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ORIGINAL PAPER

Presence of *Batrachochytrium dendrobatidis* in feral populations of *Xenopus laevis* in Chile

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Abstract *Batrachochytrium dendrobatidis* (*Bd*) is a causal agent of disease and population decline of amphibian populations, and the extinction of several anuran species worldwide. Diverse hypotheses have been provided for the emergence of this fungus in different continents, ranging from global climate change to the vectoring of *Bd* via the international trade in amphibian species. In order to address these hypotheses, it is important to assess the current distribution of *Bd* in the context of introduced non-native amphibian species. We sampled several populations of the African clawed frog *Xenopus laevis* across its distribution in Chile in order to detect the presence of *B. dendrobatidis* and evaluate the role of this frog as a potential vector. In three of ten sites sampled, individuals harbored *B. dendrobatidis* infection, with an overall prevalence of infection across the studied populations of 24% (14 positive out of 58 analyzed specimens). The rapid spread exhibited by this frog within Chile suggests that transpecific transmission of the pathogen is possible, perhaps jeopardizing native species. This finding indicates the urgent need to establish long-term monitoring population programs in order to allow early detection disease-driven changes in the sizes of native populations, allowing the prompt application of conservation practices.

Keywords Amphibian · *Batrachochytrium dendrobatidis* · Chile · Feral populations · Vector species · *Xenopus laevis*

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Introduction

Much concern has been raised in the last few years about the worldwide decline in amphibian populations (Alford and Richards 1999; Stuart et al. 2004; Mendelson et al. 2006). Among the possible causes (Blaustein et al. 2003) the emergence of the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) has been involved in mass mortalities or the apparent extinction of numerous anuran species in different continents (e.g. Bosch et al. 2001; Daszak et al. 2003; Stuart et al. 2004; Bosch and Martinez-Solano 2006). Furthermore, recent evidence establishes a link between climate change and outbreaks of this pathogenic fungus in Europe and Central and South America (Bosch et al. 2007; Pounds et al. 2006).

Given the impact of this pathogen on amphibian populations worldwide it has been considered "the worst infectious disease ever recorded among vertebrates in terms of the number of species impacted, and its propensity to drive them to extinction" (ACAP 2005; cited by Fisher and Garner (2007)).

Two hypotheses have been stated regarding the origin of this emerging infectious disease. First, the "novel pathogen hypothesis" states that the disease has recently spread into new geographic areas. Secondly, the "endemic pathogen hypothesis" suggests that it has been present in the environment, but recently has increased in host range or pathogenicity (Rachowics et al. 2005). However, balance of evidence shows that both hypotheses may be contributing to the spread of *Bd*, the expression of the disease chytridiomycosis and the declines of amphibian populations worldwide (Walker et al. 2008; Fisher and Garner 2007).

The highest number of enigmatic amphibian declines (i.e., declines with an unknown cause) are known from the Neotropics, particularly among stream-dwelling species in the high Andes (Stuart et al. 2004; Ron and Merino 2000). In South America the emergence of chytridiomycosis has been linked to the international trade in amphibians and the introduction of non-native infected species into native lowland populations (<2,500 masl). For example, in Uruguay (Mazzoni et al. 2003; Laufer et al. 2007) and Venezuela, *Bd* has been detected on bullfrogs (*Rc*) introduced for farming and food (Hanselmann et al. 2004). Another introduced species which is an effective carrier of *Bd* is the African clawed frog *Xenopus laevis*, a species which has been intensely traded for research. Africa has been proposed as the origin of *Bd* and specimens of *X. laevis* from 1938 are known to have been infected (Morehouse et al. 2003; Weldon et al. 2004). Around this time, an important international trade of this species commenced because of its use as a pregnancy assay for humans (Shapiro and Zwarenstein 1934). Since then, feral populations of *X. laevis* have been established in Europe, the United States and South America (Tinsley and McCoid 1996; Measey 2001), providing a means by which *Bd* may have been introduced to native amphibian species (Fisher and Garner 2007; Weldon et al. 2004).

