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Cryptic diversity of a widespread global pathogen reveals expanded threats to amphibian conservation

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Biodiversity loss is one major outcome of human-mediated ecosystem disturbance. One way that humans have triggered wildlife declines is by transporting disease-causing agents to remote areas of the world. Amphibians have been hit particularly hard by disease due in part to a globally distributed pathogenic chytrid fungus (*Batrachochytrium dendrobatidis* [*Bd*]). Prior research has revealed important insights into the biology and distribution of *Bd*; however, there are still many outstanding questions in this system. Although we know that there are multiple divergent lineages of *Bd* that differ in pathogenicity, we know little about how these lineages are distributed around the world and where lineages may be coming into contact. Here, we implement a custom genotyping method for a global set of *Bd* samples. This method is optimized to amplify and sequence degraded DNA from noninvasive skin swab samples. We describe a divergent lineage of *Bd*, which we call *Bd*ASIA3, that appears to be widespread in Southeast Asia. This lineage co-occurs with the global panzootic lineage (*Bd*GPL) in multiple localities. Additionally, we shed light on the global distribution of *Bd*GPL and highlight the expanded range of another lineage, *Bd*CAPE. Finally, we argue that more monitoring needs to take place where *Bd* lineages are coming into contact and where we know little about *Bd* lineage diversity. Monitoring need not use expensive or difficult field techniques but can use archived swab samples to further explore the history—and predict the future impacts—of this devastating pathogen.

Batrachochytrium dendrobatidis | amphibian | conservation | genetic monitoring

Emerging infectious diseases are increasingly recognized as a threat to both human and wildlife health (1–3). One reason emerging infectious diseases are on the rise is the facilitated spread of pathogen propagules via globalized trade. With the aid of modern shipping, pathogens have been introduced to naïve remote areas (4). These new introductions can have grave consequences, in some cases causing mass mortality in wildlife populations (e.g., refs. 4 and 5). Understanding the pathways for disease spread is critical to predicting and addressing disease outbreaks (1).

Amphibians have been hit particularly hard by emerging infectious disease in the last century. Hundreds of amphibian species have been impacted by the pathogenic chytrid fungus *Batrachochytrium*

Significance

Batrachochytrium dendrobatidis [*Bd*] is one of the most devastating wildlife pathogens ever documented. Most surveys for *Bd* report only the presence/absence of the pathogen. However, *Bd* has distinct genetic lineages that vary in geographic extent and virulence, thus reporting *Bd* presence alone is not particularly informative. Our study uses a custom method for genotyping degraded *Bd* DNA samples, such as those non-destructively collected from live animal or museum specimen skin swabs, and presents the discovery of a divergent lineage of *Bd*—*Bd*ASIA3. This study advances our understanding of the evolutionary origins of *Bd*, highlights areas of the world where *Bd* lineages are coming into contact, and opens the door to affordable, rapid genetic monitoring of this pathogen.

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The authors declare no conflict of interest.

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Data deposition: The data reported in this paper has been deposited in the National Center for Biotechnology Information Sequence Read Archive, <https://www.ncbi.nlm.nih.gov/sra> (Bioproject PRJNA555719).

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dendrobatidis [*Bd*] (6, 7). *Bd* invades the keratinized tissue of amphibians (skin in adults, mouthparts in tadpoles), disrupting critical skin functions such as the regulation of osmotic pressure and electrolyte balance (8). The resulting disease—chytridiomycosis—can be deadly to susceptible amphibians once the pathogen reaches a critical infection load (9). However, some host species can maintain *Bd* infection without disease development (10, 11). These tolerant species can be important reservoir vectors of *Bd*. For example, the American bullfrog (*Rana catesbeiana*) is a highly traded, *Bd*-tolerant species that has been implicated in spreading *Bd* throughout the Western United States, Brazil, and Korea (12–14).

Molecular studies have played an important role in illuminating the evolutionary history of *Bd*, patterns of spatial and temporal spread, and pathogen genetic diversity (e.g., refs. 15–17). There are currently 4 documented major *Bd* lineages based on the most recent whole-genome phylogeny (14). First the “global panzootic lineage,” *Bd*GPL, is globally distributed and associated with most mass mortalities in wild amphibian populations (17). Second, *Bd*CAPE was first described from an isolate collected in Cape Province, South Africa, and has since been found in Cameroon, Mallorca, and the United Kingdom (14, 17). Third, *Bd*ASIA1 was recently described from 8 isolates collected in South Korea but also includes the previously-named *Bd*CH lineage collected in Switzerland (14). Fourth, *Bd*Brazil/ASIA2 was first described from samples collected in Brazil (18) and renamed to include the clade previously known as *Bd*Korea after whole-genome sequencing revealed their close relationship (14). The observed phylogenetic relationship among the 4 currently described *Bd* lineages suggests that the earliest diverging lineage is *Bd*ASIA1, and the most recent is *Bd*GPL (14).

In addition to the 4 major lineages, some hybridization between lineages has been reported. Schloegel et al. (18) documented the first evidence of sexual recombination in *Bd*, a finding later supported by whole-genome sequencing (15). Alarming, one of the recombinant lineages studied (an F1 hybrid of *Bd*GPL and *Bd*Brazil) was found to be more virulent than either parental lineage when tested against a native Brazilian host (19). Thus, the spread and recombination of different *Bd* lineages can lead to unpredictable and potentially dangerous outcomes. Therefore, documenting the spatial distribution of *Bd* genotypes is a high priority for amphibian conservation.

