1 August 2005 Disk Used

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biological Conservation xxx (2005) xxx–xxx
BIOLOGICAL CONSERVATION
www.elsevier.com/locate/biocon

Short communication

Infection of an invasive frog *Eleutherodactylus coqui* by the chytrid fungus *Batrachochytrium dendrobatidis* in Hawaii

Karen H. Beard [a,*], Eric M. O'Neill [b]

[a] *Department of Forest, Range, and Wildlife Sciences and Ecology Center, Utah State University, 5230 Old Main Hill, Logan, UT 84322-5230, USA*
[b] *Department of Biology, Utah State University, Logan, Utah 84322-5305, USA*

Received 8 May 2005

Abstract

The chytrid fungus *Batrachochytrium dendrobatidis* has contributed to declines and extinctions of amphibians worldwide. *B. dendrobatidis* is known to infect the frog *Eleutherodactylus coqui* in its native Puerto Rico. *E. coqui* was accidentally introduced into Hawaii in the late 1980s, where there are now hundreds of populations. *B. dendrobatidis* was being considered as a biological control agent for *E. coqui* because there are no native amphibians in Hawaii. Using a DNA-based assay, we tested 382 *E. coqui* from Hawaii for *B. dendrobatidis* and found that 2.4% are already infected. We found infected frogs in four of 10 study sites and on both the islands of Hawaii and Maui. This is the first report of *B. dendrobatidis* in wild populations in Hawaii. As the range of *E. coqui* expands, it may become a vector for the transmittance of *B. dendrobatidis* to geographic areas where *B. dendrobatidis* does not yet exist.

Keywords: Amphibian declines; Biocontrol; Chytridiomycosis; *Eleutherodactylus coqui*; Hawaii; Emerging diseases; Frog; Invasion; Puerto rico

1. Introduction

Nearly one-third of all amphibians are threatened with extinction (Stuart et al., 2004). Chytridiomycosis, a disease caused by the pathogenic fungus *Batrachochytrium dendrobatidis*, has been identified as a causal agent of amphibian declines in the Americas, Europe, and Australia (e.g., Bell et al., 2004; Berger et al., 1998; Bosch et al., 2001; Lips et al., 2004; Muths et al., 2003), and has been found on every continent with amphibians, except Asia (Weldon et al., 2004). *B. dendrobatidis* is a waterborne pathogen that primarily infects keratinized tissues in the epidermis of amphibians and spreads through colonization by motile, aquatic zoospores (Longcore et al., 1999). Because *B. dendrobatidis* does not survive desiccation (Johnson and Speare, 2003), amphibians are thought to be the primary means by which the disease is transported to new areas (Daszak et al., 2003; Hanselmann et al., 2004; Weldon et al., 2004).

Some invasive amphibians (e.g., *Rana catesbeiana*) are relatively resistant to chytridiomycosis, yet are efficient carriers of the pathogen (Daszak et al., 2004). The Puerto Rican terrestrial frog, *Eleutherodactylus coqui*, is a notable amphibian invader that has not been tested for *B. dendrobatidis* outside of its native range. *E. coqui* has invaded Florida and several islands in the Caribbean, and was accidentally introduced to Hawaii via nursery plants in the late 1980s (Kraus et al., 1999). Direct development and year-round breeding are thought to contribute to its rapid spread. There are now over 250 known populations on the islands of Hawaii and Maui, located mostly in lowland forests on the windward sides (from 0

* Corresponding author. Tel.: +1 435 797 8220; fax: +1 435 797 3796.
E-mail addresses: karen.beard@usu.edu (K.H. Beard), kbeard@cc.usu.edu (K.H. Beard).

0006-3207/$ - see front matter © 2005 Published by Elsevier Ltd.
doi:10.1016/j.biocon.2005.07.004

to 1100 m altitude), with new populations being reported weekly (Kraus and Campbell, 2002).

In Hawaii, *E. coqui* appears to establish populations that have greater densities than those in their native range (20,000 frogs/ha on average in Puerto Rico, Stewart and Woolbright, 1996; K. Beard, unpublished data). The invasion threatens Hawaii's unique ecological communities because *E. coqui* predates upon endemic invertebrates, which comprise the large majority of Hawaii's endemic fauna (Beard and Pitt, 2005). The invasion also threatens Hawaii's multi-million dollar floriculture and nursery industries due to quarantine restrictions and frog de-infestation measures (Kraus and Campbell, 2002). Likewise, property value and tourism are threatened because of its loud (80–90 dBA at 0.5 m) mating calls.

