Presence of the chytrid fungus *Batrachochytrium dendrobatidis* in a Vulnerable frog in Trinidad, West Indies


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ABSTRACT: Amphibian chytridiomycosis occurs on a small proportion of West Indian islands, but the entire Caribbean region, including Trinidad, offers a suitable environment for the infection. We report the presence of the causative agent *Batrachochytrium dendrobatidis* (*Bd*) in 2 out of 12 populations sampled of the Vulnerable Trinidad stream frog, *Mannophryne trinitatis*. We analyzed 184 skin swabs collected from wild frogs using real-time PCR analysis. Follow-up sampling determined a prevalence of *Bd* infection of 3 and 23% in these 2 populations. We did not find any evidence of associated clinical disease. *Bd*-positive populations were located at the highest elevations studied (425 to 450 m). These 2 populations had more juveniles than other populations, and juveniles were more likely to be infected than adults. Our results suggest that sampling juveniles may provide the greatest sensitivity for any future monitoring for the presence of *Bd* in *M. trinitatis* populations in Trinidad.

KEY WORDS: *Mannophryne trinitatis* · Aromobatidae · Juveniles · Amphibian chytrid · Caribbean

INTRODUCTION

Chytridiomycosis, a disease caused by infection with the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), threatens many amphibian species around the world (Skerratt et al. 2007), but especially in the Caribbean region (Wilson et al. 2006, Bielby et al. 2008, Hailey et al. 2011). The chytrid fungus completely devastated amphibian communities in the Neotropics (Lips et al. 2006), and is now reported present in all mainland countries bordering the Caribbean (Lips et al. 2003a, 2004, 2006, Mendelson et al. 2005, Lampo et al. 2006, Puschendorf et al. 2006, Ruiz & Rueda-Almonacid 2008, Chatfield et al. 2012, Kaiser & Pollinger 2012), with the exception of Nicaragua. Knowing the current distribution of this pathogen is extremely important, because the Caribbean region has suitable climatic conditions for the occurrence of *Bd* (Ron 2005), and has an exceptionally high level of amphibian endemism (Hedges 2006). *Bd* is thought to be originally endemic to Africa, from where it appears to have spread by the international trade of clawed frogs *Xenopus* spp. (Weldon et al. 2004, Soto-Azat et al. 2010).

Reports of *Bd* on 6 Caribbean islands indicate that West Indian amphibians are highly susceptible to infection. In Dominica, Montserrat, and Puerto Rico, *Bd* is implicated in population declines of anurans including the mountain chicken frog *Leptodactylus fallax*, and several species of direct-developing frogs
In Cuba, the only report of Bd has been associated with toad mortality (Díaz et al. 2007); and in Dominican Republic and Tobago, the infection is present, but its effects on frog populations are unknown (Joglar & Burrowes 2005, Alemu et al. 2008). Here, we report the results of the first survey for Bd infection in a threatened frog species in Trinidad.

We studied the aromobatid Trinidad stream frog Mannophryne trinitatis, both as a threatened species of conservation concern in its own right, and as a suitable monitor for Bd in the amphibian fauna of Trinidad. Although M. trinitatis is still currently listed as Vulnerable by the International Union for Conservation of Nature (IUCN) (La Marca et al. 2004, Angulo 2010), this category was partly based on a broad distribution in Venezuela as well as in Trinidad. However, populations in Venezuela are now described as a separate species, M. venezuelensis. Therefore, M. trinitatis is now a single island endemic species of Trinidad, and as such is likely to be reassessed to a higher threat level (Hailey & Cazabon-Mannette 2011).

Mannophryne trinitatis exhibits several ecological risk factors for the development of chytridiomycosis, such as close association with water (Lips et al. 2003b), stream-dwelling tadpoles (Hero et al. 2005, Kriger & Hero 2007), and more frequent contact between individuals than is usual among anurans (Rowley & Alford 2007). Female M. trinitatis are territorial and attack other female conspecifics (Wells 1980), and the male transports tadpoles on his back for several days (Downie et al. 2001), potentially increasing vertical and horizontal transmission. Bd has already been found in its congener M. olmonae in Tobago, at a relatively high prevalence of 25% (Alemu et al. 2008).

