 2008, 246 in 2009, 125 in 2010, and 158 in 2011. Samples were broadly distributed throughout the park in 2009 and 2010; samples collected in 2008 and 2011 targeted previously undersampled regions. Toads were captured at wetlands in 17 randomly selected catchments as a part of an amphibian monitoring program (Corn et al. 2005). We also sampled toads opportunistically in terrestrial habitats because it can be difficult to find adults even at breeding sites; therefore, not all sampled toads were associated with a wetland. DNA was collected by swabbing the buccal cavity with a foam-tipped or cotton-tipped swab (Pidancier et al. 2003). Buccal swabs were stored in 95 % ethanol until DNA extraction.

DNA isolation and microsatellite amplification

Tissues were digested using a detergent-based cell lysis buffer followed by ammonium acetate protein precipitation and isopropyl alcohol DNA precipitation. Isolated DNA was resuspended in 100 µl TE buffer. DNA was not diluted for polymerase chain reaction (PCR). PCR were carried out in a PTC-200 thermocycler (MJ Research Inc., Waltham, MA) using the QIAGEN Multiplex PCR Kit (QIAGEN, Valencia, CA.). All multiplex PCR reactions used a total volume of 10 µl. We amplified 11 microsatellite loci that were developed for *Bufo boreas* (*BBR29, BBR17, BBR86, BBR87b, BBR36, BBR4, BBR292, BBR281, BBR34-2, BBR16, BBR201*; (Simandle et al. 2005). PCR conditions

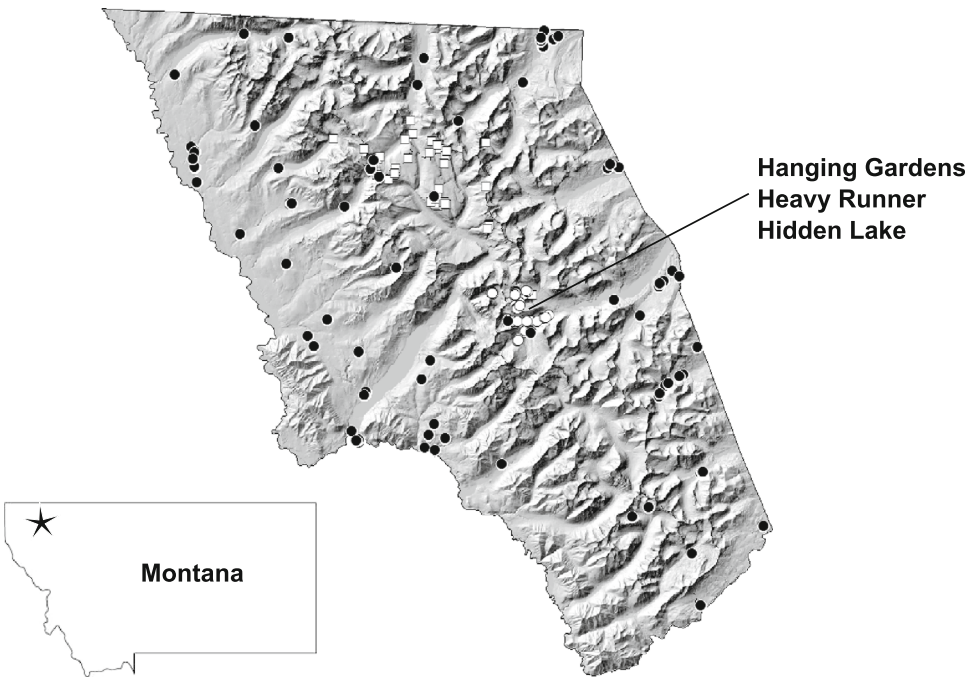


Fig. 1 Locations of individual boreal toad genetic samples ($n = 551$) from GNP. *Symbols* represent genetic groups identified by STRUCTURE analysis. *Open squares* and *circles* represent high elevation groups 1 and 2, respectively. *Closed circles* represent the low elevation group 3. *Symbols* overlap where multiple toads were sampled

followed Murphy et al. (2010). PCR products were visualized on an ABI3130xl Genetic Analyzer (Applied Biosystems Inc., Foster City, CA). Allele sizes were determined using the ABI GS600LIZ ladder (ABI) and called using Genemapper version 3.7 (ABI). Only 6 % of individuals had missing genotypes, and merely 2 % were missing more than one genotype. The mean number of loci genotyped per individual was 10.8.

