has constituted a helpful tool for orientating explorations for *Bd* infections detection, these prediction have been made on the bases of only 44 points over the New World, and none of these points was either from Argentina or neighboring countries, resulting in a low precision. Further information is needed about the prevalence and habitat requirements of *Bd* infecting anurans inhabiting Argentina for modeling an actualized and more precise *Bd* distribution map. An increase in the number of amphibian species monitored will help us to estimate the health of wild populations. The collection of environmental and biological data will provide us with valuable tools to predict new scenarios and implement adequate and specific conservation policies in order to manage disease outbreaks in Argentina.

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Detection of *Batrachochytrium dendrobatidis* in Amphibians from the Great Smoky Mountains of North Carolina and Tennessee, USA

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Amphibians in the southern Appalachian Mountains of the United States comprise a diverse and globally significant biological resource (Dodd 2004). There are ample reasons to be concerned about the welfare of amphibian populations in the Southern Appalachians, given expected changes in environmental conditions with climate change (Bernardo and Spotila 2006; Corser 2001; Dodd 1997) and reports of population declines (Corser 2001; Highton 2005). Furthermore, the pathogenic fungus, *Batrachochytrium dendrobatidis* (hereafter called *Bd*), has been detected in many sites throughout the southeastern U.S. (e.g., Daszak et al. 2005; Rothermel et al. 2008). Although Rothermel et al. (2008) detected *Bd* infection in 10 species within the families Ranidae, Hylidae, and Salamandridae, none of the 143 amphibians collected in Great Smoky Mountains National Park in 1999–2001 were infected. Importantly, this sample included only two individuals in the family Plethodontidae. Other studies have reported *Bd* infections in wild-caught plethodontid salamanders of two terrestrial species (*Plethodon neomexicanus*, Cummer et al. 2005; *P. cinereus*, Lauer et al. 2007) and three stream-associated species (*Eurycea cirrigera*, Byrne et al. 2008; *E. bislineata* and *Desmognathus fuscus*, Grant et al. 2008). Researchers have also collected morbid terrestrial plethodontid species (*Oedipina* spp. and *Bolitoglossa* spp.) during mass mortality events in Mexico and Panama (Lips et al. 2003; Parra-Olea et al. 2005). Therefore, there is an urgent need for more information regarding *Bd* occurrence and susceptibility within the range of the many endemic species of salamanders in the Southern Appalachians.

Three methods have been used to detect the presence of *Bd* in amphibians: histological examination, conventional polymerase chain reaction (PCR), and quantitative (real-time) PCR (qPCR). The relative benefits of histological examination versus the more sensitive molecular methods have been described previously (Smith 2007). Since the publication of Boyle et al. (2004), qPCR has become widely adopted in studies seeking to detect or quantify the presence of *Bd* in amphibians. A recent study has supported the increased sensitivity of qPCR over conventional PCR in detecting *Bd* (Kriger et al. 2006), but there are few empirical tests

comparing the two methods (Kriger et al. 2006). The objectives of this study were: 1) to determine the presence of *Bd* in the Great Smoky Mountains of North Carolina and Tennessee; 2) to compare the incidence of *Bd* in groups of amphibians with different life history strategies (i.e., pond-breeding, stream-breeding, and fully terrestrial); and 3) to assess repeatability of *Bd* detection by verifying a subset of our conventional PCR results using qPCR.

Methods.—Field surveys for amphibians were conducted in Great Smoky Mountains National Park, located in western North Carolina and eastern Tennessee. We sampled pond-breeding amphibians from 10 sites in April and May 2007, and collected 377 larval and 60 post-metamorphic samples. We sampled stream-breeding and fully terrestrial salamanders from 25 sites in May–July 2006 and April–July 2007, and collected one larval and 221 post-metamorphic samples. Larvae were euthanized by immersion in a concentrated solution of MS-222, which does not destroy *Bd* (Webb et al. 2005). The sampling method for post-metamorphic amphibians entailed swabbing the ventral skin (and hind-toe webbing of anurans) 15–20 times, following the protocol of Livo (unpubl.; http://wildlife.state.co.us/NR/rdonlyres/710BBC95-2DCF-4CF9-8443-D4561DBC3B69/0/PCRsampling2004.pdf). Euthanized larvae and swab samples were preserved in 70% ethanol for subsequent genetic assays.