We therefore tested the hypothesis that if Bd occurs in Trinidad, it is likely to be found in Mannophryne trinitatis, and the null hypothesis (for sampling design) was a prevalence of about 25% as in M. olmonae in Tobago. The sampling design also utilized the hypotheses that Bd is most likely to be found (1) where there are indications of frog population declines; (2) at higher elevations (Gründler et al. 2012); or (3) where visiting scientists or tourists provide a possible route of entry of the pathogen onto the island.

**MATERIALS AND METHODS**

**Study sites**

Our study sites were spread across the known geographic range of Mannophryne trinitatis in the Northern Range and Central Range hills of Trinidad (Fig. 1), and covered a wide range of geographical and ecological conditions and disturbance levels. Only 4 sites with M. trinitatis are known in the Central Range (Jowers & Downie 2004). The population of one of these sites, a stream near Tamana Cave (our Site 9; see Table 1 & Fig. 1), has been studied over a long period (Kenny 1979, Cummins & Swan 1995, Downie et al. 2001); it had apparently declined in the years before 2007, but no quantitative data were available (M. Jowers pers. comm. 15 Feb 2007). The other 11 sites were in the Northern Range, where M. trinitatis is widespread, and were selected to give a range of ecological conditions and disturbance levels, as follows. Sites at the North Coast Road (Site 2), Maracas Falls (Site 3), Mount St. Benedict (Site 4), and the Arima Road below the Asa Wright Nature Centre (Site 6) were frequented by visitors, and M. trinitatis is known to have been studied in those areas previously (Downie et al. 2001, Jowers et al. 2006). Sites at Edith Falls (Site 1), Aripo River (Site 7), and Salybia Falls (Site 11) were on or close to trails to places frequented by tourists. Sites at Blanchisseuse Road (Site 5), Cumaca (Site 8), Grande Riviere...
Alemu et al.: *Batrachochytrium dendrobatidis* in Trinidad

Table 1. *Batrachochytrium dendrobatidis*. Presence in skin swab samples of *Mannophryne trinitatis* from 12 sites in Trinidad. Only Sites 5 and 7 were sampled in 2009. See Fig. 1 for map.

<table>
<thead>
<tr>
<th>Site no.</th>
<th>Site name</th>
<th>Elevation (m)</th>
<th>Pos./total samples in 2007</th>
<th>Pos./total samples in 2009</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Edith Falls</td>
<td>107</td>
<td>0/10</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>North Coast Road</td>
<td>257</td>
<td>0/12</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>Maracas Falls</td>
<td>300</td>
<td>0/10</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>Mount St. Benedict</td>
<td>247</td>
<td>0/11</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>Blanchisseuse Road</td>
<td>427</td>
<td>1/10</td>
<td>7/30</td>
</tr>
<tr>
<td>6</td>
<td>Aripa Road</td>
<td>405</td>
<td>0/10</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>Aripo River</td>
<td>447</td>
<td>1/10</td>
<td>1/30</td>
</tr>
<tr>
<td>8</td>
<td>Cumaca</td>
<td>378</td>
<td>0/10</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>Tamana Cave</td>
<td>200</td>
<td>0/10</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
<td>Grande Riviere</td>
<td>35</td>
<td>0/10</td>
<td>–</td>
</tr>
<tr>
<td>11</td>
<td>Salybia Falls</td>
<td>64</td>
<td>0/10</td>
<td>–</td>
</tr>
<tr>
<td>12</td>
<td>Mission</td>
<td>71</td>
<td>0/10</td>
<td>–</td>
</tr>
</tbody>
</table>

(Site 10), and Mission (Site 12) were difficult to access and were likely undisturbed by visitors.

