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Presence of *Batrachochytrium dendrobatidis* in anurans from the Andes highlands of northern Chile

Rigoberto Solís¹, Mario Penna², Ignacio De la Riva³, Matthew C. Fisher⁴ & Jaime Bosch³

¹Facultad de Ciencias Veterinarias y Pecuarias, Universidad de Chile, Casilla 2 Correo 15, La Granja, Santiago, Chile

²Facultad de Medicina, Universidad de Chile, Casilla 2 Correo 15, Santiago, Chile

³Museo Nacional de Ciencias Naturales, CSIC, José Gutiérrez Abascal 2, 28006 Madrid, Spain

⁴Department of Infectious Disease Epidemiology, Imperial College London W2 1PG, UK

The chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) is a causal agent of infectious disease and decline of anuran populations inhabiting mountain systems in Central and South America. The chytrid is believed to have spread from Ecuador southward, as has recently been detected in the Andean cordilleras of Peru, Bolivia and Argentina. However, since the status of anuran populations from the Chilean Altiplano is unknown, we undertook an intensive survey of amphibian populations inhabiting high elevations in northern Chile. *Bd*-infected individuals were detected only in the northernmost localities sampled suggesting an ongoing process of *Bd* spread southward along the Andes.

Key words: Andes, anuran, *Batrachochytrium dendrobatidis*, Chile, highland, prevalence

The chytrid *Batrachochytrium dendrobatidis* (*Bd*) is a skin-infecting fungus that causes chytridiomycosis in amphibians and is considered a proximate driver of declines of this class of vertebrates in all continents where they are found (Fisher et al., 2012). Amphibian declines have principally occurred at high altitudes in relatively undisturbed natural areas and for a broad range of taxa throughout the world in both temperate and tropical regions (Hero et al., 2005). In South America, the highest number of enigmatic amphibian declines (i.e. declines with an unknown cause) are known particularly among stream-dwelling species in remote highlands in the Andes (Young et al., 2001; Stuart et al., 2004). The first report of chytridiomycosis from this part of the world came from Ecuador, where specimens of five species collected in the Andes (altitude range 3100–4000 m) were found to be infected (Ron & Merino, 2000). Since then, research has implicated *Bd* as the proximate cause of amphibian declines at high elevation sites (above 2000 m) in the Andes of Venezuela (Sánchez et al., 2008), Colombia (Ruiz & Rueda-Almonacid, 2008), Peru (Catenazzi et al., 2011), Bolivia (Barrionuevo et al., 2008) and Argentina (Barrionuevo & Mangione, 2006), and it

has been hypothesised that the chytrid is spreading as an epidemic wave along the Andean cordilleras (Catenazzi et al., 2011).

In Chile, *Bd* has been detected in three native lowland species of southern populations of *Rhinoderma darwinii*, *Batrachyla leptopus* and *Pleurodema thaul* (Bourke et al., 2010; 2011), and in feral populations of the introduced African clawed frog *Xenopus laevis* (Solís et al., 2010) from the central zone of the country (Olson et al., 2013). More recently, eight other species infected with *Bd* were reported in south-central Chile and one individual of *R. darwinii* was found dead with chytridiomycosis (Soto-Azat et al., 2013). However, to our knowledge no live specimens showing clinical signs of chytridiomycosis have been reported up to date.

Knowledge of the anuran species inhabiting upland areas of northern Chile is scarce (Veloso & Navarro, 1988; Formas et al., 2005; Ortíz & Díaz-Páez, 2006). Most species belong to the telmatobid genus *Telmatobius* (*T. peruvianus*, *T. marmoratus*, *T. zapahuirensis*, *T. pefauri*, *T. fronteriensis*, *T. halli*, *T. philippii*, *T. dankoi*, *T. vilamensis* and *T. chusmisensis*). Eight species of this genus are endemic to Chile and seven are known only from their type locality (Correa et al., 2011), being limited to small bodies of water within desert habitats (Benavides et al., 2002). In addition, two other frog species occur at high altitudes in these areas, *Rhinella spinulosa* and *Pleurodema marmoratum*, both of which have an extensive latitudinal distribution throughout parts of the Andes. In Chile, *P. marmoratum* is present in the northern region only, in an altitudinal range of 3200–5400 m, while *R. spinulosa* occurs from latitude 18°S to 38°S, and from almost sea level in Azapa to 4600 m in Chungará in the Altiplano in the north of the country (Correa et al., 2010).