Genetic analyses

Most population genetic studies of amphibians assume individuals at a breeding pond represent a population (e.g., Tallmon et al. 2000; Spear et al. 2005; Moore et al. 2011). However, these delineations may not represent genetic populations, leading to bias in subsequent analyses (e.g., Funk et al. 2005; Giordano et al. 2007). We used the Bayesian clustering model in STRUCTURE 2.3.3 (Pritchard et al. 2000) to delineate genetic groups (K) in our sample to avoid potential bias in discriminating populations, and because not all sampled individuals were associated with breeding sites. STRUCTURE 2.3.3 tests the likelihood of each K value and assigns individuals to groups so that departures from Hardy–Weinberg proportions and gametic disequilibrium are minimized. Preliminary STRUCTURE analyses with a burn-in period of 10,000 and 10,000 Markov Chain Monte Carlo (MCMC) repeats converged on $K < 6$. Therefore, for the final analyses, 10 independent runs for $K = 1$–6 were tested with a burn-in period of 100,000 and 100,000 MCMC repeats to identify the major genetic clusters, and a burn-in length of 300,000 and 300,000 MCMC repeats were used in subsequent rounds to test for further substructure within each of the major clusters. We assumed an admixture model with correlated allele frequencies.

The mean of replicate STRUCTURE runs for each K with the highest posterior probability is often used to infer the number of K in the sample; however, this statistic can sometimes be misleading, as once the real K is reached the posterior probability may continue to increase slightly at higher K (Evanno et al. 2005). Evanno et al. (2005) found that the ad-hoc statistic ΔK, which is based on the second order rate of change of the likelihood with respect to K, is a more accurate estimator of the true number of genetic clusters. However, because ΔK is a second order statistic, it cannot differentiate between $K = 1$ and $K = 2$, thus we used the ΔK method in conjunction with the highest mean posterior probability to infer the most likely K. STRUCTURE provides the proportion of membership to each cluster (q) for each individual, and individuals were assigned to a population according to their highest q-value.

We also used a second Bayesian clustering program, GENELAND 3.2.4 (Guillot et al. 2005a), to corroborate the STRUCTURE results. For the GENELAND analysis, K is not a fixed parameter and 10 independent runs with 100,000 MCMC iterations were performed allowing K to vary from 1 to 9. We used the correlated allele model to maintain consistency with STRUCTURE model choice and because it is better at detecting subtle genetic structure (Guillot et al. 2005b). The maximum rate of the Poisson process was fixed at 551 (the number of individuals; Guillot et al. 2005b), the maximum number of nuclei in the Poisson-Voronoi tessellation was set at 1653 (3 times the number of individuals; Guillot et al. 2005b), and the uncertainty of spatial coordinates was set at 500 m. The most likely number of clusters was inferred as the modal K with the highest posterior probability. Varying the spatial uncertainty did not alter the most likely number of K.

Genetic structure was also visualized using a principle coordinate analysis (PCA) implemented in GenAlEx version 6.4 (Peakall and Smouse 2005). The PCA was constructed from a pairwise, individual-by-individual genotypic distance matrix. To visualize genetic group patterns, we used MINITAB (Version 16) to conduct a principal component analysis based on the covariance matrix among allele frequencies, omitting the largest allele at each locus to account for the non-independence of allele frequencies within each locus.

We tested for gametic disequilibrium and departures from Hardy–Weinberg proportions using exact tests in GENEPOP version 4.0 (Raymond and Rousset 1995). *P* values were calculated using Markov Chain permutations (1000 dememorizations, 100 batches, 1000 iterations per batch) according to the algorithm of Guo and Thompson (1992). Genetic variation within populations was calculated as observed heterozygosity (H_O), expected heterozygosity (H_E), and allelic richness. H_O and H_E were calculated in GenAlEx version 6.41 (Peakall and Smouse 2005) and allelic richness was determined using Fstat 2.9 (Goudet 2001). Discrepancies between observed and expected heterozygosity were quantified using F_{IS} and were calculated in Fstat 2.9 (Goudet 2001). Genetic differentiation among populations was assessed using pairwise F_{ST} calculated in GENEPOP version 4.0 (Wright 1931; Weir and Cockerham 1984; Raymond and Rousset 1995), and we generated 95 % confidence intervals by bootstrap sampling over loci (Goudet 2001). We used a Mantel test (Mantel 1967), implemented in GenAlEx version 6.41, to estimate the correlation between individual genetic distances and geographical distances (Peakall and Smouse 2005).

