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Chytrid fungus infection in alpine tree frogs is associated with individual heterozygosity and population isolation but not population-genetic diversity

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Abstract

Chytridiomycosis, a disease caused by the emerging fungus *Batrachochytrium dendrobatidis* (Bd), has been implicated in the decline of over 500 amphibian species. Population declines could have important genetic consequences, including reduced genetic diversity. We contrasted genetic diversity among both long-Bd-exposed and unexposed populations of the south-east Australian alpine tree frog (*Litoria verreauxii alpina*) across its range. At the population level, we found no significant differences in genetic diversity between Bd-exposed and unexposed populations. Encouragingly, even Bd-infected remnant populations that are now highly isolated maintain genetic diversity comparable to populations in which Bd is absent. Spatial genetic structure among populations followed an isolation-by-distance pattern, suggesting restricted movement among remnant populations. At the individual level, greater heterozygosity was associated with reduced probability of infection. Loss of genetic diversity in remnant populations that survived chytridiomycosis epidemics does not appear to be a threat to *L. v. alpina*. We suggest several factors underpinning maintenance of genetic diversity: (1) remnant populations have remained large enough to avoid losses of genetic diversity; (2) many individuals in the population are able to breed once before succumbing to disease; and (3) juveniles in the terrestrial environment have low exposure to Bd, providing an annual 'reservoir' of genetic diversity. The association between individual heterozygosity and infection status suggests that, while other work has shown all breeding adults are typically killed by Bd, males with greater heterozygosity may survive longer and obtain fitness benefits through extended breeding opportunities. Our results highlight the critical role of life history in mitigating the impacts of Bd infection for some amphibian species, but we infer that increased isolation as a result of disease-induced population extirpations will enhance population differentiation and thus biogeographic structure.

Highlights:

- The decline and population fragmentation of species affected by emerging wildlife diseases potentially drive losses of genetic diversity and hence adaptive potential.
- We measured genetic diversity at single nucleotide polymorphism loci in Australian alpine tree frog populations (*Litoria verreauxii alpina*) affected by chytridiomycosis to those unexposed to the disease-causing pathogen *Batrachochytrium dendrobatidis* (Bd).
- Despite major declines in abundance of the species and contraction to isolated, permanent waterbodies, genetic diversity was similar in Bd-exposed and Bd-unexposed populations.
- Although the disease typically kills all breeding individuals during the breeding season, males with higher individual heterozygosity were less likely to be infected at the time of sampling, indicating that heterozygous males may have greater breeding opportunity due to slower development of infection.
- Comparative studies of amphibian life histories as they relate to Bd exposure may shed further light on the observed variation between species in the impacts of disease on demography and genetic diversity.

Keywords: amphibian, Australia, bottleneck, disease, fungus, heterozygosity-fitness correlation, *Litoria*

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Introduction

In recent decades amphibians have been declining faster than any other vertebrate taxon, both in terms of numbers of individuals and numbers of species, and at least one third of all species are now threatened with extinction (Stuart et al. 2004). One of the greatest immediate threats to amphibians is the disease chytridiomycosis, which has been implicated in the decline of over 500 species, including 90 extinctions (Scheele et al. 2019). Chytridiomycosis is an epidermal disease caused by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd; Berger et al. 1998). *Batrachochytrium dendrobatidis* was discovered in 1998, in both Costa Rica and Australia (Berger et al. 1998) and has since been detected on all continents where amphibians are found (Olson et al. 2013).

While chytridiomycosis has already caused the extinction of many species (Scheele et al. 2019), a major conservation challenge lies in preventing further extinctions in species that have suffered declines from Bd, but persist in remnant populations (Scheele et al. 2014, Scheele et al. 2016, Scheele et al. 2017b). This challenge is evident across the world, in species such as the boreal toad (*Anaxyrus boreas*) (Muths et al. 2011) and yellow-legged frog (*Rana muscosa*) in North America (Vredenburg et al. 2010), the midwife toad (*Alytes obstetricans*) in Europe (Rosa et al. 2013), and tropical rainforest frogs in Australia, including the armoured mist frog (*Litoria lorica*) (Puschendorf et al. 2011).

Concerns have been raised about the genetic viability of species that have faced severe declines (Jamieson et al. 2008). Loss of genetic diversity through population bottlenecks caused by disease may pose an additional challenge for the persistence and recovery of such species, with negative effects on individual fitness, disease resistance, and adaptive potential (Spielman et al. 2004). For amphibian species where chytridiomycosis is associated with population extirpation, remnant populations are often more isolated than pre-decline, and occur at low densities, which is likely to lead to stronger biogeographic structure.

Eastern Australia is a hotspot for chytridiomycosis-induced amphibian declines (Scheele et al. 2017c, Skerratt et al. 2007). One species that has experienced major declines associated with the emergence of Bd is the alpine tree frog (*Litoria verreauxii alpina*). *Litoria v. alpina* was historically common in parts of southeastern Australia but declined in the 1980s with the emergence of Bd (Osborne et al. 1999, Skerratt et al. 2016). The impacts of Bd on *L. v. alpina* include large reductions in range and abundance, and contraction of remnant populations to perennial water bodies (Osborne et al. 1999, Skerratt et al. 2016); essentially habitat loss and fragmentation. The disease could, therefore, potentially impact genetic diversity through genetic drift in the relatively small and isolated surviving populations (Keyghobadi 2007). Remnant *L. v. alpina* populations now appear stable, but disease impact remains high, with Bd prevalence often exceeding 90% in adults during the breeding season, resulting in near complete annual adult mortality (Brannelly et al. 2015, Scheele et al. 2016). Importantly, population persistence is dependent on young adults breeding for the first time, depositing eggs before succumbing to chytridiomycosis (Scheele et al. 2015).

The few studies that have investigated the genetic impacts of chytridiomycosis on amphibian populations have yielded diverse and sometimes contrasting results for genetic diversity at the individual and population levels (Addis et al. 2015, Luquet et al. 2012, Savage and Zamudio 2011, Wagner et al. 2017). For example, in lowland leopard frogs (*Lithobates yavapaiensis*) higher heterozygosity is associated with lower risk of Bd-associated mortality (Savage et al. 2015). In contrast, in boreal toads (*Bufo boreas*) more heterozygous individuals had increased risk of Bd infection (Addis et al. 2015). Variable species responses likely reflect that chytridiomycosis influences the demography and ecology of amphibian species in diverse ways, potentially leading to different genetic impacts, and different opportunities for evolutionary responses. Thus, to develop an understanding of the ecological and evolutionary consequences of this globally emerging pathogen, biogeographic studies across species with contrasting responses are required.

