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Chytrid Fungus in Frogs from an Equatorial African Montane Forest in Western Uganda

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ABSTRACT: *Batrachochytrium dendrobatidis*, the causative agent of chytridiomycosis, was found in 24 of 109 (22%) frogs from Kibale National Park, western Uganda, in January and June 2006, representing the first account of the fungus in six species and in Uganda. The presence of *B. dendrobatidis* in an equatorial African montane forest raises conservation concerns, considering the high amphibian diversity and endemism characteristic of such areas and their ecological similarity to other regions of the world experiencing anuran declines linked to chytridiomycosis.

Key words: Africa, amphibians, Anura, *Batrachochytrium dendrobatidis*, Chytridiomycota, Uganda.

Chytridiomycosis, an emerging infectious disease caused by the fungus *Batrachochytrium dendrobatidis,* is a major cause of amphibian population declines (Daszak et al., 1999). Its global distribution, responsiveness to microenvironmental conditions affected by climate change, and association with anuran extinctions in Central America and Australia make it a disease of special concern for conservation (Daszak et al., 1999; Pounds et al., 2006). Despite growing information about *B. dendrobatidis* elsewhere in the world, comparatively little is known about the fungus in Africa, where it is thought to have originated (Weldon et al., 2004).

This report presents the results of a survey for *B. dendrobatidis* in Kibale National Park, Uganda. Kibale is a 795-km^2 park located in western Uganda near the foothills of the Rwenzori Mountains (0°13′–0°41′N, 30°19′–30°32′E), consisting primarily of moist semideciduous and evergreen forest, transitional between lowland rainforest and montane forest (elevation ranging from approximately 1,100 to 1,600 m), and interspersed with grassland, woodland, lakes and wetlands, colonizing forest, and plantations of exotic trees (Chapman et al., 1997; Chapman and Lambert, 2000). Mean daily minimum and maximum temperatures in Kibale were recorded as 14.9 C and 20.2 C, respectively, from 1990 to 2001, with mean annual rainfall during the same period of 1749 mm, distributed across distinct, bimodal wet and dry seasons (Chapman et al., 1999, 2005). Kibale has experienced marked climate change over the last approximately 30 yr, with increasing annual rainfall, increasing maximum mean monthly temperatures, and decreasing minimum mean monthly temperatures (Chapman et al., 2005).

Medium- and high-altitude equatorial African forests such as Kibale hold a great diversity of herpetofauna, including many endemic anuran species (Channing and Howell, 2006). Surveys by Vonesh between 1995 and 1997 identified 28 anuran, 15 lizard, and 32 snake species in Kibale, an assemblage that shows an affinity with the herpetofauna of the Guinea–Congolean rain forests to the west (Vonesh, 2001). The herpetofauna of Kibale is markedly different from that of the coastal mountains of Tanzania, which contain many local endemic anuran species, and where *B. dendrobatidis* notably contributed to the recent decline of the Kihansi spray toad, *Nectophrynoides asperginis* (Krajick, 2006; Lee et al., 2006). To our knowledge, ours is the first multispecies survey for *B. dendrobatidis* in Kibale or in any similar equatorial African montane forest site.

Frogs were collected from locations near Makerere University Biological Field

TABLE 1. Prevalence and intensity of infection with *B. dendrobatidis* in frogs collected from Kibale National Park, Uganda.

Species	Abundance[a]	Habitat type[b]	Number tested	Number positive	Prevalence (%)[c]	Mean intensity[d]
Bufo funereus	U	1	5	3	60 (23–88)	2.22±2.00
Hyperolius kivuensis	C	2	22	3	14 (4–34)	1.62±1.40
Leptopelis christyi	U	1, 3	7	2	29 (8–65)	1.28±1.20
Leptopelis kivuensis	U	3, 4, 5	36	9	25 (14–41)	2.17±1.92
Phrynobatrachus graueri	C	2	1	0	0.0	N/A
Ptychadena mascareniensis	U	3, 5	18	2	11 (2–34)	2.40±2.30
Xenopus wittei	U	5	20	5	25 (11–47)	0.63±0.32
All species			109	24	22 (15–31)	2.02±1.58

[a] Relative abundance descriptors are taken from Vonesh (2001); C=common (one can find many specimens); U=usual (one can find when looking in the proper habitat during the appropriate season).

[b] Numbers indicate habitat types from which frogs were captured: 1=forest interior, near stream; 2=forest interior, in/near permanent pond; 3=forest interior, in/near ephemeral pool; 4=forest interior, far from water; 5=forest edge, in/near permanent pool. All frogs were captured in habitat types typical for each species (Vonesh, 2001).

[c] Numbers in parentheses indicate 95% confidence intervals calculated with the modified Wald method (Agresti and Coull, 1998) and are given only where samples sizes are ≥5.

[d] Mean intensities are expressed as $\log_{10}$ zoospore equivalents per positive toe clip±standard error of the mean, calculated according to the methods of Boyle et al., 2004.

Station (elevation approximately 1500 m) in Kibale National Park in January and June 2006. Sites were chosen that were known to have high amphibian densities, both from anecdotal local reports and from previous amphibian surveys (Vonesh, 2001). Frogs were captured by hand or with hand nets, placed individually into single-use plastic bags until a toe clip was obtained from the right rear foot, and released at the site of capture. Toe clips were placed in approximately 5 volumes of RNAlater® stabilization solution (Ambion, Inc., Austin, Texas, USA) and stored at 4 C in the field and at −20 C after transport to the laboratory. Equipment and hands were disinfected with 10% bleach and alcohol wipes, respectively, between individuals.