Disease sampling and analyses

A subset of toads sampled for DNA were also tested for Bd in 2009 ($n = 109$). These samples were broadly distributed

throughout the GNP (Online Resource 1). We also re-sampled the Hanging Gardens region of the park in 2011 ($n = 90$) because it was under-sampled relative to the rest of the park in 2009. Toads from this region were both genotyped and sampled for Bd infection. We sampled for Bd by swabbing the pelvic patch and undersides of legs and feet with a sterile cotton swab, using standardized, clean procedures (Hossack et al. 2013a). Each swab was stored in a sealed vial with 95 % ethanol until analysis for Bd presence using a PCR assay (Annis et al. 2004). Samples were categorized as either infected (Bd = 1) or uninfected (Bd = 0).

We tested for an association between individual multiple-locus heterozygosity and Bd infection using a hierarchical logistic regression model with year modeled as a random effect (lme4 package in program R 2.12.2; R Development Core Team 2011). Individual multiple-locus heterozygosities were calculated as the proportion of heterozygous typed loci/mean heterozygosity of typed loci (Coltman et al. 1999) using the R package GENHET (Coulon 2009) because not all loci could be scored in all individuals. We included a term for STRUCTURE group in the model to test whether infection prevalence differed according to genetic group. We also included a term for elevation because Bd infection is commonly reported at high elevations worldwide (Ron 2005; Fisher et al. 2009) and infection increases with elevation in GNP (Hossack et al. 2013a). Females may experience reduced exposure to Bd compared to males and juveniles because they tend to be less aquatic (Bartelt et al. 2004), so we included a term for sex in our model. Previous work showed no relationship between infection of toads and other co-occurring amphibians in GNP (Hossack et al. 2013a); therefore, we did not consider the effects of community structure on infection prevalence.

Results

Population structure

We did not know whether wetlands that we sampled represented discrete populations, and many sampled toads were not sampled within a specific wetland. Therefore, we used the Bayesian clustering model in STRUCTURE 2.3.3 (Pritchard et al. 2000) to delineate genetic groups. The first round of STRUCTURE analysis including all 551 individuals revealed the most likely number of clusters was 2. ΔK was highest at K = 2, but the mean estimated logarithm probability of the data [ln Pr (X|K)] continued increasing at K = 3 through K = 6, a phenomenon which may occur after the true K is reached (Online Resource 2; Evanno et al. 2005). Because the difference in mean ln Pr (X|K) between K = 2 and K = 6 is much less than the difference between K = 1 and K = 2 (1707 vs. 3544, respectively), we concluded the most likely K to be 2.

The mean capture elevations of the two genetic groups (± 1 SD) were significantly different (1763 m ± 232 vs. 1484 m ± 228, $t = 14.17$, df = 550, $P < 0.01$). However, there were several instances where individuals assigned to the low elevation group were sampled at high elevations, and vice versa. In many of these cases, outlier individuals had low q-values of 0.5 – 0.7, indicating weak assignment to either major group. There was a group of 76 individuals that strongly assigned to the high elevation group but were captured at low elevations (Online Resource 3). These individuals were spatially clustered in the high elevation group (close to the Continental Divide, approximately 4 km south of Hanging Gardens; Fig. 1) but are actually located in a valley directly adjacent to other high elevation toads. Thus, the division of individuals into two elevation-based groups is rough. Nonetheless, there is a clear pattern indicating that elevation is important in structuring genetic groups, whether acting directly or in conjunction with correlated environmental conditions.

We continued STRUCTURE analysis in a hierarchical manner until no further substructure was detected (when K = 1). Within the high elevation group, ΔK was highest at K = 3, but the ΔK values for K = 2 and K = 3 were very similar (Online Resource 2). The mean ln Pr (X|K) values were again highest for K = 6, but the difference between K = 1 and K = 2 was greater than between K = 2 and K = 3 (865 vs. 417). Pritchard et al. (2000) recommend choosing the smallest value of K that captures the major structure in the data, thus K = 2 within the high elevation group. These two groups are roughly arranged along a north–south gradient (Fig. 1), dividing the high elevation group into a northern group and a southern group. All pairwise F_{ST} comparisons between the three groups were significant, ranging from 0.11 to 0.23 (Table 2).