Here, we contrasted genetic diversity in remnant populations of *L. v. alpina* exposed to Bd since the mid-1980s with Bd-naïve populations. We investigated whether infected frog populations contained lower genetic diversity than uninfected populations and whether heterozygosity-fitness correlations might be associated with Bd-infection status among individuals. We hypothesised that reduced genetic diversity could result from major population crashes, and from population fragmentation following the local extinction of populations in interlinking ephemeral wetland complexes (Scheele et al. 2016). Given *L. v. alpina* has effectively experienced a shift from iteroparity to semelparity (Scheele et al. 2016), we compared the degree of genetic differentiation among age cohorts in Bd-infected and uninfected populations as rates of genetic drift are expected to differ in populations with overlapping and non-overlapping generations (Hill 1979, Rogers and Prügel-Bennett 2000).

Finally, we investigated potential heterozygosity-fitness associations (Acevedo-Whitehouse et al. 2003, Coltman et al. 1999) with Bd infection status of individuals to test the hypothesis that, within a given remnant population, individuals with greater heterozygosity would be less likely to be infected.

Methods

Study species and sample collection

Litoria v. alpina is a medium sized (snout-urostyle length = 25–50 mm) Australian tree frog (family Hylidae) that breeds in ponds in spring. Males form breeding aggregations and use advertisement calls to attract females that arrive throughout spring. *Litoria v. alpina* was historically widespread and abundant throughout sub-alpine areas of New South Wales, Victoria, and the Australian Capital Territory in south-eastern Australia (Gillespie et al. 1995). However, like many amphibian

species in eastern Australia, this species experienced severe population declines in the late 1980s due to chytridiomycosis and is now restricted to a small number of isolated remnant populations (Osborne et al. 1999, Scheele et al. 2017c). Comprehensive surveys in the mid-1990s documented *L. v. alpina* occurrence at only nine of 140 known historical sites in NSW and the ACT (Osborne et al. 1999). Post-decline surveys have been less thorough in Victoria, but the species disappeared from many of its former sites (Gillespie et al. 1995).

Litoria v. alpina is currently considered a subspecies of the more widely distributed whistling tree frog, occurring above 1,000 metres elevation. The other subspecies, *L. v. verreauxii*, is found throughout much of southeast Australia at lower elevations (Figure 1) and also experienced declines associated with Bd emergence (Scheele et al. 2014). For the current study, samples were collected from 10 sites, including eight sites covering the range of the *L. v. alpina* subspecies (Figure 1) and two sites in the range of *L. v. verreauxii*.

Although recognised as distinctive subspecies, a study of skin colouration, morphology, and call variation revealed geographic variation in these traits but did not distinguish the subspecies on these characteristics (Smith et al. 2003). Further, there has been no genetic evaluation of the distinctiveness of the subspecies. Therefore, we included all samples in our dataset and used a set of genetic clustering analyses to investigate whether the partitioning of these samples into two major groups corresponding to the subspecies classification was warranted. Sampling was approved under ANU Ethics permits A2011/51 and A2013/32, and scientific licences SL100411, T2011501 and 10006052.

Sample collection

Two of the *L. v. alpina* populations, Grey Mare and Bullfight, were naïve to Bd at the time of sample collection (Figure 1) (Hunter et al. 2009, Scheele et al. 2016). These two sites are the only known unexposed sites for the species. The other eight sites were long-exposed to Bd. Exact dates of Bd emergence are unknown, but amphibian declines occurred throughout the mid-1980s, consistent with the spatio-temporal spread of Bd in eastern Australia (Scheele et al. 2014a; Scheele et al. 2017b).

Samples (toe tips) were collected from up to 50 individuals at eight of the study sites in 2011. At the remaining two sites, samples were collected using buccal swabs in 2011 and 2013 (Table 1). DNA yields and quality (based on spectrophotometry and gel electrophoresis) were similar from both methods. A new pair of disposable gloves was used for each frog to avoid infection transmission between frogs. Buccal samples were collected by gently inserting a sterile cotton swab inside the mouth of each frog and rolling it over all areas several times to obtain adequate genetic material.

Batrachochytrium dendrobatidis in study populations

Extensive field and laboratory research has investigated Bd dynamics, population impacts, and Bd susceptibility in *L. v. alpina*. In Bd-infected populations, large increases in Bd prevalence and infection intensity occur during the breeding season in adults (Scheele et al. 2015). High Bd prevalence results in very high (>95%) annual mortality of first-time

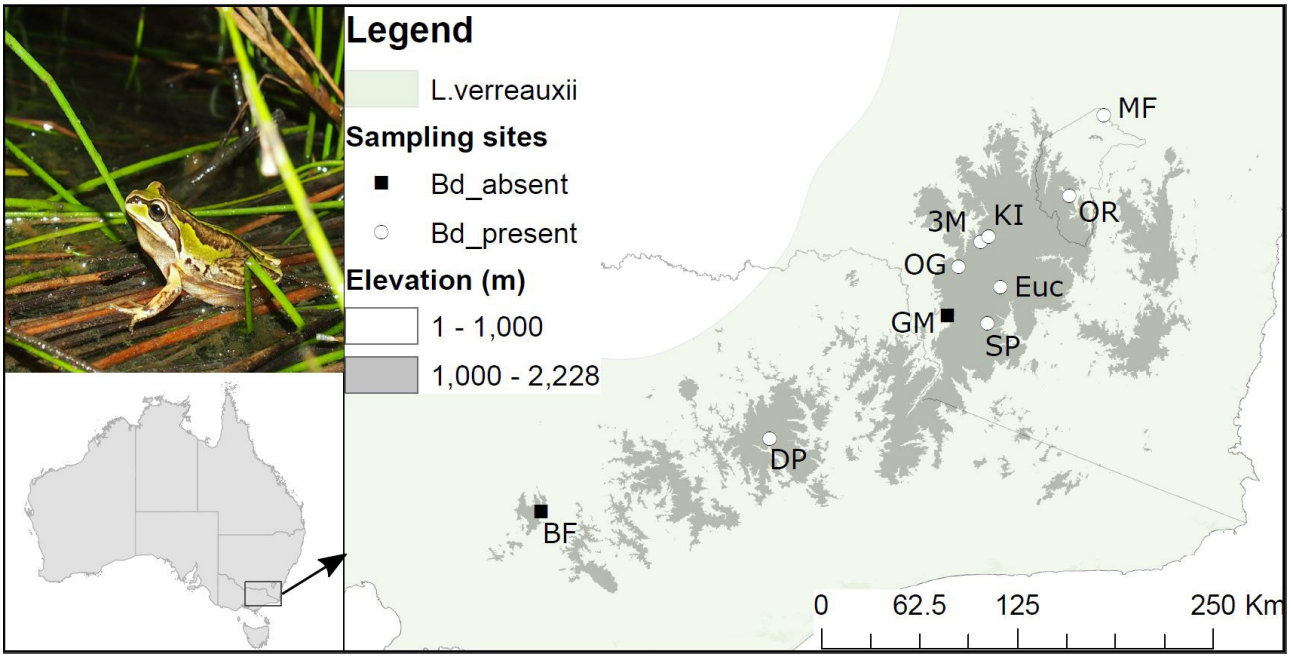

Figure 1. Map of sampling sites that were exposed and unexposed to Bd based on Bd detection at the time of sampling (right), location of the study region within Australia (bottom left), and sampled *Litoria verreauxii* at the Mulligan's Flat sampling site (top left; photo credit Sam Banks). The distribution of *L. verreauxii* in south-eastern Australia is shown on the map. The currently recognised *L. v. alpina* subspecies typically occurs at elevations over 1,000 m.