Numerous methods for managing *E. coqui* populations have been developed in Hawaii; yet, there has been no report of a successfully eliminated population. Biological control based on amphibian diseases is considered an attractive option because Hawaii has no native amphibians. *B. dendrobatidis* has been found to infect *E. coqui* in Puerto Rico dating back to 1978 and is thought to contribute to declines at high elevations (Burrowes et al., 2004). Thus, it has been suggested that *B. dendrobatidis* could be used to control *E. coqui* (Hawaii State Department of Agriculture, 2004). Our objective was to determine whether *B. dendrobatidis* is already present in *E. coqui* populations in Hawaii.

2. Materials and methods

E. coqui were collected from seven locations on the island of Hawaii and three locations on Maui in May and August 2004, respectively (Table 1). Locations were selected to maximize diversity in forest-type, elevation, and geological history. For one night at each location, subadult [snout-vent length (SVL) < 24 mm (Woolbright, 1985)] and/or adult frogs [SVL ⩾ 24 mm] were collected by slowly and systematically walking in a 20 × 20 m plot between 2000 and 2200 h. For each frog, SVL and perch height were recorded. Frogs were collected using standard protocols for testing for *B. dendrobatidis* infection [as outlined in O'Neill et al. (in review)] and were preserved in 70% ethanol. *E. coqui* demonstrated no overt clinical signs of chytridiomycosis when collected, such as unusual sloughing of the skin or mortality.

We tested 175 subadults and 207 adults for *B. dendrobatidis* using the DNA-based assay described by Annis et al. (2004). This assay uses species-specific primers (*B. dendrobatidis*1a and *B. dendrobatidis*2a) located within ITS1 and ITS2 to amplify the 5.8S region of nuclear rDNA. Tissue samples ranged from a whole foot (subadults) to a half toe (adults). DNA was extracted using the protocol from Schizas et al. (1997) with the following modifications: the digestion reaction contained 20–30 µl Te (10 mM Tris, 0.1 mM EDTA), and 1.0 µl Proteinase K (20 mg/ml). Samples were digested and periodically vortexed for 3 h at 55 °C. PCR protocols were the same as those described in Annis et al. (2004) including the use of Platinum® *Taq* DNA Polymerase (Invitrogen Corporation, Carlsbad, California, USA).

Positive controls were both pure *B. dendrobatidis* DNA extracted from culture (Joyce Longcore, unpublished data) and DNA extracted from *Rana muscosa* that had previously tested positive for *B. dendrobatidis* (Jessica Morgan, unpublished data). Negative controls consisted of purified water in the PCR reaction and reanalyses of DNA from animals that previously tested negative for *B. dendrobatidis*. PCR products were visualized on a standard 1.4% agarose gel. Samples that contained a band at 330 base pairs (BP) in length were presumed to be positive for *B. dendrobatidis* infection (Annis et al., 2004). Three samples resulted in a faint band at 330 BP. In these cases, samples were PCR amplified a second time using the first PCR product as the template. To create comparable negative controls in

Table 1
Number of *Eleutherodactylus coqui* examined and diagnosed with the chytrid fungus *Batrachochytrium dendrobatidis* from 10 locations in Hawaii

Location	Island	Coordinates	Elevation (m)	(No. with fungus/No. examined)	
				Subadults	Adults
Hawaiian Paradise Park	Hawaii	N19°36′W154°59′	50	1/42	–
Humane Society	Hawaii	N19°36′W155°01′	135	0/13	–
Kurtistown	Hawaii	N19°36′W154°05′	310	1/18	–
Lava Tree State Park	Hawaii	N19°29′W154°54′	180	4/26	1/75
Manuka Natural Area Reserve	Hawaii	N19°07′W155°50′	560	0/24	–
Puainako/Safeway	Hawaii	N19°42′W155°04′	45	0/4	–
Waipio Overlook	Hawaii	N20°07′W155°35′	305	0/7	–
Kihei Nursery	Maui	N20°44′W156°27′	400	–	0/51
Maliko Gulch	Maui	N20°52′W156°19′	440	2/41	0/75
Miles Makawao	Maui	N20°53′W156°19′	20	–	0/6
Total				8/175	1/207

Frogs were collected in the summer of 2004.

these cases, PCR products scored as negative in the first round were retested with a second PCR amplification. Tests were conducted blindly and 8% of each run were controls. To confirm our results, tissues (not DNA) from eight specimens that tested positive and 10 specimens that tested negative in our laboratory were analyzed by Pisces Molecular LLC (Boulder, Colorado, USA).