DNA isolation techniques for pond-breeding amphibians varied depending on whether the sample was a whole larva or a skin swab. For larvae, oral discs were excised and DNA was isolated using standard phenol-chloroform techniques. Briefly, oral discs were initially incubated in 100 μl of 1 mg/ml collagenase (Sigma-Aldrich Chemical Company, St. Louis, Missouri) in phosphate buffered saline (PBS) for 4 h at 37°C followed by an additional incubation using 100 μl of proteinase K for 16 h at 37°C. After incubation, the digested tissue samples were triturated to disrupt cellular matrixes and an equal volume of a 1:1 ratio of phenol:chloroform was added. The sample was vortexed thoroughly for 10 sec and allowed to set at room temperature for approximately 5 min. The sample was then centrifuged at 10,000 × g for 5 min and the top, aqueous layer was moved to a new tube. Approximately five volumes of 100% ethanol and 100 μl of 3 M sodium acetate were added to the sample for DNA precipitation. The samples were allowed to sit at -80°C for 5 min and then centrifuged at 10,000 × g for 10 min. The resulting DNA pellet was washed once in 80% ethanol and subsequently centrifuged at 10,000 × g for 5 min. The DNA pellet was air dried and resuspended in 20 μl of molecular grade water.

Larvae collected in the field were either kept separate for individual analysis or pooled. The oral discs of separated individuals (N = 90) were kept isolated and processed separately throughout. However, some pooled samples (N = 293 individuals) of larvae were large (as many as 73 tadpoles per jar) and had to be divided into subsamples of three to seven oral discs per tube (N = 66 reactions). Pooled samples of pond-breeding amphibian larvae consisted of only a single species. In addition to testing the oral discs, we also retained and tested the ethanol from each pooled sample of larvae (N = 8). The ethanol was passed through a 0.22 μm filter to collect possible free-floating *Bd* zoospores. The cellular debris was reverse-osmosed to collect the debris in a new tube. The sample was then boiled in a hot water bath and the resulting cell lysate was frozen until further DNA analysis. To isolate DNA from skin swabs (N = 199), we pressed the swab against the sides of its 2-ml vial to remove most of the retained ethanol (and any *Bd*) from the cotton. The tubes containing ethanol were then centrifuged to create pellets of swab-debris and possible zoospores. The swab-debris pellet was re-suspended in water and boiled. The resulting cell lysate was frozen until used directly for PCR analysis; cell lysates of *Bd* were not found inhibitory to PCR reactions (C. S. Brooks, unpubl. data). All pond-breeding amphibian samples were analyzed using conventional PCR following Annis et al. (2004). As controls, the above procedures were repeated using *Bd*-free swabs and frog tissues intentionally inoculated with serially diluted zoospores to determine minimum detection limits. These controls indicated that as few as 10 zoospores could be detected using the described procedures (C. S. Brooks, unpubl. data).

DNA was extracted from swabs collected from stream-breeding and fully terrestrial salamanders following the methodology of Hyatt et al. (2007). Swab samples were tested for the presence of *Bd* DNA using qPCR Taqman assays and species-specific primers developed by Boyle et al. (2004). With our limited funding, we chose to use this more expensive (and presumably more sensitive) analytical method for only the samples of plethodontids. Plethodontids are of greater concern from a conservation standpoint and use of qPCR ensured a high degree of confidence in negative results. An internal control (VIC_{TM} dye, Applied Biosystems) was added to every sample well of the assay, and was used to determine the presence of PCR inhibitors, which may result in false negatives (Hyatt et al. 2007). Additionally, a sample known to contain *Bd* was run concurrently as a positive control. To avoid the high costs associated with qPCR, samples from identical locations (but not necessarily the same species) were pooled, with up to three samples per reaction well (Hyatt et al. 2007). To further avoid high costs, all assays were performed in singlicate (following Kriger et al. 2006) rather than the widespread method of performing assays in triplicate (following Boyle et al. 2004). Actual detection probability is not expected to decline significantly with these cost-cutting measures (Kriger et al. 2006; Hyatt et al. 2007).