Table 1. Sample sizes and genetic diversity statistics in *Litoria verreauxii* in south-eastern Australia calculated from (1) pairwise genetic distances across all 42668 variants and (2) AIC-based genotype calls (using genotype likelihoods calculated from read depth). Dataset (2) uses the 50 SNPs with highest sample coverage filtered by F_{IS} (absolute $F_{IS} < 0.2$).

		***Bd*-absent**			***Bd*-present**						
	Site location	**Mt Bullfight**	**Grey Mare**	**Three Mile Dam**	**Dinner Plain**	**Eucumbene**	**Kiandra**	**Mulligan's Flat**	**Ogilvies Creek**	**Orroral River**	**Sponar's**
	Site code	BF	GM	3M	DP	Euc	KI	MF	OG	OR	SP
	Individuals sampled	50	50	50	50	49	50	45	50	47	50
	Post filtering sample size	46	42	41	47	43	46	32	44	41	43
(1)	Mean multivariate distance to site centroid	22997	24422	24524	24960	25258	24748	23407	24658	23498	24467
(2)	Mean sample size	18.5	20.8	17.2	15.2	15.0	26.9	16.4	16.8	23.1	21.7
	Mean # of alleles	1.37	1.77	1.77	1.63	1.81	1.91	1.67	1.74	1.84	1.77
	Mean effective # alleles	1.13	1.38	1.34	1.26	1.36	1.34	1.31	1.35	1.33	1.32
	Mean PIC	0.17	0.35	0.34	0.26	0.36	0.35	0.29	0.34	0.34	0.33
	Mean H_O	0.12	0.25	0.24	0.18	0.25	0.22	0.21	0.25	0.23	0.26
	Mean H_E	0.11	0.23	0.22	0.16	0.23	0.22	0.19	0.22	0.21	0.21
	Mean unbiased H_E	0.11	0.24	0.23	0.17	0.23	0.22	0.20	0.23	0.22	0.21

breeders (Brannelly et al. 2015, Scheele et al. 2015). Mortality occurs during spring or early summer, and infection intensity is not related to body condition (Brannelly et al. 2015). Importantly, adults reproduce before succumbing to chytridiomycosis and recruitment, aided by low Bd prevalence in tadpoles, is sufficient to facilitate population persistence (Scheele et al. 2015). The demography of Bd-infected populations and Bd naïve populations differ substantially: Bd-infected populations typically contain no individuals older than 3 years, whereas studied Bd-free populations have a diverse age structure, with some individuals documented surviving until at least 7 years of age (Scheele et al. 2016). This shift in demography has resulted in a major reduction in niche breadth, with the species only persisting in or nearby perennial ponds, where drought-associated recruitment failure is absent (Scheele et al. 2017a, Scheele et al. 2017c). Earlier maturation at small body size has also been documented in Bd-infected populations (Scheele et al. 2017d). Under laboratory conditions, individuals raised from wild collected eggs from Bd-infected populations demonstrated very high susceptibility following experimental exposure to Bd, with near complete mortality (Bataille et al. 2015).

DNA extraction and genotyping by sequencing (GBS) library preparation

DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit following the manufacturer's protocol. For swab samples, the entire swab tip was cut off using ethanol-flamed surgical scissors and placed directly in the well of a 96-well plate for extraction. Following DNA extraction, DNA was eluted in 200 uL of TE buffer.

GBS library preparation was carried out according to a modified version of the protocol described by Elshire et al. (2011). Genomic DNA was digested with PstI and DNA was ligated with uniquely barcoded sequencing adaptor pairs (Elshire et al. 2011). Samples were then individually amplified with 25 PCR cycles in a 50 uL reaction (25 uL Bioline 2x Taq Master Mix, 10 pmoles forward and reverse primer, 13 uL H_2O and 10 uL template) and pooled at 40 – 50 ng DNA per sample. We sequenced fragments between 250 and 450 bp in size in a single HiSeq2000 lane using a 100-bp paired-end protocol at the Biomolecular Resource Facility at the Australian National University.

SNP calling and filtering

The TASSEL GBS UNEAK pipeline was used to call variants using a *de novo* approach (Glaubitz et al. 2014, Lu et al. 2013). From an initial 248,771 bi-allelic SNPs (restricted to single SNPs per sequence) over 485 samples, we removed SNPs that were genotyped in less than 10% of samples or greater than 90% of samples (putative paralogs) or were missing in more than two populations. We removed samples with fewer than 2000 SNPs then removed 3104 rare SNPs that did not pass a paralog threshold of 5. The filtered genotype matrix had 42,668 variants across 425 samples. Sequence data are available through Sequence Read Archive[1] (project PRJNA592019; details in Table S1, Supplementary Material).

1 https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA592019

Analyses of genetic structure

Due to the low sequencing depth in our dataset (20% sample coverage and 0.35 mean sequencing depth in the full dataset), we used three different approaches for the analysis of genetic structure, including analyses based on pairwise individual genetic distances using allele presence/absence on the full suite of post-filtering loci. This approach uses all of the available information but is potentially influenced by a high degree of missing data. Therefore, we followed the 'first pass' analysis of the full data set with two other approaches, including analyses of genetic structure based on genotype likelihoods (10,071 SNPs with 38% sample coverage) (White et al. 2013) and analyses based on genotypes at the 50 SNPs with greatest sample coverage after Akaike's Information Criterion (AIC)-based genotype calling, using a delta AIC > 2 (between the best-supported and second-ranked genotype) criterion for genotype acceptance (as a qualitative validation of the results using the first approach) (White et al. 2013).

The first suite of analyses of genetic structure used all 42,668 loci to calculate pairwise genetic distances using a binary distance metric based on allele presence/absence at shared SNP loci between each pair of individuals. We used the multi-response permutational procedure (*mrpp*) and permutational multivariate analysis of variance (*adonis*) functions in the vegan package (Oksanen et al. 2016) in R (R Development Core Team 2016) to test for genetic structure among populations (using 1,000 permutations for each). We then used a pairwise *adonis* analysis to test for structure among all site pairs. We also used a standard AMOVA analysis implemented in GenAlEx (Peakall and Smouse 2012) to estimate PhiPT, an analogue of F_{ST} derived from distance data (Excoffier et al. 1992) among populations and among population pairs.

We used the *adonis* approach to test the null hypothesis of no genetic structure among age cohorts within each population, with and without an interaction between population Bd status and age. For this analysis, permutation of individuals among age cohorts was conducted within populations, and we tested for significant structure explained by age cohorts alone and by the population Bd status and age interaction.