Statistical analyses were conducted using SAS v.9 for Windows (SAS Institute, Cary, North Carolina, USA). To determine if there was a difference in the number of subadult and adults infected, we compared the number of infected and uninfected individuals using Pearson's χ^2 exact test. To determine if there was a difference between adult and subadult perch heights, we conducted a *t*-test. A folded *F*-test suggested that group variances were unequal; thus, a Satterthwaite approximation was used. Significant differences were accepted at $p < 0.05$.

3. Results

Of the 382 individuals tested, nine showed positive bands for *B. dendrobatidis* (Table 1). No bands of other sizes were observed in any samples. All positive and negative controls were correctly scored. Sixteen of the 18 specimens that tested either positive or negative in our laboratory were confirmed by Pisces Molecular LLC (Table 2). Two samples (one positive and one negative) were scored differently by the two laboratories.

B. dendrobatidis was detected at four of the 10 study sites (Table 1). We detected *B. dendrobatidis* in populations on both the islands of Hawaii and Maui. *B. dendrobatidis* was found to infect frogs from locations ranging from 50 to 440 m in elevation (Table 1).

Subadults measured 14.50 ± 0.26 mm (SE) SVL and adults measured 30.52 ± 0.15 mm SVL. We found a greater infection rate in subadults than adults (4.6% vs. 0.5% tested positive) ($\chi^2 = 6.51$, df = 1, $p = 0.013$). Subadults perched closer to the forest floor than adults (0.45 ± 0.035 m vs. 0.87 ± 0.027 m) (df = 414, $t = 9.16$, $p < 0.0001$).

Table 2
Eleutherodactylus coqui samples that scored positive and negative for *Batrachochytrium dendrobatidis* in our laboratory compared to scoring from Pisces Molecular LLC (Boulder, Colorado, USA)

Population	Island	(No. positive/No. negative)			
		Subadults		Adults	
		Our laboratory	Pisces	Our laboratory	Pisces
Hawaiian Paradise Park	Hawaii	1/1	1/1	–	–
Kurtistown	Hawaii	1/1	1/1	–	–
Lava Tree State Park	Hawaii	4/4	4/4[a]	0/2	0/2
Maliko Gulch	Maui	2/2	2/2	–	–

[a] One of our positives was scored negative by Pisces and one of our negatives was scored positive by Pisces.

4. Discussion

We found that the chytrid fungus *B. dendrobatidis* is present in Hawaii and infects *E. coqui*. Like other notable amphibian invaders (Daszak et al., 2003; Hanselmann et al., 2004; Pessier et al., 1999; Weldon et al., 2004), *E. coqui* is now known to be a carrier of *B. dendrobatidis* in locations outside of its native range. Because *E. coqui* is unlikely to be eradicated from Hawaii (Beard and Pitt, 2005), these populations may represent a stable source of *B. dendrobatidis* in the Pacific. *E. coqui*, apparently traveling in nursery plants from Hawaii, have already reached another Pacific island, Guam (Beard and Pitt, 2005). The potential for *E. coqui* to transmit *B. dendrobatidis* with future introductions adds to its capacity to threaten native communities.

E. coqui could have transported *B. dendrobatidis* to Hawaii; however, because the location of source population(s) and number of introductions are not known, it is not presently possible to consider the status of *B. dendrobatidis* in these populations. Alternatively, *E. coqui* could have acquired *B. dendrobatidis* from non-native amphibians already in Hawaii, some were purposely introduced as biological control agents (i.e., *Bufo marinus* and *Dendrobates auratus*), while another was brought in for culinary purposes (*R. catesbeiana*). At two of the four locations where *E. coqui* was found to be infected, we observed large *B. marinus* populations. Instead, *B. dendrobatidis* could have been transported in infected water (Johnson and Speare, 2003) or been carried there by some mechanical vector. It is interesting that *B. dendrobatidis* has now been found on an island with no native amphibians, suggesting that it can survive in incipient amphibian populations, or that it can arrive and survive in these locations without amphibians.