To assess repeatability of *Bd* detection, 15 samples from the pond-breeding group that tested positive and 20 that tested negative using conventional PCR were rerun using qPCR. Five positive controls of differing concentrations (undiluted and dilution factors of 1/20, 1/400, 1/8000 and 1/160000) were also run using qPCR. Samples were run in triplicate at 1/10 and 1/100 dilution factors (for a total of six replicates per sample). Samples were scored as negative if all six of the replicates tested negative, positive if three to six replicates tested positive and equivocal if one or two of the replicates tested positive. These tests were conducted blindly, such that the number and identity of samples that tested positive using conventional PCR was not known during the qPCR analysis.

Results.—Using conventional PCR, we detected *Bd* in samples from four of the 10 localities where pond-breeding amphibians were collected. All positive samples using this method were larval anurans, with five to 116 infected Wood Frog (*Lithobates sylvaticus*) larvae, one infected toad (*Anaxyrus* sp.) larva, and one to seven chorus frog (*Pseudacris* sp.) larvae being detected (Table 1). The above ranges of infected *Lithobates* and *Pseudacris* larvae are large because it is impossible to determine how many individuals within a pooled set of samples were infected. At sites where *Bd* was detected, prevalence was apparently very low. We can

TABLE 1. Post-metamorphic (P) and larval (L) amphibians tested for *Batrachochytrium dendrobatidis* (*Bd*) infection in Great Smoky Mountains National Park, North Carolina and Tennessee, USA, in 2006–2007.

Species	Life Stage	N*	*Bd* Detected
Pond-breeding			
Ambystoma maculatum	P	3 (3)	No
Hemidactylium scutatum	P	3 (3)	No
Notophthalmus viridescens	P	37 (37)	Yes
Anaxyrus sp.	L	122 (11)	Yes
Anaxyrus sp.	P	6 (6)	No
Pseudacris sp.	L	7 (2)	Yes
Pseudacris crucifer	P	6 (6)	No
P. feriarum	P	1 (1)	No
Lithobates catesbeianus	P	1 (1)	No
L. clamitans	L	4 (4)	No
L. clamitans	P	3 (3)	No
L. sylvaticus	L	244 (45)	Yes
Stream-breeding			
Cryptobranchus alleganiensis	P	1 (1)	No
Desmognathus sp.	P	4 (4)	No
D. imitator	P	1 (1)	No
D. imitator/D. ocoee	P	6 (4)	No
Eurycea sp.	L	1 (1)	No
E. wilderae	P	11 (10)	No
Pseudotriton ruber	P	1 (1)	No
Fully terrestrial			
D. wrighti	P	5 (5)	No
Plethodon jordani	P	35 (15)	No
P. metcalfi	P	23 (8)	No
P. serratus	P	12 (11)	No
P. teyahalee	P	17 (7))	No
P. jordani x *P. metcalfi*	P	94 (32)	No
P. jordani x *P. metcalfi* x *P. teyahalee*	P	11 (6)	No

* Numbers in parentheses indicate number of samples tested after pooling.

infer prevalence from samples in which large numbers of larvae were collected from a given site on the same date, and individual larvae were preserved and analyzed separately. At Finley Cane Sinkholes (35.61027°N, 83.74388°W), only 1.6% of toad larvae were infected. At Stupka's Sinkhole (35.58972°N, 83.84777°W), 3.7% of Wood Frog larvae were infected. Likewise, the one positive subsample of Wood Frog larvae from Finley Cane Sinkholes contained three larvae (out of a total of 72), which suggests prevalence was ≤ 4%. These sites are in, or within 10 km of, the heavily visited Cades Cove area in the western section of the park.