To assess how topography influences genetic connectivity at a local scale at high elevations, we used STRUCTURE to test whether toads in Hanging Gardens ($n = 30$) and toads in two neighboring basins (Heavy Runner [$n = 17$] and Hidden Lake [$n = 23$]) were genetically distinct (Fig. 1). The ΔK method (Evanno et al. 2005) revealed the most likely number of K to be 3 (Fig. 2), indicating that the three basins are clearly genetically differentiated. However, STRUCTURE plots of q values (proportion of membership to each cluster) show evidence of first-generation migration between Heavy Runner and Hidden Lake, indicating movement does occur between basins. These putative migrants are genotyped at all loci, so it is unlikely that this pattern is the result of misassignment. F_{ST} values between the 3 basins were significant, ranging from 0.11 to 0.14.

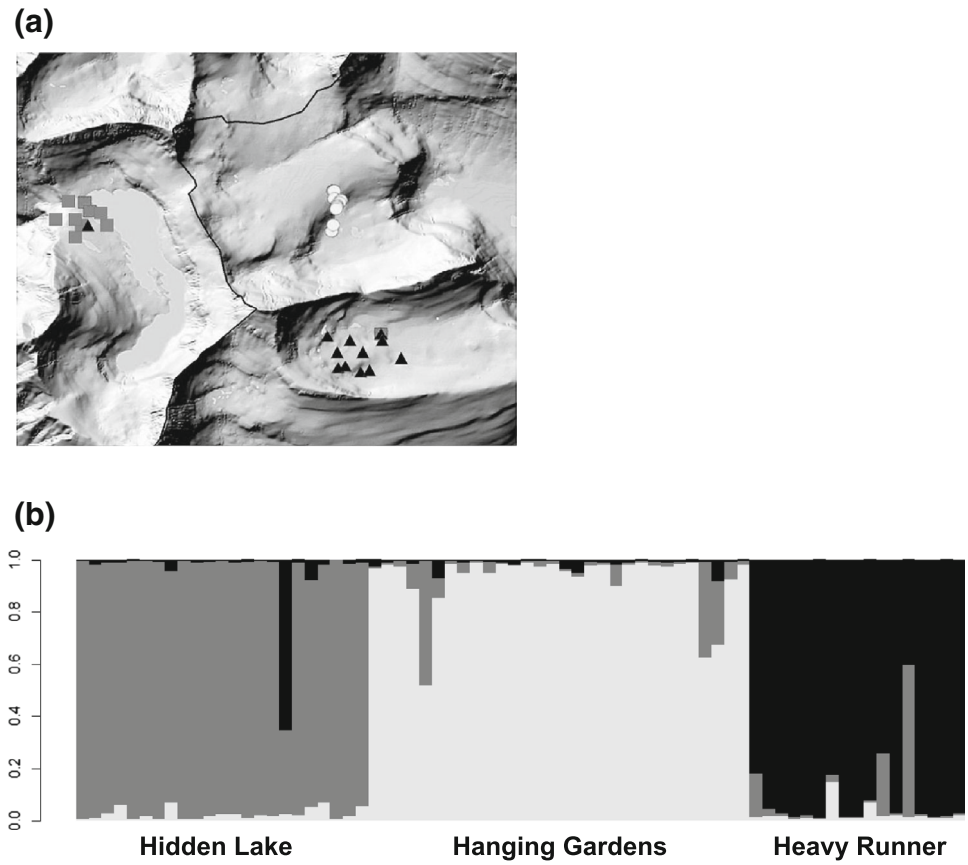


Fig. 2 **a** Population assignments from STRUCTURE analysis including toads from Hanging Gardens ($n = 30$), Heavy Runner ($n = 17$), and Hidden Lake ($n = 23$) basins. Each population is indicated by a *different symbol*, and *symbols* overlap where multiple toads were sampled. The *black line* indicates the Continental Divide. **b** Plot of q values from STRUCTURE simulations when K = 3