We found that infection rates of *B. dendrobatidis* were greater in subadults than adults. This may have occurred because of one or more of the following hypotheses: (1) infected subadults have a lower survival rate than uninfected subadults, (2) we sampled a greater proportion of each subadult than of each adult, or (3) subadults are more vulnerable to infection than adults (assuming they recover). As has been found in previous studies (Beard et al., 2003), we found that subadults perch heights were closer to the forest floor than that of adults. This preference is thought to result in part from the greater moisture requirements of subadults (Pough et al., 1983). Because *B. dendrobatidis* is an aquatic pathogen (Longcore et al., 1999), the high moisture environment found closer to the forest floor could contribute to greater infection in subadult frogs.

Overall, we found a low infection rate (2.4%, $n = 382$), even compared to other studies of the same species (7.1% infected in Puerto Rico, $n = 28$) (Burrowes et al., 2004). There are several potential explanations for this low infection rate. Because an infection of *B. dendrobatidis* may be localized (Berger and Speare, 1998), and different tissues (sometimes different feet) from the same specimen may be analyzed, we believe that DNA tests for *B. dendrobatidis* can produce false negatives. Additionally, we believe that low-level infections may lead to inconsistent results between different samples from one specimen. This is supported by the fact that the one specimen that we scored positive and Pisces Molecular scored negative had a light band that was barely detectable with a single round of PCR in our laboratory. Because we isolated DNA from one tissue sample per specimen and the test may fail to detect low-level infections, we believe our estimate of *B. dendrobatidis* infection of *E. coqui* in Hawaii is conservative.

Some studies suggest that *E. coqui* is not particularly susceptible to *B. dendrobatidis* infection and chytridiomycosis. Because *E. coqui* does not congregate in a breeding chorus or have an aquatic life stage, it would be expected to have a low prevalence of infection (Lips et al., 2003). In addition, in contrast to other species (e.g., *Bufo boreas*), laboratory tests using different levels of exposure to *B. dendrobatidis* have shown that *E. coqui* have no significant response in mortality (Cynthia Carey, personal communication.). Alternatively, the low prevalence might simply reflect a recent invasion of the fungus into these populations. Further research is needed to determine the susceptibility of *E. coqui* to *B. dendrobatidis*. We believe that *B. dendrobatidis* should not be used as a biological control agent because it is not a species-specific pathogen, many amphibians are highly susceptible, alternative hosts have not yet been identified, and it has been shown to be readily dispersed by human activities.

Acknowledgments

Funding was provided by the US Fish and Wildlife Service, Hawaii Department of Land and Natural Resources Invasive Species Council, and Jack Berryman Institute. We thank K.E. Mock and M. Pfrender for laboratory space and equipment. J. Chong for laboratory assistance. This manuscript was improved by comments from the Herpetology Group at Utah State University.

References

Annis, S.L., Dastoor, F., Ziel, H., Daszak, P., Longcore, J.E., 2004. A DNA-based assay identifies *Batrachochytrium dendrobatidis* in amphibians. Journal of Wildlife Diseases 40, 420–428.

Beard, K.H., Pitt, W.C., 2005. Potential consequences of the coqui frog invasion in Hawaii. Diversity and Distributions.

Beard, K.H., McCullough, S., Eschtruth, A., 2003. A quantitative assessment of habitat preferences for the Puerto Rican terrestrial frog, *Eleutherodactylus coqui*. Journal of Herpetology 37, 10–17.

Bell, B.D., Carver, S., Mitchell, N.J., Pledger, S., 2004. The recent decline of a New Zealand endemic: how and why did populations of Archey's frog *Leiopelma archeyi* crash over 1996–2001?. Biological Conservation 120, 189–199.

Berger, L., Speare, R., 1998. Chytridiomycosis: a new disease of wild and captive amphibians. Australia and New Zealand Council for the Care of Animals in Research and Teaching Newsletter 11, 1–3.

Berger, L., Speare, R., Daszak, P., Green, D.E., Cunningham, A.A., Goggin, C.L., Slocombe, R., Ragan, M.A., Hyatt, A.D., McDonald, K.R., Hines, H.B., Lips, K.R., Marantelli, G., Parkes, H., 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proceedings of the National Academy of Sciences of the United States of America 95, 9031–9036.

Bosch, J., Martinez-Solano, I., Garcia-Paris, M., 2001. Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. Biological Conservation 97, 331–337.