Reports of declines of highland anuran populations in neighbouring countries include *P. marmoratum* and *T. marmoratus* and have been attributed to chytridiomycosis (Barrionuevo & Mangione, 2006; Seimon et al., 2006; De la Riva & Lavilla, 2008; Catenazzi et al., 2011). However, the disease status of these

Correspondence: Rigoberto Solís (rsolis@uchile.cl)

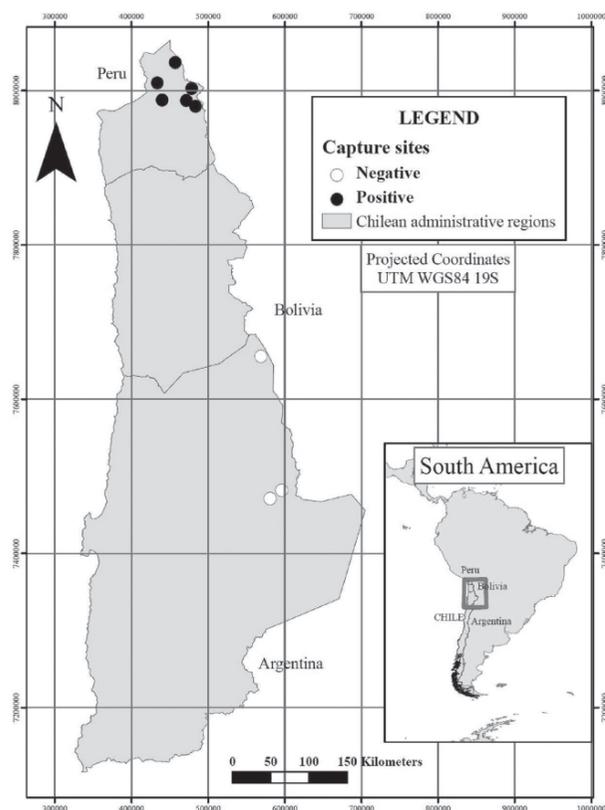


Fig. 1. Map of northern Chile showing the location of anuran populations sampled for *Batrachochytrium dendrobatidis* (*Bd*). Black circles and white squares represent populations in which individuals that tested positive and negative for *Bd* were found, respectively.

species and the endemic *Telmatobius* that occur in the Chilean Altiplano is unknown (De la Riva & Lavilla, 2008; IUCN, 2012). Eight out ten of the Chilean *Telmatobius* species are endemic and confined to permanent water bodies, streams and high altitude peatlands (bofedales) where larval stages often spend extended periods of time, a condition which has been reported to facilitate the presence and permanence of *Bd* in highland environments (Catenazzi et al., 2013). Here we report the results of a *Bd* survey in the Andes of northern Chile to obtain a better understanding of the distribution and spread of this pathogen in South America.

During February 2005 and 2007 populations of highland Andean anurans were sampled opportunistically, covering recognised type localities for taxa occurring in two northern administrative regions of Chile (Formas et al., 2005; Veloso, 2006): Arica and Parinacota (XV Region, 17°30' to 19°14' S latitude, and W longitude from 68°50' to the Pacific Ocean; see Fig. 1) and Antofagasta (II Region, 20°56' to 26°05' S latitude and W longitude from 67°00' to the Pacific Ocean), at elevations between 2400 and 4600 m. Tadpoles and aquatic adult frogs of *Telmatobius* were searched for visually and manually under rocks in the streams and by rummaging along the edges of streams and banks of wetlands and pools. The terrestrial frogs *R. spinulosa* and *P. marmoratum* were detected under stones near water bodies, and captured by hand or using nets. At each capture site, water or substrate temperature was measured ($\pm 0.1^\circ\text{C}$). Using

disposable gloves, 2–5 mm of toe was clipped from each adult individual and preserved in a 2 ml sterile tube filled with 70% ethanol for molecular analysis. After each sample was collected, instruments were cleaned with a 99% ethanol-soaked tissue and the blades were held over an open flame to destroy any remaining DNA from the previous sample.