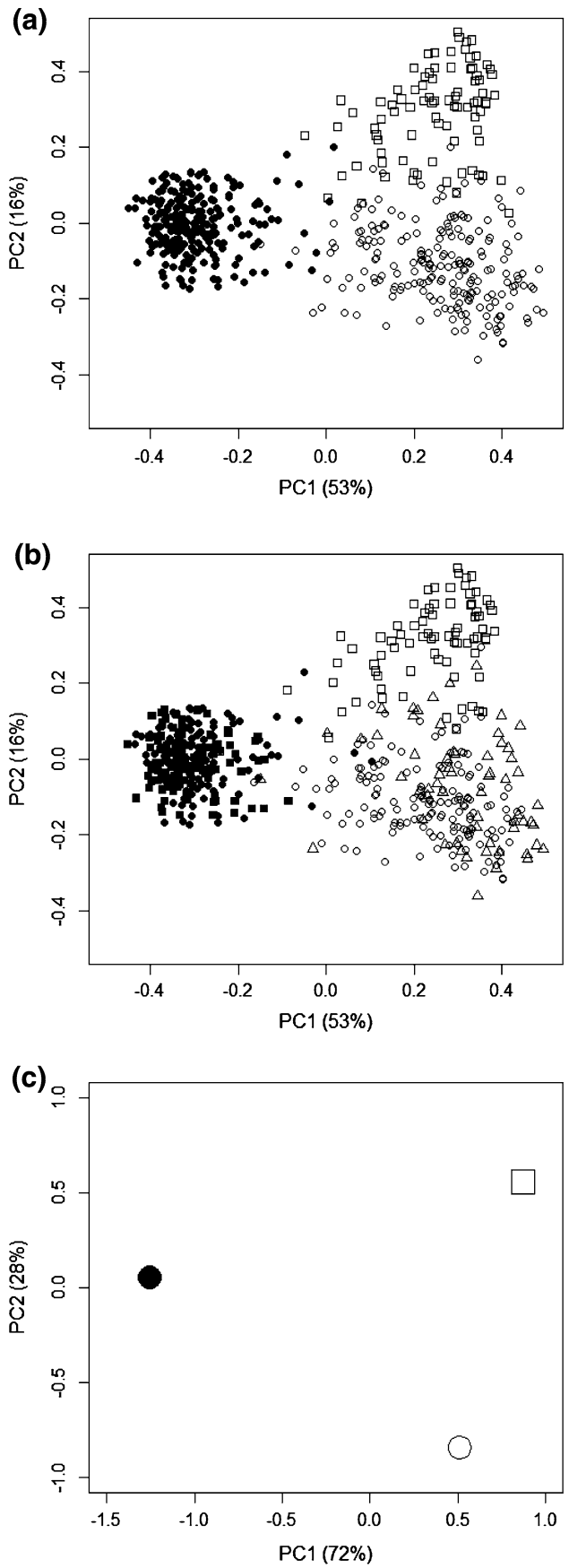
Within the low elevation group, ΔK was highest at K = 2 (Online Resource 2). However, the height of the modal value of the distribution of ΔK was low, indicating the strength of the signal was fairly weak. The plotted mean ln Pr (X|K) values indicate no clear break in slope and are instead more representative of a single population exhibiting isolation by distance (Schwartz and McKelvey 2009). Additionally, Mantel tests revealed a significant pattern of isolation by distance parkwide (Mantel $r = 0.30$, $P < 0.01$), suggesting no evidence of further substructure within the low elevation group.

STRUCTURE results were broadly supported by GENELAND and PCA (Fig. 3). Ninety-nine percent of individuals (543) were assigned to the same primary group (high or low) as STRUCTURE, but GENELAND detected 6 groups in the high elevations and 3 groups in the low elevations. PCA supported the presence of 3 genetic groups (Fig. 3). PC1 clearly separated individuals into the same high (positive coordinate 1 values) and low (negative coordinate 1 values) elevation groups detected by both STRUCTURE and GENELAND (Fig. 3a, b). Coordinate 2 differentiated between the two groups within the high elevation cluster detected by STRUCTURE (Fig. 3a, c), but did not clearly differentiate between the 6 high elevation groups and 3 low elevation groups detected by GENELAND (Fig. 3b). The GENELAND results may, therefore, overestimate population structure. For further analyses, individuals were grouped according to the results of STRUCTURE. We acknowledge that this is likely a conservative estimate of population structure, and these groups are probably not true populations in a biological sense (Waples and Gaggiotti 2006). F_{IS} values indicate a deficit of heterozygotes, likely due to the Wahlund effect.

Variation within groups

The number of alleles per locus ranged from 4 alleles at *BBR29* to 40 alleles at *BBR16*. Genetic variation was high; mean expected heterozygosity within groups ranged from 0.68 to 0.74, and mean allelic richness ranged from 9.89 to 13.04 alleles per locus (Table 1).