In Chile, the African clawed frog was introduced in the 1970s (Jaksic 1998; Lobos and Measey 2002; Lobos and Jaksic 2005) and in the 1980s the first feral populations were recognized (Veloso and Navarro 1988). Since then, these invasive frogs have spread in central Chile, today occupying artificial water bodies as well as natural ponds and streams in which they may coexist with native anurans (Lobos and Jaksic 2005). However, given the lack of formal surveillance schemes for the endemic fauna, the extent of any declines among native species is hard to quantify. Recognizing the threat posed by the presence and potential spread of *X. laevis* to endemic Chilean toads and frogs, we report here the results of a survey that identifies the proportion of *Bd*—infected animals with among feral populations of *X. laevis* in Chile.

Materials and methods

During 2005–2007 populations of *X. laevis* were opportunistically sampled from populations across their known range in central Chile, covering four of the administrative regions (IV, V, VI and Metropolitan Region; from 29°20′ to 34°45′S; see Fig. 1). Samples were obtained from lentic waters (lagoon, lake and streams) and agricultural ponds where individuals were captured using simple funnel traps (Lobos and Measey 2002). By using disposable gloves, a piece (2–5 mm) of toe was clipped from each individual and preserved in a 2 ml sterile tube filled with 70% ethanol for further molecular analysis. After each sample was collected, instruments were cleaned with a 99% ethanol-soaked tissue and the blades were then held over an open flame to destroy any remaining DNA from the previous sample.

All tissue samples were stored in 70% ethanol and subsequently screened using a quantitative real-time polymerase chain reaction protocol (qPCR, Boyle et al. 2004). Toe clips were extracted using a bead-beating protocol as outlined in Boyle et al. (2004). Extractions were diluted 1/10 in dH_2O before being used, in duplicates, in real time qPCR. For the purpose of quantification 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 estimate of less than