All tissue samples were subsequently screened using a quantitative real-time polymerase chain reaction protocol (qPCR, Boyle et al., 2004). DNA was extracted from toe clips using a bead-beating protocol as outlined in Boyle et al. (2004). Extractions were diluted 1/10 in dH_2O before being used, in duplicate, in real time qPCR. For the purpose of quantification and to assess the intensity of infection a standard curve using *Bd* genomic equivalent (GE) of 100, 10, 1 and 0.1 was used. If only one of the duplicates generated an amplification profile, the sample was provisionally scored as positive. If comparison of the amplification profiles to the standard curve generated by the GE standards yielded an average GE estimation of less than 0.1 and/or standard errors greater than the estimate itself, the sample was scored as negative. All samples generating average GE estimates of 0.1 GE or higher (with standard errors less than the average score) were scored as positive. The proportion of animals infected with *Bd* in each population was calculated by dividing the number of positive cases by the total number of frogs sampled, and its respective 95% confidence interval was calculated ($\pm 1.96 \cdot \text{SE}$).

The sample consisted of three larvae in stages <42, 42–46, and >46 (Gosner, 1960), 21 post-metamorphics, 42 juveniles and 67 adult individuals. None of the individuals sampled showed visible signs of chytridiomycosis. In total 133 animals were sampled, with an overall proportion of infected individuals across all the sites surveyed of 0.18 (± 0.06). Positive cases were detected in all sites where animals were captured in the most northerly region, Arica and Parinacota (see Table 1), with a proportion of infection ranging between 0.05 and 0.50 detected for individuals of *P. marmoratum* in Lake Chungará and *T. peruvianus* in Putre, respectively (Table 1). The highest value of intensity of infection (243.37 *Bd* GE) was measured in a metamorphic stage of *T. marmoratus* captured in Caquena and the lowest in a juvenile of the same species and an adult of *R. spinulosa* (both with 0.01 *Bd* GE) in Quebrada de Allanes.

At the species level, *T. peruvianus* showed the highest prevalence (50%, at however only two samples) followed by *T. marmoratus* (35%) and *R. spinulosa* (11%); *P. marmoratum* showed the lowest level of infection (5%). No differences were detected in level of infection associated with ontogenic stage in comparisons between the proportions of infected adult frogs and sub-adult stages (0.47/0.53 for *T. marmoratus* and 0.5/0.5 for *R. spinulosa*). In the region of Antofagasta (Latitude >21° S), all frogs of the two species sampled (*T. philippi*, $n=1$ and *R. spinulosa*, $n=30$) were negative for the *Bd* diagnostic assay. Neither altitude nor water temperature showed a significant relationship with variations in the proportion of infected individuals ($r_s=0.378$, $p=0.402$ and $r_s=-0.285$, $p=0.534$, respectively, Spearman's rank correlation

Table 1. Localities and species tested for *Batrachochytrium dendrobatidis* in highlands of the Andes of northern Chile. (1) LHS: Life History Stage, A: adult, J: juvenile, M: metamorph, L: larvae; (2) IA: infected animals; (3) Expressed as *Bd* genomic equivalents found on infected animals (mean, min and max). *: temperature measured on the substrate (below stones) alongside streams

Locality	Lat. (°S)	Long. (°W)	Elev.	°t _{water} at capture	Species	n	LHS (1)	IA (2)	Proportion infected (± 1.96 SE)	Intensity of infection (3)	
Cosapilla	17.749	69.408	4400	10.2–11.0	<i>R. spinulosa</i>	1	A	0	0.40±0.30	22.32 (1.67–66.36)	
						9	M	4			
				10.2–11.0	<i>T. marmoratus</i>	11	A	5	0.46±0.27	1.67 (0.33–2.37)	
						1	M	1			
Quebrada de Allanes	17.988	69.628	3250	17.7–26.5	<i>R. spinulosa</i>	2	A	1	0.33±0.53	0.01	
						1	M	0			
				17.7–27.4	<i>T. marmoratus</i>	10	A	1	0.18±0.22	0.42	
						1	J	1			
Caquena	18.055	69.206	4409	18.9–19.3	<i>T. marmoratus</i>	6	A	1	0.38±0.26	0.12 (0.09–0.15)	
						1	J	1			
						4	M	2			195.69 (148.02–243.37)
						2	L	1			
Putre	18.188	69.568	3424	12.6–14.7	<i>R. spinulosa</i>	12	A	3	0.20±0.20	1.85 (0.17–2.39)	
						2	J	0			
				(15.3–15.7)*	<i>T. peruvianus</i>	2	J	1	0.50±0.69	0.63	
						1	M	0			
Parinacota	18.196	69.268	4392	12.6	<i>T. marmoratus</i>	1	J	0	0.33±0.53	0.35	
						2	M	1			
						2	A	1			
Lago Chungará	18.264	69.156	4589	(8.6–10.9)*	<i>P. marmorata</i>	12	A	1	0.05±0.10	0.04	
						4	J	0			
				5.9–11.8	<i>R. spinulosa</i>	12	A	0	0		
						1	J	0			
						1	M	0			
Amincha	21.191	68.339	3865	18.5–21.5	<i>T. philippii</i>	1	A	0	0		
Río Vilama(A)	22.757	68.070	3211	17.7–24.9	<i>R. spinulosa</i>	8	J	0	0		
Río Vilama (B)	22.854	68.217	2485	15.6–20.1	<i>R. spinulosa</i>	22	J	0	0		