Most of the sites are first-order streams with patchy stretches of flowing water among rocks, gravel, and leaf litter. Edith Falls, Maracas Falls, Aripo River, Cumaca, and Grande Riviere are second-order streams, with continuous water flow ca. 1 m wide, and Salybia Falls is possibly a third-order stream, 2 to 3 m wide. We recorded GPS coordinates at each site as precisely as possible; however, heavy forest cover prevented precise measurement at some sites. All coordinates were within 200 m of the exact site. We used GPS coordinates for mapping purposes only (using ESRI ArcMap 8.2 and ArcView GIS 3.1) and do not report them in detail.

**Bd sampling and analysis**

We sampled 10 to 12 *Mannophryne trinitatis* at each of 12 study sites in July and August 2007, during the wet season (Table 1). We aimed to provide a ca. 95% probability of detection of *Bd* for an infection prevalence of 25%, as found in *M. olmonae* in Tobago (Alemu et al. 2008). We calculated that this would be achieved by taking skin swabs from about 10 individuals in each population, since the probability of a single sample being negative is then 0.75, the probability of all 10 samples being negative is 0.75^10 = 0.056, and the probability of detecting the infection by obtaining at least 1 positive sample is 0.944 or 94.4%. Thusfield (1995, their Table 13.4) also indicates that a sample size of 10 is sufficient to give a 95% probability of detection of a prevalence of 25% in a population of 120, or a sample size of 11 is sufficient in an infinite population.

Frogs were caught by hand or using small (10 × 7 or 15 × 12 cm) aquarium dip nets. We rubbed the skin of each frog 25 times using a sterile rayon-tipped swab, as described by Alemu et al. (2008). Each frog was swabbed over its entire body, but with an emphasis on the ventral pelvic patch, which *Bd* shows a predilection for infecting (Berger et al. 2005). The rayon tip of each swab was then placed in a 2 ml microcentrifuge tube with screw cap and rubber O-ring containing 1 ml 70% ethanol. After swabbing, we measured the snout–vent length (SVL) of each frog with calipers. In order to minimize the risk of disease spread between frogs or between study sites, we wore new disposable latex gloves when handling each animal caught, and only used nets for 1 frog per day. We sterilized the nets by immersing them in a 10% solution of Clorox bleach, equivalent to 0.5% sodium hypochlorite (Johnson et al. 2003) for 3 h, then washed them in running water for 1 min, and immersed them 25 times in each of 4 changes of clean water to thoroughly remove the bleach. We analyzed for the presence of *Bd* using real-time Taqman PCR with *Bd*-specific primers, as described by Boyle et al. (2004). Each PCR run included positive and negative controls, and each sample was analyzed in duplicate on the same plate.

Follow-up sampling of *Bd*-positive sites was conducted in August 2009. We sampled 30 *Mannophryne trinitatis* at each site in order to verify the positive *Bd*-infection status of the population and to obtain further information on the prevalence of infection. We used the same capture, sampling, and swab-analysis methods as in 2007.

Statistical analysis used Minitab or GraphPad, and 95% confidence intervals for percentages were from Table 23 of Rohlf & Sokal (1981). *χ²* analysis was considered valid when the minimum expected value was ≥5; samples were pooled if necessary to meet this criterion.

**RESULTS**

Only 2 of the 123 samples from 2007 gave positive results for *Bd* using real-time PCR (Table 1). Because both wells on the PCR plate gave weakly (<1 genome equivalent) positive results for each positive sample, we repeated the analysis in duplicate to confirm the results. All other swabs were negative for *Bd* in both wells on the PCR plate. The 2 positive samples were from different watersheds (Fig. 1). Sample sizes were
increased at both these sites in 2009 compared to 2007, and further positive results were obtained from both sites (Table 1), with <1 to 98 genome equivalents being detected in these positive samples. No animals showed any evidence of clinical chytridiomycosis, such as lethargy, skin lesions, or sloughing.

The resurvey of the 2 populations in 2009 revealed *Bd* prevalences of 3.3% (95% CI: 0.1 to 17.2%) and 23.3% (95% CI: 9.9 to 42.3%) respectively. At both these sites, we found a higher proportion of juveniles (SVL < 18.5 mm) infected with *Bd* compared to adults, although sample sizes were too small to analyze separately. Overall, the proportion of juveniles positive for *Bd* infection (21.1% of 38) was marginally significantly greater than for adults (4.8% of 42), combining these 2 sites (Fisher’s exact test, p = 0.041).