We used Discriminant Analysis of Principal Components using the *dapc* function in the *adegenet* R package (Jombart 2008) to further investigate patterns of genetic structure within our dataset. We generated principal components with the *dudi.pco* function of the *ade4* R package (Dray and Dufour 2007) and conducted the DAPC analysis using the discrete sampling locations as pre-defined groups. We chose the number of principal components to retain in the analysis by optimising the α-score, which optimises the trade-off between the power of discrimination and over-fitting.

We used a further-refined genotype dataset to compare the *dapc* outputs with an *NGSadmix* (Skotte et al. 2013) analysis of population structure to estimate individual population ancestry coefficients based on genotype likelihoods from the full dataset. We calculated genotype likelihoods for each possible genotype at each SNP from read depth information[2] (White et al. 2013). We standardised the genotype likelihoods to sum to 1 across all three possible genotypes. We excluded loci that contributed little information (fewer than 100 individuals with genotypes satisfying the delta AIC > 2 criterion, and minimum minor allele frequency <0.02), resulting in a dataset of 411 individuals and 10,071 loci. We ran *NGSadmix* models for scenarios of 2,4,6,8 and 10 groups.

Comparisons of genetic variation within populations

We used the betadisper function of the vegan R package (Oksanen et al. 2016) to test for homogeneity of group dispersions (in this case, mean distance to population centroids). We used a permutation test (1,000 permutations) to determine if the population groupings were equally dispersed and used the Tukey HSD test to compare among population pairs.

We complemented the analysis based on pairwise individual genetic distances with more 'traditional' metrics of genetic diversity calculated from a subset of 50 SNPs. The loci used were the 50 SNPs with the greatest coverage of individuals after calling individual genotypes using AIC scores of genotype likelihoods. These were calculated from read depth information as described above, with genotypes called using a delta AIC criterion of >2, selecting the 50 loci with greatest sample coverage (mean coverage 74%) and F_{IS} values not less than -0.2 or greater than 0.2. We used GenAlEx (Peakall and Smouse 2006) to estimate the number of alleles, number of effective alleles, observed and expected heterozygosity across these loci in each population.

Is heterozygosity of individuals associated with Bd infection status?

We tested whether individual heterozygosity was associated with the Bd infection status of individuals. We used a data subset of 207 individuals from the six Bd-exposed populations for which we had individual Bd infection status data. This included the Kiandra (n = 45), Ogilvies (11), Three Mile (37), Eucumbene (41), Sponars (26), and Dinner Plain (47) sites (KI, OG, 3M, EU, SP and DP in Figure 1, respectively). We did not include samples from populations where Bd was not known to be present for this analysis, although Bd testing data were collected from these sites (Scheele et al. 2016), and we did not have Bd status data from the MF and OR sites although Bd is known to be present at these sites. We used sterile swabs (Medical Wire and Equipment Co. MW 100–100) to sample for Bd. Individuals were sampled in a standardised way with three strokes on each side of the abdominal midline, the inner thighs, hands and feet. Samples were analysed using real-time quantitative polymerase chain reaction (qPCR) (Boyle et al. 2004, Hyatt et al. 2007). Samples were analysed in triplicate and were considered positive if all three wells returned a positive reaction (Hyatt et al. 2007).

2 https://github.com/mgharvey/GBS_process_Tom_White, last accessed 08 December 2019

We generated a number of individual heterozygosity estimates to investigate the trade-off between precision (stricter genotype filtering to avoid under-calling heterozygotes) and quantity of data from a low sequencing depth dataset. We used three simple estimators based on the proportion of heterozygous SNPs with minimum sequencing read depths of 4, 6, 8, and 10 reads (corresponding to 55,878, 22,026, 10,904 and 6,836 SNP genotypes across the 207 individuals, respectively). We used R to fit binomial GLMs with Bd status as the response variable and population and H_O as the explanatory variables. This was repeated for each H_O estimator and the models were compared using AIC. We used Poisson GLMs to evaluate similar models using Bd infection intensity (qPCR-estimated zoospore-equivalent score) as the response variable.

Results

Analyses of genetic structure among individuals and populations

Broadly, our SNP data revealed that individuals clustered strongly into genetically similar groups within sampling locations, and genetic affinities among sampling locations reflected geographic location. The *mrpp* analysis identified significant differences among sites (P = 0.001). The observed *delta* (weighted mean of mean within-site genetic distances) was 34840 and expected *delta* (under the null hypothesis of no structure) was 36720, corresponding to an α statistic (chance-corrected estimate of the proportion of the distances explained by group identity) of 0.051. Likewise, the *adonis* analysis identified significant differentiation among groups (R^2 = 0.018, P = 0.001). All pairwise *adonis* comparisons except MF-OR (P = 0.143) identified significant differentiation. In contrast, the AMOVA (total PhiPT = 0.057, P = 0.001) identified significant pairwise PhiPT values among all population pairs, which corresponded broadly to an isolation-by-distance (IBD) pattern with some genetic grouping of sampled populations among the northern sites (Figure 2). The IBD pattern was non-significant (Mantel *r* = 0.36, P = 0.07) when the distant Mt Bullfight (BF) samples were removed from the analysis. This may be due to the presence of historical biogeographic structure in the alpine region of southern New South Wales indicated by the strong genetic clustering of a group of sites from west-flowing catchments in the northern region of the Snowy Mountains (GM, KI, 3M, OG), which clustered separately from a group comprising the remaining sites except BF (Figure 2).

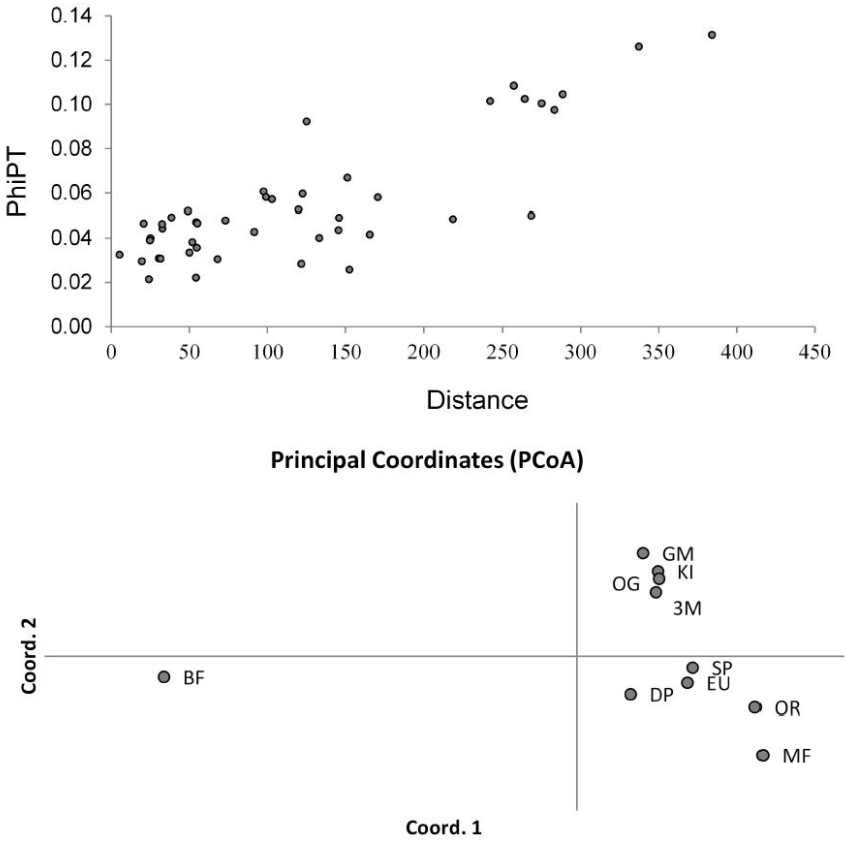

Figure 2. Spatial patterns of genetic differentiation among sampling site for *Litoria verreauxii* across 10 sites and 425 individuals, showing (top) the isolation-by-distance pattern of AMOVA PhiPT among sites and (bottom) a plot of the first two principal components of a PCoA analysis of genetic differentiation among sites (explaining 70% and 18% of variation, respectively) using PhiPT as a non-standardised genetic distance metric among populations.