coefficient). A similar absence of correlation occurred when these variables were analysed at the species level.

Our results extend the known range of *Bd* and its potential to develop chytridiomycosis southward along the western slopes of the high Andes of South America. In addition, the occurrence of infected individuals in all populations sampled in the most northern sites indicates that the rate of spread southward from Peru might be faster than previously thought (Catenazzi et al., 2011) and the appearance of *Bd* at the more southerly sites which were negative in this survey is likely. We add two new *Bd*-infected species to the previously reported from high elevations in the Altiplano (*T. peruvianus* and *R. spinulosa*, Olson et al., 2013), which might reflect their declining status of small populations in Putre (IUCN, 2012). The highest prevalences occurred in individuals of *R. spinulosa* and *T. marmoratus* captured above 4400 m, but the highest intensities of infection were measured in samples from metamorphic individuals of the latter species collected in Caquena. In this locality water temperature ranged between 18.2 and 19.3°C, values which are within the optimal range for growth of *Bd*. (Piotrowski et al., 2004).

Among the species studied, *R. spinulosa* has an extensive latitudinal and altitudinal distribution, ranging to central Chile and also elsewhere in the Andean slopes of Peru, Bolivia and Argentina. This distributional pattern makes this species a likely *Bd* vector, potentially putting at risk anuran species that inhabit the southern part of the range of *R. spinulosa*. Moreover, it has been reported that this toad shows noticeable phenotypic plasticity to environmental temperature conditions (Méndez & Correa-Solís, 2009), which may facilitate *Bd* infection and a role as a reservoir host (Catenazzi et al., 2013; Sapsford et al., 2013).

Species of the genus *Telmatobius* may be especially vulnerable to chytridiomycosis due to their aquatic habits and the year-long presence of infected tadpoles (Catenazzi et al., 2013). Nevertheless, in Bolivia it appears that species of *Telmatobius* from the dry puna and Altiplano habitats can persist even under high *Bd* infection loads, while *Telmatobius* species from the humid forests of the eastern sectors of the Andes suffer severe declines or even extinctions (De la Riva & Burrowes, 2011). Mapping the global distribution of *Bd* has shown that detected *Bd* infections are related to amphibian biodiversity

and local environmental variables (Olson et al., 2013). High humidity and reduced temperature variation may increase zoospore survival, forcing the epidemiology of chytridiomycosis in the eastern Andes (De la Riva & Burrowes, 2011). Therefore, as all species of Chilean *Telmatobius* inhabit dry environments, it may be that although they affected by *Bd*, severe chytridiomycosis is unlikely unless local conditions change.

The introduction and establishment of feral infected populations of *Xenopus laevis* may potentially have vectored the chytrid fungus into the central zone of Chile in the 1970s (Solís et al., 2010). The source of infection for species inhabiting high elevations in the northern part of the country may be related to the epidemic wave which is hypothesised to have spread southwards along the Andean cordilleras from Ecuador (Catenazzi et al., 2011), where *Bd* was probably introduced in the 1970s to 1980s (Lips et al., 2008), however the accuracy of this temporal and geographical sequence has not been proven. Therefore, the appearance of *Bd* in Bolivia and Argentina predates that of Peru, making the north-south wave scenario more complicated than previously thought (De la Riva & Burrowes, 2011). Moreover, *Bd*-infected species were recently found in lowlands of central-south of Chile (Soto-Azat et al., 2013) and in environments located at southern latitudes in temperate wetlands of the Argentinian Patagonia (Ghirardi et al., 2014). Thus the source of Chilean *Bd* might be multiple, a hypothesis that can be tested when different strains of the fungus are isolated and analysed using contemporary genomic technologies and molecular epidemiology (Farrer et al., 2011).

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