According to the qPCR assays, every stream-dwelling and terrestrial salamander swab sample was negative for the presence of *Bd* (Table 1). By determining the presence of *Bd* in the positive control, we confirmed that the reactions worked. However, the standard dilution of 1/10 (Hyatt et al. 2007) of the swab extract yielded a mere 20% success rate in internal control reactions, indicating PCR inhibition was a significant problem. To overcome the presence of PCR inhibitors, all samples were rerun with a 1/100 dilution. The success rate of internal control reactions was 100% following the increased dilution factor.

Results of samples analyzed using both conventional PCR and qPCR were largely discordant. Of the 35 samples that were run using conventional PCR and subsequently rerun using qPCR, 14 were discordant with the original result, 13 were concordant and 8 were equivocal. In total, seven additional samples unequivocally tested positive using qPCR: two *Lithobates sylvaticus* larvae from The Sinks (35.66944°N, 83.66138°W; ca. 14 km from Cades Cove), one *L. sylvaticus* larva from Finley Cane Sinkhole, one *L. sylvaticus* larva from Tremont Road (35.65222°N, 83.69722°W; ca. 10 km from Cades Cove), and three post-metamorphic *Notophthalmus viridescens* from Stupka's Sinkhole (Table 1). Importantly, *Bd* infection would not have been detected in *N. viridescens* had the samples not been retested using qPCR. Conversely, 7 samples that tested positive using conventional PCR tested negative using qPCR. It is also important to note that positive controls had predictable results. The undiluted positive control and the control diluted to 1/20 tested positive, the control diluted to 1/400 was equivocal (consistent with a very low concentration) and the controls diluted to 1/8000 and 1/160000 tested negative (consistent with concentrations too low to detect).

Discussion.—Our sampling revealed the presence of *Bd* in a limited number of sites and species within Great Smoky Moun-

tains National Park. The detection of *Bd* in the Park is one of a growing list of occurrences in the southeastern U.S. For example, recent surveys have also found *Bd*-infected amphibians at Congaree National Park in South Carolina, Chattahoochee River National Recreation Area in Georgia, and sites in the Blue Ridge Mountains of North Carolina and Georgia (Rothermel et al. 2008).

Another goal of this study was to provide preliminary information regarding *Bd* prevalence and which species are infected. We detected *Bd* in the larval stages of pond-breeding anurans in the genera *Anaxyrus*, *Lithobates*, and *Pseudacris*, as well as in post-metamorphic pond-breeding salamanders (*Notophthalmus*). Overall prevalence in the park appears to be low (1–4%), but exact measures of prevalence are not possible because we had low sample sizes for many sites and individual larvae were often pooled by species and site. We could not assess the influence of elevation on *Bd* occurrence because elevation and taxonomic associations were confounded; that is, samples (including positives) of pond-breeding species tended to be from lower-elevation sites and samples (all negative) of stream-dwelling and terrestrial salamanders tended to be from higher-elevation sites. Woodland salamanders that are direct-developing may have a low likelihood of infection simply because they do not inhabit streams and other aquatic habitats where *Bd* occurs. However, at least one species (*Plethodon metcalfi*) has been shown to be susceptible to chytridiomycosis following experimental exposure to *Bd* (V. M. Vazquez and B. B. Rothermel, unpubl. data). Resistance to *Bd* in some species derives from antimicrobial peptides produced by skin glands (Rollins-Smith et al. 2005) or cutaneous bacteria that inhibit growth of fungi, in some cases (Harris et al. 2006; Lauer et al. 2007). Different species and populations possess various combinations of these inhibitory factors, resulting in variation in resistance to chytridiomycosis.

The simplest explanation for the discordance in conventional PCR versus qPCR results is that *Bd* occurs with such low prevalence in the Great Smoky Mountains that repeated tests on the same sample yield only occasional positives. The implications of this finding are: 1) that large sample sizes may be necessary to detect the presence of *Bd* where present; and 2) current estimates of prevalence may be underestimates. Our comparative tests also suggest that conventional PCR detection rates may not be significantly lower than those of qPCR, although additional studies with larger sample sizes are needed.

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