When we repeated the *adonis* analysis to test for significant differentiation among age cohorts within sites, we found a marginally non-significant effect (R^2 = 0.007, P =0.072). Likewise, there was no significant Bd-status * age cohort interaction that would support a scenario of greater genetic structure among age cohorts in the effectively semelparous Bd-exposed populations compared to the iteroparous non-exposed populations (Table 2).

In Figure 3, we show 'assignment' plots from a DAPC analysis where eight principal components were retained based on α-score optimisation. This resulted in a scenario where individuals clustered into groups based on sampling sites. Indeed, the individual group membership coefficients (Figure 3) show that where individuals were assigned partial membership of multiple groups, this was typically the genetic group corresponding to their sampled population and the group corresponding to the next-nearest sampled population. The greatest degree of 'cross-assignment' of membership coefficients was among the two closest sites (3M and KI). Notably, the recognised subspecies (*L. v. verreauxi*: MF and OR; *L. v. alpina*: all other sites) were not distinguished by this analysis.

The *NGSadmix* analysis of genotype likelihoods from 10,071 loci identified highest support for the K=10 model and largely assigned individuals to genetic clusters corresponding to the population in which they were sampled (Figure 4). There was some degree of 'multiple membership' of the individual admixture coefficients, but the patterns of partial assignment of individuals to neighbouring patterns were less apparent than with the DAPC analyses.

Analyses of genetic variation within populations

The comparisons of genetic variation among sites based on inter-individual genetic distances and population allele frequencies provided no evidence for a reduction in genetic variation in Bd-exposed populations relative to unexposed populations. Site-level genetic diversity estimates are presented in Table 1. In fact, according to all measures of genetic variation used, one of the Bd-unexposed populations (Mt Bullfight [BF], the most geographically distant sampling site) had the lowest genetic variation. Samples from the other Bd-unexposed site, Grey Mare [GM], had amongst the highest genetic diversity of the 10 sites sampled (Table 1). The *betadisper* test rejected the null hypothesis of homogeneity of genetic distances within populations (F=8.31, df = 9, P = 0.001). The principal coordinates analysis underpinning the *betadisper* test is plotted in Figure 3a. The Tukey HSD pairwise comparisons identified significantly lower diversity within the BF samples compared to all other sampling sites (Figure 5), and lower genetic diversity in the Mulligan's Flat [MF] and Orroral River [OR] samples relative to a number of other sampling sites (Figure 5).

Is heterozygosity of individuals associated with Bd infection status?

We found no relationship between infection intensity (a measure of infection severity) and individual heterozygosity, but a marginally significant relationship between individual heterozygosity and the probability of Bd infection (binomial model coefficient [logit scale] = -11.2 ± 5.7 s.e., z = -1.97, P = 0.048). The best model of the probability of individual infection featured an effect of population and a heterozygosity estimator based on the proportion of heterozygous loci after excluding all loci with sequencing read depth < 6. The models featuring other simple heterozygosity estimators (minimum read depth of 4, 8 and 10) were ranked within 2 ΔAIC units of the best model but featured marginally non-significant negative effects of individual heterozygosity on infection probability. According to the best model, frogs with greater mean observed heterozygosity were less likely to be infected with Bd (Figure 6). There was also a large population effect, with very high infection rates at Ogilvies Creek (OG), and relatively low infection rates at Three Mile Dam (3M) and Sponar's Lake (SP).

Table 2. Adonis analyses of genetic diversity in *Litoria verreauxii* by partitioning genetic distances within and among groups based on population Bd status and individual age cohorts within populations. P-values are based on 1,000 permutations.

Variable	DF	Sums of Squares	Mean squares	F	R^2	P
Permutation of individuals among age cohorts within populations (Sites)						
Age (within Site)	1	1.46×10^9	1.45×10^9	2.139	0.008	0.068
Residuals	242	1.65×10^{11}	6.82×10^8	0.991		
Total	243	1.67×10^{11}	1			
Permutation of individuals among age cohorts within populations, contrasting Bd-infected and uninfected populations						
Bd (Site-level)	1	4.45×10^9	4.45×10^9	6.641	0.026	0.054
Age (within Site)	1	6.89×10^8	6.89×10^8	1.029	0.004	0.092
Bd * Age	1	7.25×10^8	7.25×10^8	1.082	0.004	0.111
Residuals	240	1.61×10^{11}	6.69×10^8	0.964		
Total	243	1.67×10^{11}		1		