Twenty-three F_{IS} values were significantly different from zero before correcting for multiple comparisons. After Bonferroni correction, *BBR29* in group 1, *BBR87b*, *BBR292*, *BBR 16*, and *BBR201* in group 2, and *BBR86*, *BBR36*, *BBR292*, *BBR281*, *BBR34-2*, *BBR16*, and *BBR201* in group 3 deviated from Hardy–Weinberg expectations. All loci were heterozygote deficient, with the exception of *BBR201*, which had an excess of heterozygotes. Because no locus deviated from Hardy–Weinberg expectations in



◀**Fig. 3** Principal coordinate analysis based on pairwise genetic distances between individuals (**a**, **b**), and principal component analysis based on covariance matrix of group mean allele frequencies (**c**). *Symbols* in **a** and **c** correspond to STRUCTURE group assignments. *Symbols* in **b** correspond to GENELAND group assignments

all groups, all loci were retained for further analyses. Significant gametic disequilibrium was detected among 28 pairs of loci after Bonferroni correction, but only 4 pairs of loci were out of equilibrium in 2 groups (*BBR17* & *BBR86*, *BBR4* & *BBR34-2*, *BBR86* & *BBR281*, *BBR87b* & *BBR36)* and no locus-pair was out of equilibrium in all groups.

We also tested whether possible restricted gene flow at high elevations resulted in lower genetic variation compared to the low elevation group (e.g., Funk et al. 2005; Giordano et al. 2007). Mean expected heterozygosity (± 1 SE) was actually higher in the high elevation group (0.77 ± 0.03 vs. 0.68 ± 0.08). However, mean allelic richness was significantly lower in the high elevation group than in the low elevation group using a randomization test (10,000 randomizations, $P < 0.01$) where individual genotypes were randomized with respect to elevation designation (high or low) and allelic richness was calculated for each randomization.

Disease prevalence

Of the 199 individuals tested for Bd, 31 out of 109 and 4 out of 90 were positive in 2009 and 2011, respectively (Online Resource 1). This difference between years is probably because samples in 2009 were broadly distributed throughout the park, whereas the 2011 samples were confined to Hanging Gardens, Heavy Runner, Hidden Lake, and the Gunsight Valley (4 km south of Hanging Gardens), regions known to have low infection prevalence (Hossack, unpublished data). Logistic regression analysis including samples from both years revealed individual standardized multiple-locus heterozygosity was positively related to Bd infection ($\beta = 2.63$, SE = 1.19, $P = 0.03$; Fig. 4). Bd infection was unrelated to STRUCTURE group ($\chi^2 = 0.45$, df = 1, $P = 0.50$), elevation ($\chi^2 = 2.44$, df = 1, $P = 0.12$), and sex ($\chi^2 = 0.56$, df = 2, $P = 0.75$).

Discussion

We found that the population genetic structure of boreal toads in GNP is primarily influenced by landscape factors (elevation, mountains). Evidence of reduced allelic richness in the high elevation group suggests there may be long-term genetic consequences of isolation at high

Table 1 Sample size, mean capture elevation, and genetic information of three groups of boreal toads in GNP

Population	N	Mean capture elevation (m)	*AR*	H_E	H_O	F_{IS}
High elevation group						
1	87	1865	9.99	0.73	0.67	0.09*
2	210	1721	9.89	0.74	0.71	0.038*
Low elevation group						
3	254	1478	13.04	0.68	0.62	0.091*

N sample size, *AR* allelic richness; H_E expected heterozygosity, H_O observed heterozygosity, F_{IS} $1 - (H_O/H_E)$

Significance * $p < 0.05$

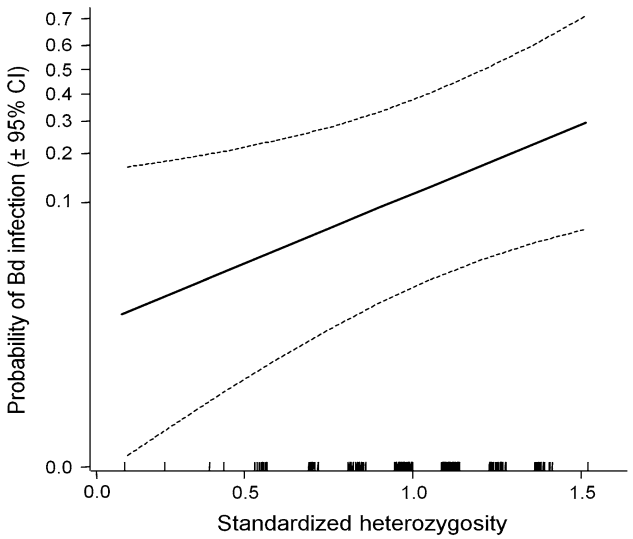


Fig. 4 Predicted probability of infection with *Batrachochytrium dendrobatidis* (Bd) relative to heterozygosity. Hatch marks on the horizontal axis show the distribution of individual standardized heterozygosities

elevations. A more immediate concern for population persistence is chytridiomycosis. We report an unexpected result that infection prevalence increases with heterozygosity, which is contrary to previous studies showing the opposite pattern. Our results suggest that dispersal facilitates disease transmission.