Burrowes, P.A., Joglar, R.L., Green, D.E., 2004. Potential causes for amphibian declines in Puerto Rico. Herpetologica 60, 141–154.

Daszak, P., Cunningham, A.A., Hyatt, A.D., 2003. Infectious disease and amphibian population declines. Diversity and Distributions 9, 141–150.

Daszak, P., Strieby, A., Cunningham, A.A., Longcore, J.E., Brown, C.C., Porter, D., 2004. Experimental evidence that the bullfrog (*Rana catesbeiana*) is a potential carrier of chytridiomycosis, an emerging fungal disease of amphibians. Herpetological Journal 14, 201–207.

Hanselmann, R., Rodriguez, A., Lampo, M., Fajardo-Ramos, L., Aguirre, A.A., Kilpatrick, A.M., Rodriguez, J.P., Daszak, P., 2004. Presence of an emerging pathogen of amphibians in introduced bullfrogs *Rana catesbeiana* in Venezuela. Biological Conservation 120, 115–119.

Hawaii State Department of Agriculture, 2004. T-STAR Hawai'i Coqui Frog Invasive Species Project, 16 November 2004, University of Hawai'i at Manoa. Available from: <http://www.ctahr.hawaii.edu/coqui/bio_control.asp>.

Johnson, M.L., Speare, R., 2003. Survival of *Batrachochytrium dendrobatidis* in water: quarantine and control implications. Emerging Infectious Diseases 9, 922–925.

Kraus, F., Campbell, E.W., 2002. Human-mediated escalation of a formerly eradicable problem: the invasion of Caribbean frogs in the Hawaiian Islands. Biological Invasions 4, 327–332.

Kraus, F., Campbell, E.W., Allison, A., Pratt, T., 1999. *Eleutherodactylus* frog introductions to Hawaii. Herpetological Review 30, 21–25.

Lips, K.R., Reeve, J.D., Witters, L.R., 2003. Ecological traits predicting amphibian population declines in Central America. Conservation Biology 17, 1078–1088.

Lips, K.R., Mendelson, J.R., Munoz-Alonso, A., Canseco-Marquez, L., Mulcahy, D.G., 2004. Amphibian population declines in montane southern Mexico: resurveys of historical localities. Biological Conservation 119, 555–564.

Longcore, J.E., Pessier, A.P., Nichols, D.K., 1999. *Batrachochytrium dendrobatidis* gen et sp nov, a chytrid pathogenic to amphibians. Mycologia 91, 219–227.

Muths, E., Corn, P.S., Pessier, A.P., Green, D.E., 2003. Evidence for disease-related amphibian decline in Colorado. Biological Conservation 110, 357–365.

O'Neill, E.M., Mendelson III, J.R., Beard, K.H., Brem, F., Practical Application of a PCR-based Assay for Chytrid Fungi in the Field and Laboratory. Ecohealth. (in review).

Pessier, A.P., Nichols, D.K., Longcore, J.E., Fuller, M.S., 1999. Cutaneous chytridiomycosis in poison dart frogs (*Dendrobates* spp.) and White's tree frogs (*Litoria caerulea*). Journal of Veterinary Diagnostic Investigation 11, 194–199.

Pough, F.H., Taigen, T.L., Stewart, M.M., Brussard, P.F., 1983. Behavioral modification of evaporative water loss by a Puerto Rican frog. Ecology 64, 244–252.

Schizas, N.V., Street, G.T., Coull, B.C., Chandler, G.T., Quattro, J.M., 1997. An efficient DNA extraction method for small metazoans. Molecular Marine Biology and Biotechnology 6, 381–383.

Stewart, M.M., Woolbright, L.L., 1996. Amphibians. In: Reagan, D.P., Waide, R.B. (Eds.), The Food Web of a Tropical Rain Forest. University of Chicago Press, Chicago, pp. 363–398.

Stuart, S.N., Chanson, J.S., Cox, N.A., Young, B.E., Rodrigues, A.S.L., Fischman, D.L., Waller, R.W., 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306, 1783–1786.

Weldon, C., du Preez, L.H., Hyatt, A.D., Muller, R., Speare, R., 2004. Origin of the amphibian chytrid fungus. Emerging Infectious Diseases 10, 2100–2105.

Woolbright, L.L., 1985. Sexual dimorphism in body size of the subtropical frog, *Eleutherodactylus coqui*. Unpublished Ph.D. Dissertation, State University of New York, Albany.