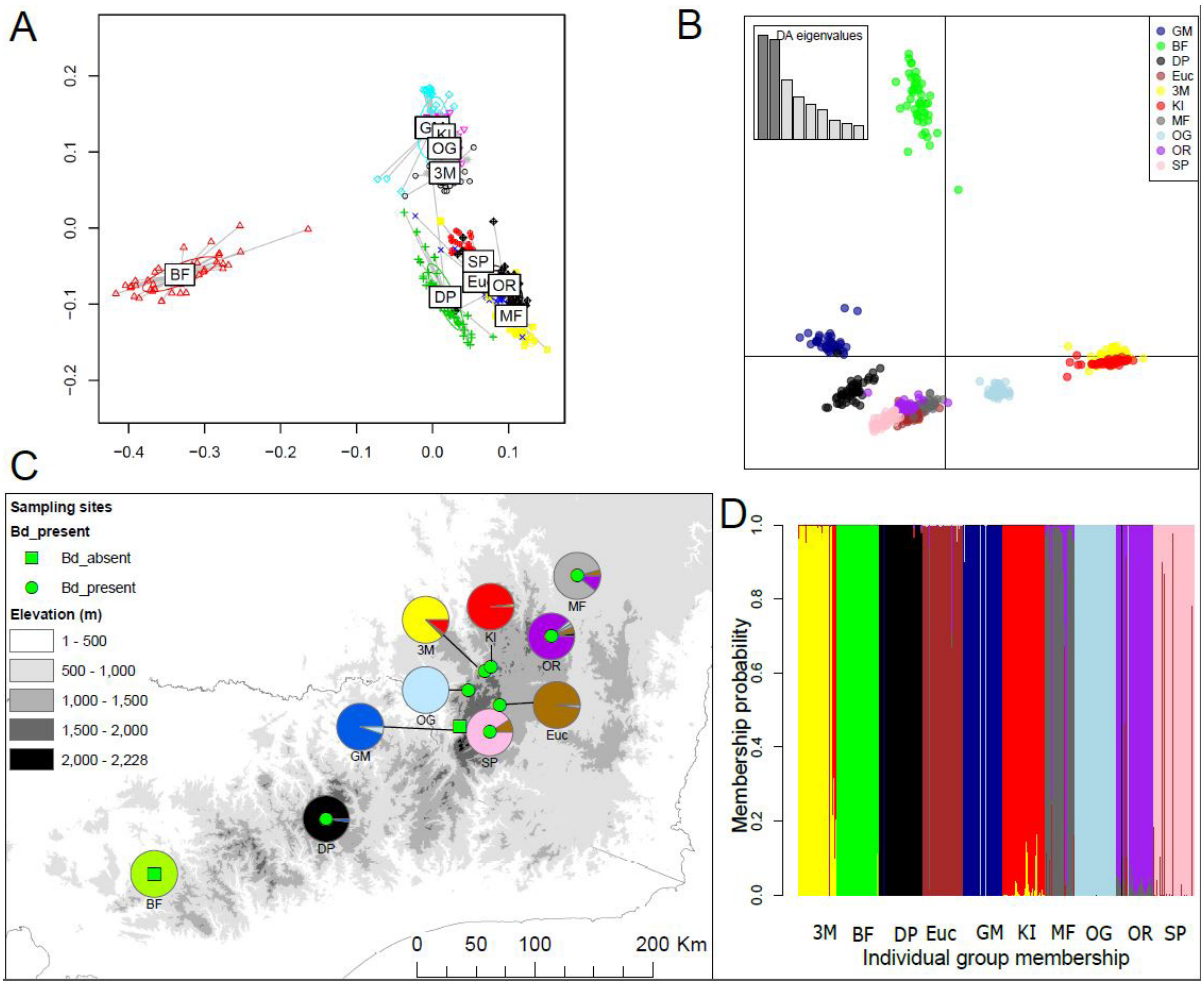

Figure 3. Principal coordinates analysis from the *betadisper* analysis of within-group variances (A) and discriminant analysis of principal components (DAPC) based on genetic distances among individual *Litoria verreauxii alpina* and an assumption of 10 genetic clusters corresponding to the 10 sampling locations. Panel B shows the DAPC scatterplot and panels C and D show the estimated group membership coefficients per population (n = 10) and individual (n = 425), respectively.

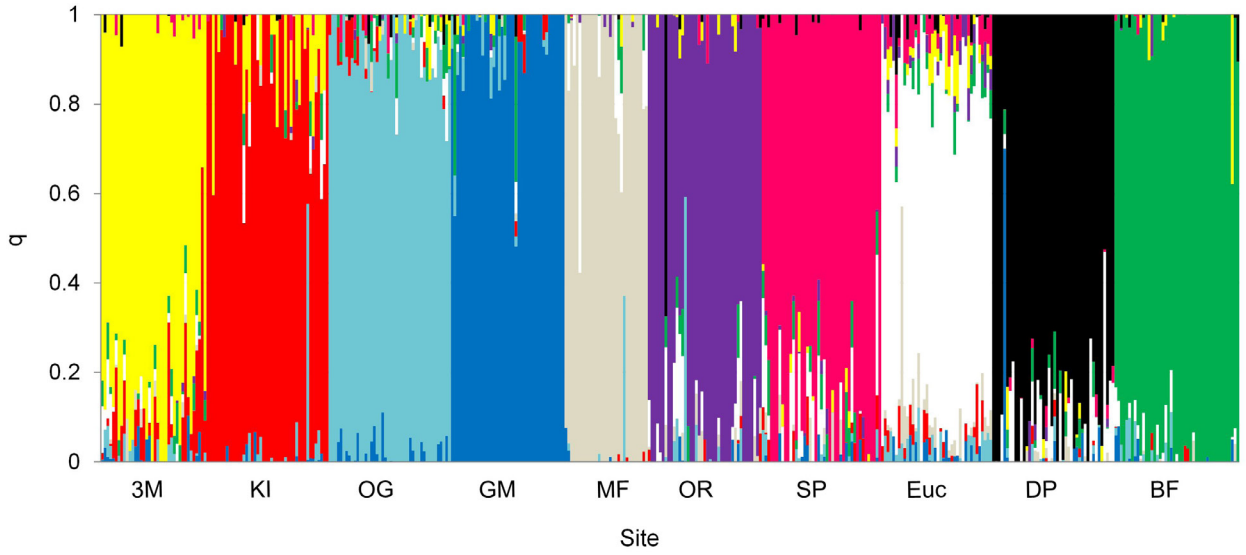

Figure 4. Results of an analysis of genetic admixture among 425 *Litoria verreauxii* from NGSadmix (Skotte et al. 2013) using a panel of 10,071 loci and assuming a model of 10 distinct populations. The vertical lines correspond to individuals with colour coding representing the estimated ancestry proportion in each population.

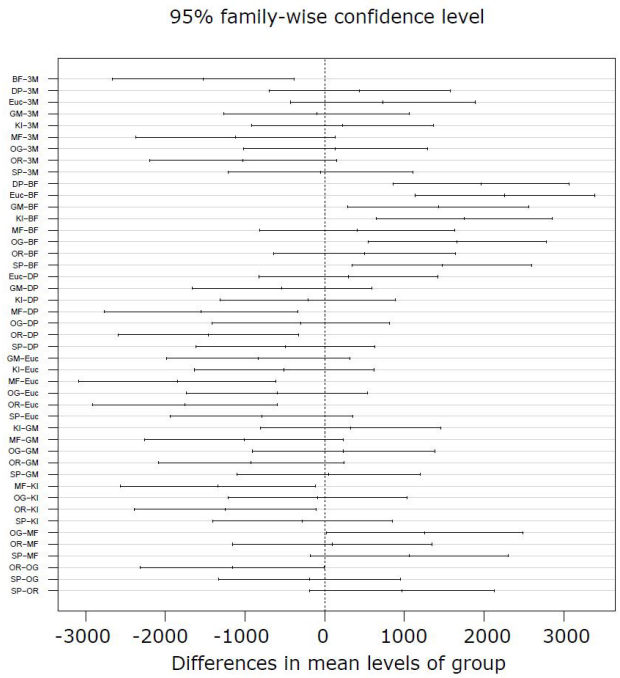

Figure 5. Pairwise Tukey HSD comparisons between *Litoria verreauxii* populations of distance-based genetic diversity estimates from a *betadisper* analysis in the vegan R package (Oksanen et al. 2016).