Parkwide genetic structure and genetic variation

Our results suggest there are three primary genetic groups of boreal toads in GNP, which are broadly coincident with elevation. STRUCTURE roughly divided individuals into a high elevation genetic group and a low elevation genetic group (Fig. 1), with further substructure in the high elevation group. These results were supported by GENELAND and PCA. However, some individuals found at low elevations assigned to the high elevation group, and vice versa. Additionally, pairwise F_{ST} values confirmed that gene flow is restricted between high and low elevation groups (Table 2).

Schwartz and McKelvey (2009) warned that patterns of spatial autocorrelation of allele frequencies due to mating with neighbors should be considered prior to population structure analyses. This is because autocorrelation along with irregular sampling can be misinterpreted as landscape features acting as barriers to gene flow. We had no a priori reason to expect autocorrelation in our data because we sampled continuously at a scale much larger than the dispersal capability of boreal toads, so it is unlikely we detected false barriers. Nevertheless, the designation of three primary discrete groups should be viewed as a coarse estimation of population structure within the park, and group F_{IS} values indicate it is likely an underestimate (Table 1). Further, though we could not resolve true biological populations at a parkwide scale, STRUCTURE analysis including Hanging Gardens, Hidden Lake, and Heavy Runner basins indicates that there are likely several local populations within the high elevation group (Fig. 2). Therefore, our analysis describes broad patterns of genetic differentiation.

There are several reasons gene flow might be restricted between low and high elevation groups. Upslope dispersal may be limited by energetic costs and heightened desiccation risk with increased elevation due to reduced forest cover (Rothermel and Semlitsch 2002; Lowe et al. 2008; Semlitsch et al. 2009). However, boreal toads are vagile compared to other amphibians (Harris 1975; Semlitsch

Table 2 Pairwise F_{ST} values (below diagonal) and 95 % confidence intervals (above diagonal) for groups of boreal toads in GNP

Group	Group		
	High		Low
	1	2	3
1	–	0.08–0.16	0.11–0.34
2	0.11	–	0.12–0.31
3	0.23	0.20	–

1981; Kleeberger and Werner 1982; Muths 2003; Adams et al. 2005), suggesting that dispersal limitation may not explain the observed pattern of genetic structure in GNP.

Rather than reflecting dispersal constraints, the genetic difference we observed between low and high elevation toads could be due to reproductive isolation based on elevation-related differences in breeding phenology. Funk et al. (2005) found restricted gene flow between Columbia spotted frog populations at low and high elevations despite evidence of long distance upslope dispersal, suggesting there may be premating barriers to gene flow between frogs at low and high elevations. In GNP, many breeding ponds are separated by >1 km of elevation, and breeding at low elevation sites can occur up to 60 days earlier than breeding at high elevations sites (B. R. Hossack, personal observation). Thus, even if dispersal is not restricted between high and low elevations, toads living at different elevations are likely reproductively isolated. Snowpack throughout the Northern Rockies is expected to decrease under future climate change scenarios (Leung et al. 2004; Mote 2006), potentially reducing these differences in phenology and genetic differentiation related to elevation. The genetic differentiation we observed between low and high elevation groups could also, in part, reflect adaptation to local environmental conditions that vary along an elevational gradient (e.g., temperature-moisture regime, length of growing season, forest cover, topographic morphology; Bonin et al. 2006; Murphy et al. 2010; Yang et al. 2012), although we acknowledge the use of neutral markers in this study prevents us from making inference about adaptive processes.

Overall, boreal toads in GNP have high genetic diversity. Expected heterozygosity ranged from 0.68 to 0.74 within groups, and allelic richness ranged from 9.89 to 13.04 alleles per locus. These values are at the upper range observed for other pond-breeding amphibians (Newman and Squire 2001; Funk et al. 2005; Manier and Arnold 2006; Moore et al. 2011). For example, expected heterozygosity of wood frog populations in the Prairie Pothole Region in North Dakota ranged from 0.44 to 0.50 and the maximum number of alleles per locus was 5 (Newman & Squire 2001). The authors hypothesized that this low genetic variation was likely due to extinction/colonization dynamics associated with periodic dry conditions. Several recent wildfires in GNP also lead to high turnover in wetlands (Hossack et al. 2013b), which could increase genetic variation because toads from outside the fire perimeter colonize burned wetlands (McCauley 1991).