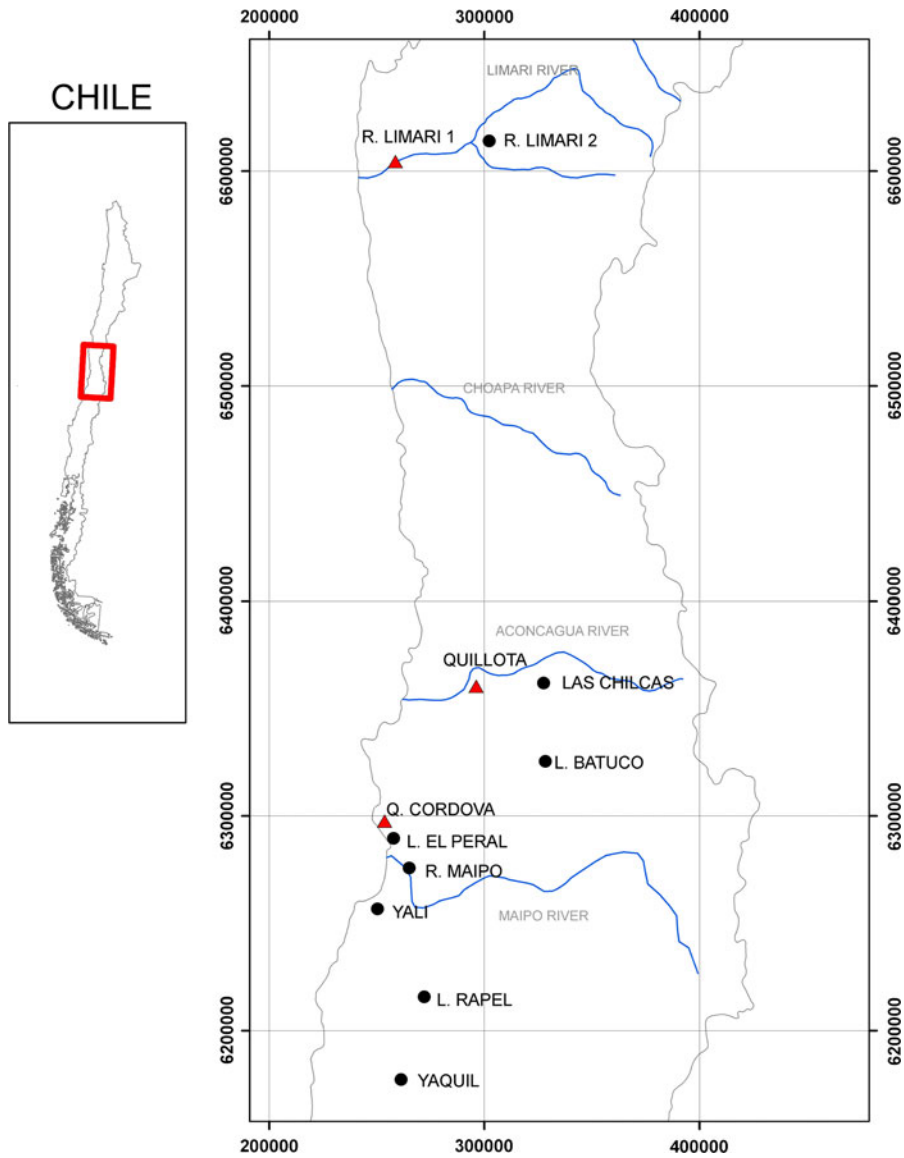

Fig. 1 Map of Central Chile showing the location of *Xenopus laevis* populations sampled for *Batrachochytrium dendrobatidis* (*Bd*). *Triangles* and *circles* represent populations in which individuals tested positive and negative for *Bd*, respectively

0.1 and/or standard errors greater than the estimate itself, the sample was scored negative. All samples generating average GE estimates of 0.1 GE or greater, and with standard errors greater than the average score, were scored as positive.

The proportion of infected animals 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 ($\pm 1.96 \times$ SE). In addition, for locations with positive cases the lowest proportion of infected animals expected to detect with 90% certainty for each sample size was calculated following DiGiacommo and Koepsell (1986). Comparison with the observed values were made using χ^2 tests.

Results

All individuals sampled were adult *X. laevis* with no clinical signs of chytridiomycosis. Despite a similar capture effort the sample size ranged from one (in the localities of El Peral lagoon and Rapel lake) to ten (Table 1). In total, 58 animals were sampled, with an overall proportion of infected animals across the populations of 24% ($\pm$15.9%). Positive cases were

detected in three of the ten locations sampled with the proportion of infected animals ranging between 80, 77, and 75% in Limarí River, Quillota and Quebrada de Córdova, respectively (Table 1). These values did not differ from the lowest proportion of infected animals expected calculated for the corresponding sample size ($\chi^2 = 2.047$, $df = 2$, $P = 0.359$).

Discussion

To our knowledge, chytridiomycosis has not previously been reported in Chile. However, our results give us reliable proof of the presence of *Bd* in this country and provide us with a possible mechanism for its introduction and dissemination in central Chile. *Xenopus laevis*, or other introduced amphibian species, have been indicated as possible carriers of this fungus to other countries (Mazzoni et al. 2003; Daszak et al. 2004; Garner et al. 2006) and specifically to Chile (Lips et al. 2008). Since its popularity as a pregnancy assay for humans (Shapiro and Zwarenstein 1934), and more recently as a model for scientific research, large numbers of African clawed frogs have been captured in the wild in southern Africa (the country that is postulated as the origin of this chytrid) and exported around the world (Weldon et al. 2004). The establishment of feral infected populations of *X. laevis* could potentially have vectored the chytrid into importing countries, thus disseminating the infection into their native amphibian species.

The overall proportion of infected *X. laevis* reported in our study (24.1%, 14 positive out of 58 specimens) is higher than the value of 2.6% reported for the archived individuals of *X. laevis* from southern Africa (15 positive out of 583 specimens; Weldon et al. 2004), but similar to the prevalence among *Xenopus wittei* (25%) and within the range of prevalences recently recorded for *Xenopus* spp. collected from South Africa (Goldberg et al. 2007).

The heterogeneous distribution of *Bd* among populations of *X. laevis* in Chile may be attributed to a range of factors including: the infection status of the population from which the amphibian samples were derived, the ecological connectivity of the site, the habitat (permanent/temporary water source, stream/pond/artificial) and the prevailing bioclimatic conditions. Notably, the highest proportion of infected animals recorded (80%) was found in the Limarí river (1), the northern most population sampled. This population is relatively isolated and has limited connectivity with other sites watercourses, being thought to have been established by the anthropogenic translocation of *X. laevis* from central Chile, wherein natural dispersal in both northerly and southerly

Table 1 Proportion of infected animals with *Batrachochytrium dendrobatidis* in populations of the African clawed frog *Xenopus laevis* in Chile