Discussion

Our findings that levels of genetic diversity in isolated remnant Bd-exposed populations can remain comparable to Bd-naïve populations in some species, and that individual heterozyogsity is associated with infection likelihood, have important implications for conservation. Furthermore, our genetic data provide no support for the *L. v. alpina* and *L. v. verreauxii* subspecies distinction that is based on skin colouration and morphology (Smith et al. 2003).

Genetic diversity of Bd-exposed versus unexposed populations

That Bd-exposed populations of *L. v. alpina* showed no difference in genetic diversity relative to unexposed populations could have two alternative explanations. First, despite high ongoing disease impact in remnant populations (Scheele et al. 2016), effective population size may have remained sufficient to avoid loss of genetic diversity through genetic drift. This may occur because after juvenile dispersal from natal ponds, the species is largely terrestrial and unlikely to be exposed to Bd outside the breeding season (Scheele et al. 2015). Because *L. v. alpina* typically take two to three years to reach sexual maturity (Scheele et al. 2015), a large proportion of the population does not return to waterbodies to breed in any given year, thus functioning as a 'reservoir' of genetic diversity unaffected by Bd. Further, because Bd infection is initially low at the start of the breeding season, a large number of individuals are able to breed successfully before succumbing to infection, thus avoiding a loss of genetic diversity from each generation. Similarly, evidence across multiple species demonstrates that population size can remain large in Bd-impacted species through compensatory recruitment offsetting low adult survival (Muths et al. 2011, Phillott et al. 2013), and this factor may also contribute to *L. v. alpina* persistence (Scheele et al. 2015). Our data indicate little movement between *L. v. alpina* populations, except for the closest neighbouring populations in our sample set (Kiandra and Three Mile Dam; Figure 3). Thus, we do not expect strong genetic 'rescue effects' whereby diversity is increased following a major bottleneck due to immigration.

The second potential explanation for our results is that our Bd-unexposed sites were too few to provide a representative group for comparison, but they were all that were available. Very few studies have compared genetic diversity in long Bd-exposed and unexposed populations of an amphibian species that has experienced major declines associated with Bd (Savage et al. 2015), perhaps as suitable cases where some populations remain naïve to infection are rare.

Our study capitalised on a unique opportunity for contrasting such populations, but we acknowledge that the number of unexposed sites is very low (only two such populations are known for this species), and the environmental factors that have kept them isolated from Bd exposure (low connectivity) may also influence levels of genetic diversity in those populations, as in a previous study of corroboree frogs (*Pseudophryne corroboree, Pseudophryne pengilleyi*) and boreal toads (*Anaxyrus boreas*) (Addis et al. 2015, Morgan et al. 2008). Indeed, the two populations where Bd was absent at the time of sampling were the only populations with no evidence of any admixture with other sampled populations (Figure 3). Similar associations between population isolation, disease occurrence, and genetic diversity have been documented in other species (Blanchong et al. 2008, Cullingham et al. 2009). Further evaluation of the above-mentioned explanations could be possible through DNA sequencing of preserved *L. v. verreauxii* museum specimens collected from across our study region prior to the emergence of Bd. However, extraction of DNA from amphibian specimens stored in formalin remains a challenge.

Heterozygosity of individuals is associated with Bd infection status

We found that, after accounting for variability in infection rates among populations in our statistical models, individuals with greater heterozygosity (as estimated from SNP loci across the genome) were less likely to be infected with Bd, although there was no association between genetic diversity and Bd infection intensity, consistent with other research (Savage et al. 2015). Low-depth genotyping by sequencing data present some challenges for heterozygosity-fitness analyses relating to confidence in heterozygote and homozygote calls. We therefore interpret our results with some caution as the findings were sensitive to the trade-off between confidence in genotype calls (sequence depth per SNP) and the number of genotypes called per individual. The direction of the estimated