We found that allelic richness decreased with elevation, suggesting there may be long-term genetic consequences of isolation at high elevations. The loss of alleles reduces the genotypic diversity in a population that is subject to natural selection (Allendorf 1986), and large populations harboring more genetic variation have a greater response to selection than small, isolated populations with reduced genetic variation (Frankham 1996). Reduced allelic richness may have important implications for chytridiomycosis resistance. MHC loci characteristically have many alleles and are associated with disease resistance in many taxa (Clarke 1979), including resistance to Bd (Savage and Zamudio 2011). We do not present data on MHC loci, but it is plausible that reductions in allelic diversity could significantly affect disease susceptibility.

Mountains ridges are barriers to gene flow

The amount of population differentiation in this study was higher than boreal toad populations elsewhere (Manier and Arnold 2006; Moore et al. 2011), which suggests there are stronger barriers to gene flow in this system. Previous population genetic studies of toads were conducted in gentler landscapes (Manier and Arnold 2006) or where the predominant landscape features promote connectivity (wetlands and lakes; Moore et al. 2011), which may explain comparatively stronger differentiation in GNP. Significant differentiation between genetic groups at high elevations observed in our study ($F_{ST} = 0.11$) adds to evidence that mountain ridges act as barriers to gene flow in amphibians (Tallmon et al. 2000; Funk et al. 2005; Giordano et al. 2007). This result was further supported by significant differentiation of Hanging Gardens, Heavy Runner, and Hidden Lake, three high elevation sites separated by >300 m tall mountain ridges (Fig. 2).

Higher disease prevalence in more heterozygous individuals

We found that more heterozygous individuals were more likely to be infected with Bd than less heterozygous individuals (Fig. 4). This result contrasts with previous studies reporting that more heterozygous individuals within local populations tend to be less susceptible to pathogens or parasites (e.g., Roelke et al. 1993; Coltman et al. 1999; Luikart et al. 2008). In addition, others have reported that local populations with greater mean heterozygosity tend to be more resistant to pathogens or parasites (Meagher 1999; Hedrick et al. 2001; Spielman et al. 2004). These relationships have been reported at both adaptive loci (i.e., MHC loci; Hedrick et al. 2001; Savage and Zamudio 2011), and neutral loci (i.e., microsatellite loci; Coltman et al. 1999), suggesting benefits of adaptive diversity in conferring resistance/lessening susceptibility, as well as benefits of genome-wide diversity in reducing the negative effects of inbreeding when parasites/pathogens represent a

significant fitness cost. To our knowledge, our study is the first to show an inverted relationship for neutral diversity, where disease prevalence increases with individual heterozygosity, invoking a demographic explanation rather than an adaptive explanation.

Differences in individual heterozygosity can result from either inter- or intra-population mechanisms (Luquet et al. 2011). Genetic drift and isolation lead to differences in heterozygosity among populations (Kimura et al. 1963). Therefore, individuals from smaller and more isolated populations will tend to have lower heterozygosity. Individual heterozygosity will also vary within populations by chance or because of matings between relatives (inbreeding). It is important to consider whether the observed correlation between heterozygosity and presence of Bd results from inter- or intra-population differences in heterozygosity because they have very different management implications (i.e., promoting gene flow or not; Luquet et al. 2011).

The relationship between heterozygosity and disease prevalence we report here likely results from a population-level effect. That is, individuals with higher heterozygosity were more likely to come from local populations with greater immigration. Populations experiencing higher immigration are expected to have greater heterozygosity and greater exposure to chytridiomycosis, resulting in a positive relationship between heterozygosity and Bd infection. This effect is likely to be most pronounced with immigration from a high prevalence population into a population that is fixed for different alleles, but may become less pronounced over time as the population reaches migration-drift equilibrium. More broadly, our results suggest that dispersal facilitates the spread of chytridiomycosis throughout GNP. Additionally, we provide empirical support for mathematical models predicting that contact among populations increases prevalence, incidence, and rate of disease spread (Hethcote 1976; Post et al. 1983; Andreasen and Christiansen 1989; Hess 1996), and may even allow disease to persist when it would otherwise decline in isolated populations (e.g., Post et al. 1983; Andreasen and Christiansen 1989).

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