Locality	Latitude (°S)	Longitude (°W)	*n*	Proportion of infected animals (±1.96 SE)	Percent-infected expected[a]	Intensity of infection[b]	Habitat type[c]	Date
Río Limarí (1)	30.667	71.516	5	80 ± 35	37	2.6 (0.1–9.2)	PS	December
Río Limarí (2)	30.730	71.678	4	0	–	–	PS	November
Las Chilcas	32.868	70.843	9	0	–	–	EP	December
Quillota	32.876	71.177	9	77 ± 27	23	1.3 (0.2–2.4)	PP	September
Batuco	33.197	70.841	9	0	–	–	EP	March
Q. Córdova	33.433	71.650	4	75 ± 42	44	6.6 (0.8–10)	PS	December
Laguna El Peral	33.507	71.608	1	0	–	–	LG	May
Río Maipo	33.633	71.533	2	0	–	–	PS	December
Yali	33.801	71.697	10	0	–	–	LG	January
Rapel	34.175	71.474	1	0	–	–	L	May
Yaquil	34.563	71.481	4	0	–	–	EP	January

[a] Lowest expected proportion of infected animals to detect with 90% certainty given sample size

[b] Expressed as *Bd* genomic equivalents found on infected animals (mean, min and max)

[c] *EP* ephemeral stream, *L* lake, *LG* lagoon, *PP* permanent pond, *PS* permanent stream

directions has been more localized (Lobos and Jaksic 2005).

The apparent absence of *B. dendrobatidis* from Batuco is interesting since this site is within 30 km of Caren lagoon (Metropolitan Region), the site with the first known feral population of *X. laevis* in Chile (Jaksic 1998). Notably, the animals from Batuco were caught from an artificial pond which is emptied periodically, a regime which is likely to reduce transmission rates due to the sensitivity of *Bd* to desiccation (Berger et al. 2004). Unfortunately, no samples were obtained from Caren lagoon, even though funnel traps were installed several times during this study, and it is possible that the original population has disappeared, or that a very few animals remain.

In Chile, *X. laevis* is typically associated with lentic and artificial water bodies such as ponds, dams and irrigation canals (Lobos and Jaksic 2005) that undergo significant variations in their level and turnover of water. According to the analyses of Kriger and Hero (2007), these characteristics suggest a low habitat suitability for *Bd*. However, although the habitat preferences of *X. laevis* may limit its potential as a vector of *Bd*, any individuals which are infected may present a threat to the native amphibian fauna owing to their high dispersal ability (including an ability to disperse overland to colonize new water bodies; Lobos and Garin 2002).

To date, *X. laevis* distribution embraces four administrative regions of the country and continues to spread at a rate that may reach 4.4–5.4 km/year, inhabiting environments with water temperature and pH values (Lobos and Jaksic 2005) that are suitable for growth of *B. dendrobatidis* (Piotrowski et al. 2004; Ron 2005). However, despite almost 40 years since its introduction, no declines of native Chilean anuran species in the present range of *X. laevis* have been noted. Although this apparent paradox may be due to the fact that Chilean species are resistant to infections by *B. dendrobatidis*, it is more likely a consequence of the lack of systematic monitoring programs. The only suspected extinction of an anuran species in Chile is *Rhinoderma rufum*, a species which has not been observed since the date *X. laevis* was introduced (Young et al. 2001). However, its probable extinction may is most likely linked to habitat destruction (Veloso 2006).

Suitable environmental conditions and anthopogenic activities have facilitated the establishment of an invasive species such as *X. laevis*, which may have acted as a transport, introducing and spreading *Bd* into Chile. No other non-native amphibian species have knowingly been introduced in Chile thus far, including the American bullfrog, however, *Bd* has been detected in other areas across the country were *X. laevis* is not present and where suitable conditions for the species don't exist (Solís et al., unpublished data). Therefore, it is reasonable to think that more than one episode of introduction may have taken place in the country, or that the infection has spread outside of the original host species. In order to distinguish between these hypotheses, it would be appropriate to use molecular genotyping techniques to compare Chilean samples of *Bd* against those recently sequenced from other regions of the globe in order to identify likely sources of introduction, including those genotypes found within South Africa. This study is the first to identify the presence of *Bd* in Chile, which presents a potentially major threat to native Chilean amphibian species and indicates the urgent need to establish long-term population monitoring programs which allow the prompt application of still nonexistent conservation practices.

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