Total DNA was extracted from toe clips with the use of the ZR Genomic DNA II Kit™ (Zymo Research Corp., Orange, California, USA). For the tissue homogenization step, a rotor-stator homogenizer with single-use individually sterilized probes was used to prevent cross-contamination. Negative-control DNA extractions were also performed with every 10 toe clip extractions. To test for *B. dendrobatidis*, a previously described real-time quantitative PCR was used that can detect a single zoospore in a diagnostic sample (Boyle et al., 2004). Samples were run in triplicate, with the use of 1 μl of extracted DNA template per reaction.

To confirm positive results, a nested PCR was used that targets the fungal cysteinyl tRNA synthase (*ctsyn1*) gene (Morehouse et al., 2004; Garner et al., 2006), although with newly designed internal PCR primers (*ctsyn1*aF: 5′-TCAGCTGCCGTCGTTTGTGAATTG -3′ and *ctsyn1*aR: 5′-CCAGAGCAGTTTG-CCAGCATCAAA -3′). Amplicons were electrophoresed in 1.5% agarose gels, which were stained with ethidium bromide and visualized under ultraviolet light. Amplicons were purified from gels with the use of the ZymoClean Gel DNA Recovery Kit (Zymo Research) and were sequenced directly and in both directions with primers *ctsyn1*aF and *ctsyn1*aR. Sequencing was performed at the University of Illinois Roy J. Carver Biotechnology Center.

A total of 109 frogs from seven species were collected (Table 1). Frogs of all species were adults, with the exception

of *Leptopelis christyi* and *L. kivuensis*, for which approximately 50% of frogs were post-metamorphic juveniles. All frogs appeared clinically normal at the time of sampling. *Batrachochytrium dendrobatidis* was detected in 24/109 (22.0%) toe clip samples from six species (Table 1). Prevalence estimates varied between 0% and 60% among species, although sample sizes within species were too small for meaningful statistical comparisons. Among infected frogs, there was no significant association between intensity of infection and species (Kruskal-Wallis rank sum test statistic=10.92; $P=0.09$). *Batrachochytrium dendrobatidis* was not detected in any negative control samples, indicating that laboratory contamination did not affect these results.

With the use of nested PCR, a 235 bp segment of the *B. dendrobatidis ctsyn1* gene was successfully amplified and sequenced in three positive field samples (two *L. kivuensis* and one *Ptychadena mascareniensis*). All three sequences matched exactly that of the published *B. dendrobatidis ctsyn1* gene (GenBank accession number BH001044), and all contained adenine at position 400, a single nucleotide A/G polymorphism previously documented in a geographically distributed sample of *B. dendrobatidis* isolates (Morehouse et al., 2003). These three samples also had among the highest intensities of infection of the field samples determined to be positive by real-time quantitative PCR (all had >1.7 log zoospore equivalents per sample; attempts to amplify field samples with infection intensities less than this were unsuccessful). These data, combined with information from amplifications of dilutions of positive control samples of known concentrations (data not shown), indicate a detection limit of approximately three zoospore equivalents per reaction for the nested PCR.

This survey represents the first account of *B. dendrobatidis* in the six positive species listed in Table 1. It is also the first account of *B. dendrobatidis* in Uganda, and it thereby significantly expands the known geographic range of the fungus in Africa. Kibale is approximately 800 km from the nearest other location where frogs infected with *B. dendrobatidis* have been documented (*P. anchietae* in Nairobi, Kenya; Speare and Berger, 2000). Kibale is approximately 1,200 km from Kihansi Gorge, Tanzania, where *B. dendrobatidis* contributed to the decline of *N. asperginis* (Krajick, 2006; Lee et al., 2006), and over 2,000 km from sites in southern Africa where infected *Xenopus* spp. have existed since at least as early as 1938 (Weldon et al., 2004).

It has been suggested that *B. dendrobatidis* may have emerged through the export of *X. laevis* from southern Africa for human pregnancy testing beginning in the 1930s (Weldon et al., 2004). We note that the prevalence of *B. dendrobatidis* in *X. wittei* in Kibale (25%) is higher than that reported in archived specimens of *X. laevis*, *X. meulleri*, and *X. gilli* collected from southern Africa between 1879 and 1999 (2.7%; Weldon et al., 2004), but is within the range of location-specific prevalence values for *Xenopus* spp. collected from South Africa more recently (Weldon, 2005). It is unclear whether this pattern represents a recent increase in *B. dendrobatidis* prevalence across Africa, or whether it merely reflects typical differences in prevalence among spatially and temporally separated populations of ecologically similar species.

No accounts exist of mass anuran mortality events in Kibale or surrounding areas. Because long-term historical records are lacking for anuran populations in Kibale and in equatorial Africa in general, it is currently unclear how *B. dendrobatidis* might be affecting amphibian health and conservation in the region. Considering the remarkable biodiversity and endemism of equatorial Africa's medium- and high-altitude forests, and considering their general ecological similarity to forests in Central America and Australia that have been profoundly affected by

chytridiomycosis, an improved understanding of the disease in equatorial Africa is exigent.

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