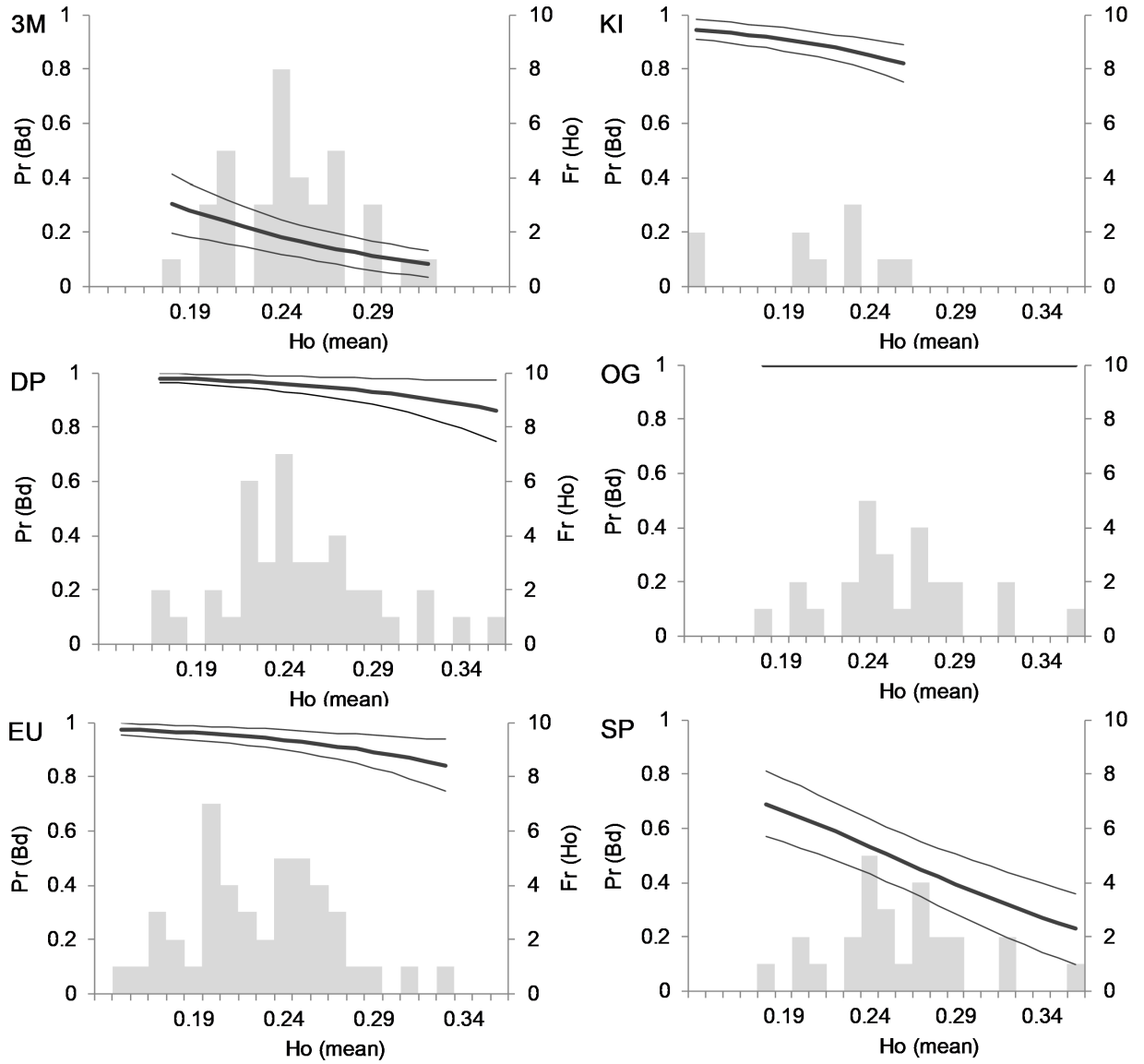

Figure 6. Predicted probability of individual *Litoria verreauxii* Bd infection status in relation to population and mean observed heterozygosity (SNP loci with minimum sequencing read depth of 6). Predictions error bars represent standard deviations, and bar plots show the observed distribution of H_O values among frogs sampled at each site. Model coefficients for the population effects were 4.7 ± 1.2 s.e. (DP), 4.2 ± 0.9 (EU), 3.2 ± 0.6 (KI), 18.6 ± >1000 (OG), and 1.6 ± 0.6 (SP), with 3M as the reference population (intercept = 1.2 ± 1.4) and H_O coefficient of -11.2 ± 5.6. Note that the H_O coefficient is identical for each site, but that the shape of the prediction curve on the linear response scale differs due to the population effect.

effect of individual heterozygosity on Bd status (infected vs uninfected) was consistent among all models but statistically significant only in the best-supported model using a depth threshold of six. Under-calling heterozygotes is a risk with this threshold, but we do not expect that to have occurred in a biased manner with respect to the response variable.

Positive associations between disease resistance and individual heterozygosity have been reported in many taxa (Acevedo-Whitehouse et al. 2003, Coltman et al. 1999), although this pattern has not been consistent in Bd-infected frog species. Genetic diversity at the population level was associated with mortality and Bd infection prevalence in lowland leopard frogs (*Lithobates yavapaiensis*) (Savage et al. 2015). No relationship was detected between individual genetic diversity and Bd infection in European tree frogs (*Hyla arborea*), but greater susceptibility to indirect parasite-mediated consequences of Bd exposure was documented in genetically depauperate tadpoles (Luquet et al. 2012). In yellow-bellied toads (*Bombina variegata*), no correlation between individual heterozygosity and Bd infection was observed (Wagner et al. 2017). The opposite pattern to our finding was observed in boreal toads (*Bufo boreas*) (Addis et al. 2015), where more heterozygous individuals were more likely to be infected with Bd. In that study, individuals were sampled from many populations across the landscape and it is

likely that both heterozygosity and Bd infection status were influenced by the effects of landscape-mediated population connectivity on gene flow and disease spread.

In the case of *L. v. alpina*, we suggest that the association between individual heterozygosity and Bd infection status could indicate two possible processes. One explanation is that observed heterozygosity at SNP loci across the genome could be indicative of diversity at (unlinked) loci related to immune responses to Bd. Indeed, associations between genetic diversity at immune-related loci and Bd resistance have been documented. Major histocompatibility complex (MHC) heterozygosity was found to be a significant predictor of survival of Bd infection in the lowland leopard frog (*Lithobates yavapaiensis*) (Savage and Zamudio 2011). In *L. v. alpina*, specific components of MHC class II allele amino acid structure are associated with Bd resistance (Bataille et al. 2015), and transcriptomic evidence identified a more robust innate and adaptive immune gene expression response at the early subclinical stage of infection in frogs from a long-exposed population compared with frogs from an unexposed population (Grogan et al. 2018). A second explanation is that, independent of the direct immune response to Bd infection, individuals with greater genome-wide heterozygosity are better able to persist under the physiological challenges imposed by Bd infection. Indeed, it has been argued that heterozygote advantage is expected to occur at a large proportion of loci with adaptive mutations (Sellis et al. 2011), and heterozygosity-fitness correlations have been noted for non-immune loci in many species (Kardos et al. 2015, Lie et al. 2009, Slate et al. 2004).

Consistent with the transcriptomics results of Grogan et al. (2018), our results indicate that genetic factors may inhibit or slow initial infection development, but that ultimately immune defences are overcome and Bd-exposed individuals have a very high rate of mortality (Bataille et al. 2015, Scheele et al. 2016). Individuals sampled in Bd-infected populations – of which 26% were uninfected in the relevant dataset – should have equal likelihood of exposure to infectious Bd zoospores in breeding habitat. However, despite the correlation between heterozygosity and infection, annual mortality is near complete in Bd-infected *L. v. alpina* populations, in contrast to uninfected populations where adults commonly survive across multiple years (Scheele et al. 2016).

Given that *L. v. alpina* is now effectively a semelparous species with near complete adult mortality during the breeding season (Scheele et al. 2016), a reduced infection rate of more heterozygous individuals might confer some, but limited, fitness benefits. Potentially, greater longevity of heterozygous males during the breeding season increases their ability to mate with multiple females. Thus, 'resistance' is not associated with long-term survival following exposure to Bd but enhances short-term survival by enabling increased reproductive output during a single breeding season. In other circumstances, particularly where infection rates are lower than in *L. v. alpina* (where high frog density and breeding activity create ideal conditions for aquatic reinfection and transmission), selection for increased resistance contributing to long-term survival may be greater.

Spatial patterns of genetic structure

Our genetic data do not support the classification of *L. verreauxii* into the two currently recognised subspecies, *L. v. verreauxii* and *L. v. alpina*. These results are concordant with previous research which found different calling patterns in the two recognised subspecies, but a gradient of call structure at sites near the boundaries of the recognised subspecies, suggesting some level of gene flow (Smith et al. 2003). With only two populations of *L. v. verreauxii* analysed within this study, however, further research is needed to assess the validity of the subspecies' status. Our data do, however, provide some insights into the processes influencing biogeographic structure in these tree frogs. We identified a high degree of genetic population structure, indicating little gene flow among all but two immediately neighbouring populations (Figure 2). The spatial pattern of genetic structure largely followed a scenario of isolation by distance. Given the recent local extinction of many populations due to chytridiomycosis, and thus increased population fragmentation, we expect that this structure will continue to strengthen with reduced gene flow. Chytridiomycosis therefore impacts biogeographic structure of surviving taxa in diverse ways, including range contraction and population isolation.

Conclusions

Concern has been raised about the genetic viability of highly isolated remnant populations following Bd declines in amphibians (Allentoft and O'Brien 2010, Morgan et al. 2008). We found, however, that genetic diversity was not lower in Bd exposed populations relative to unexposed *L. v. alpina* populations. Importantly, within Bd-exposed populations, individuals with greater heterozygosity were less likely to be infected with Bd. Our results provide a positive genetic outlook for species that persist in remnant populations with endemic Bd infection but suggest that maintenance of genetic diversity in those populations will be important for persistence in the presence of Bd.

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Supplementary Materials

The following materials are available as part of the online article from https://escholarship.org/uc/fb

Table S1. Sample information including data accessibility from Sequence Read Archive.

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