There was a lower proportion of juveniles in our 2007 sample of *Mannophryne trinitatis* in Trinidad (17.1% of 123) compared to *M. olmonae* in Tobago (61.9% of 126), a highly significant difference (p < 0.001, as reported by Alemu et al. 2007). However, the population structure of *M. trinitatis* at the 2 sites where *Bd* was found showed many juveniles, and was more similar to that of *M. olmonae*. Juveniles accounted for 47.5% of 80 individuals at these 2 sites, highly significantly different from the value (18.4% of 103) at sites where *Bd* was not found (χ² = 17.7, 1 df, p < 0.001).

Tadpoles of *Mannophryne trinitatis* were observed either in water or transported on the backs of male frogs. At some sites, tadpoles were particularly abundant (Aripo River). However, only metamorphosed animals were sampled, and few individuals of other species were observed during the present study. We sampled 1 individual of the Endangered frog *Pristimantis urichi* at the Blanchisseuse Road site in 2009. This frog was negative for *Bd* infection.

**DISCUSSION**

We found *Bd* infection in the Vulnerable Trinidad stream frog *Mannophryne trinitatis* at 2 of 12 sites sampled in Trinidad, although with a lower prevalence (3 to 23%) than previously found in *M. olmonae* in Tobago (Alemu et al. 2008). The infection was associated with juveniles; juveniles had higher prevalence than adults, and populations with *Bd* had more juveniles than other populations. Several hypotheses might explain this association between population structure and presence of *Bd* infection: density-dependence (Briggs et al. 2010), greater infectivity of juveniles (Kriger et al. 2007, Longo & Burrowes 2010), or pathogen-induced change of behavior making juveniles stay near water rather than dispersing into the terrestrial habitat. Most juveniles were not infected, so the latter is a less likely explanation. Together, the greater prevalence in juveniles than adults and the association with population structure suggest that sampling juveniles would be the most efficient way of monitoring *Bd* in *M. trinitatis*, particularly in populations with many juveniles.

No *Bd* infection was detected at either Tamana Cave, the only site where a decline in a population of *Mannophryne trinitatis* has been reported, or at sites previously used for the study of *M. trinitatis*. There was thus no indication that *Bd* is currently causing population declines in *M. trinitatis*, or that *Bd* was introduced to Trinidad as a result of the study of amphibians. The 2 *Bd*-positive sites were the highest sites sampled, at elevations of 425 to 450 m above sea level (Table 1); high elevation is a risk factor for amphibian declines due to chytridiomycosis (Bielby et al. 2008). The species of amphibian of greatest conservation concern in Trinidad is the Critically Endangered golden tree frog *Phytotriades auratus* (Hailey & Cazabon-Mannette 2011), which is restricted to the area above 800 m around the 2 peaks in the Northern Range, El Tucuche (936 m, north of Site 3) and Cerro del Aripo (941 m, north of Site 7). The daily maximum temperature at the summit of Cerro del Aripo is about 18°C (Beard 1946), within the optimum range for the growth of *Bd* (17 to 25°C; Piotrowski et al. 2004), and similar to temperatures causing higher mortality in frogs in the laboratory (17°C: Andre et al. 2008, *Rana muscosa*; Bustamante et al. 2010, *Atelopus zeteki*). The golden tree frog has only been studied at Cerro del Aripo, and there is evidence that it has declined in abundance there in recent years (M. Jowers pers. comm. 15 Feb 2007, M. Moore pers. comm. 28 Mar 2008). The presence of *Bd* infection at the 2 highest-elevation sites studied here, close to one of the locations of *P. auratus*, is thus of great concern. Testing for the presence of *Bd* infection in *P. auratus* populations, and preventing spread of *Bd* to that species (if not yet present), are now major goals for amphibian conservation in Trinidad.

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LITERATURE CITED


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