Recent genetic and genomic studies have provided unprecedented insight into the evolutionary history of *Bd*. However, a comprehensive understanding of historical and contemporary patterns of *Bd* diversity and spread has been limited by the lack of robust *Bd* genotype data in many areas of the world. One key limitation in using molecular tools to study *Bd* has been the need for pure cultures, which yield high-quality and -quantity DNA and have been required for whole-genome sequencing. However, the process of isolating and maintaining live *Bd* cultures is time-consuming and challenging, particularly in remote areas. In contrast, skin-swab samples are plentiful because they are easy to collect and are part of a standardized protocol to detect the presence of *Bd* via qPCR (20). Swab samples provide ample DNA for sensitive PCR techniques but often do not have enough high-quality DNA for whole-genome sequencing. Some studies have attempted to address this problem by sequencing small, hypervariable loci present in high copy number—specifically the ribosomal intragenic spacer (ITS-1) region. However, phylogenetic inferences made from this region produce relationships that are highly discordant with those inferred from high-coverage, whole-genome sequencing (14). These challenges have resulted in a large gap in knowledge between our robust understanding of *Bd* presence and prevalence in many parts of the world and our patchy knowledge of genetic variation and lineage distributions.

Thus, many critical questions remain in the *Bd*–amphibian system. First, are there additional undiscovered *Bd* lineages present in wild amphibian populations? Second, what is the current distribution of known *Bd* lineages in unsampled or undersampled areas of the world? Third, where are divergent *Bd* lineages coming into contact? Answering these questions would provide a truly global understanding of the threat that *Bd* poses to amphibians around the world and identify geographic centers of high conservation urgency. Here, we employ a microfluidic PCR genotyping method targeting almost 200 loci across the *Bd* genome from swab samples (21). With this technique, we can now leverage a global library of amphibian skin swabs that have never been genotyped. Our analysis provides a deeper understanding of the diversity and distribution of *Bd* globally and highlights cryptic variation in this pathogen around the world.

Results

Global *Bd* Diversity. We used our swab genotyping assay to assign 222 samples from 24 different countries to major *Bd* clades (Dataset S1). The dataset includes 189 field-collected swabs, 18 museum swabs, and 15 pure *Bd* isolates collected between 1984 and 2017 (Dataset S1 and SI Appendix, Fig. S2). The samples represent all continents where *Bd* occurs and were chosen to target areas of the world where genotype data are lacking and explore localities where lineages may be coming into contact. We first describe our findings at the global scale, integrating our dataset with 47 previously published *Bd* whole genomes (14, 15, 17), some of which we resequenced using our method (SI Appendix, Table S1). Fig. 1A shows the most current and complete global survey of *Bd* lineage distributions. Our global phylogeny (Fig. 1B) recapitulates the structure of a recent whole-genome phylogeny (14), with the addition of a divergent *Bd* clade found only in Asia that we name *Bd*ASIA3. Below, we highlight results from each of 4 regions of the world. The regional results are summarized in Fig. 2, where we show a separate phylogeny for each region of the world.

Asia. Our most significant finding in Asia is a unique and divergent *Bd* lineage that we name *Bd*ASIA3 (Fig. 2A). This lineage is clearly differentiated in the phylogenetic analyses and appears to be widespread in the Philippines, Indonesia, and parts of China. *Bd*ASIA3 co-occurs with *Bd*GPL in all 3 countries. In the Philippines, 56% (19/34) of samples harbored the *Bd*ASIA3 lineage and 41% (14/34) of samples had the *Bd*GPL lineage. In Java, Indonesia, 62% (8/13) of samples were in the *Bd*ASIA3 lineage and 38% (5/13) were *Bd*GPL. In China, 43% (3/7) of samples were *Bd*ASIA3 and 57% (4/7) were *Bd*GPL. Thus, this previously undescribed *Bd* lineage appears to be relatively common in samples collected from various parts of Asia.

One additional sample from the Philippines had a unique genetic signature and could not be confidently assigned to a known *Bd* lineage (RMB10661). To assess whether this sample represents a mixed infection or a hybrid between 2 lineages, we plotted the average number of alleles per locus (Fig. 3). RMB10661 has a similar degree of heterozygosity as the average for each of the major lineages, so it does not appear to be a hybrid or mixed sample. In addition, this sample was sister to the *Bd*ASIA3 clade in the phylogeny and has unique haplotypes at some loci. Therefore, this sample appears to be distinct from currently named lineages and possibly represents another undescribed, early branching lineage.

Europe. In Europe, we report the presence of 3 major lineages: *Bd*GPL, *Bd*CAPE, and *Bd*ASIA1 (Fig. 2B), reinforcing the key finding that multiple divergent *Bd* lineages are now commonly found at the regional scale. Of our genotyped samples from Europe, 90% (38/42) belong to *Bd*GPL and 10% (4/42) belong to the *Bd*CAPE lineage. The presence of *Bd*ASIA1 in Europe was

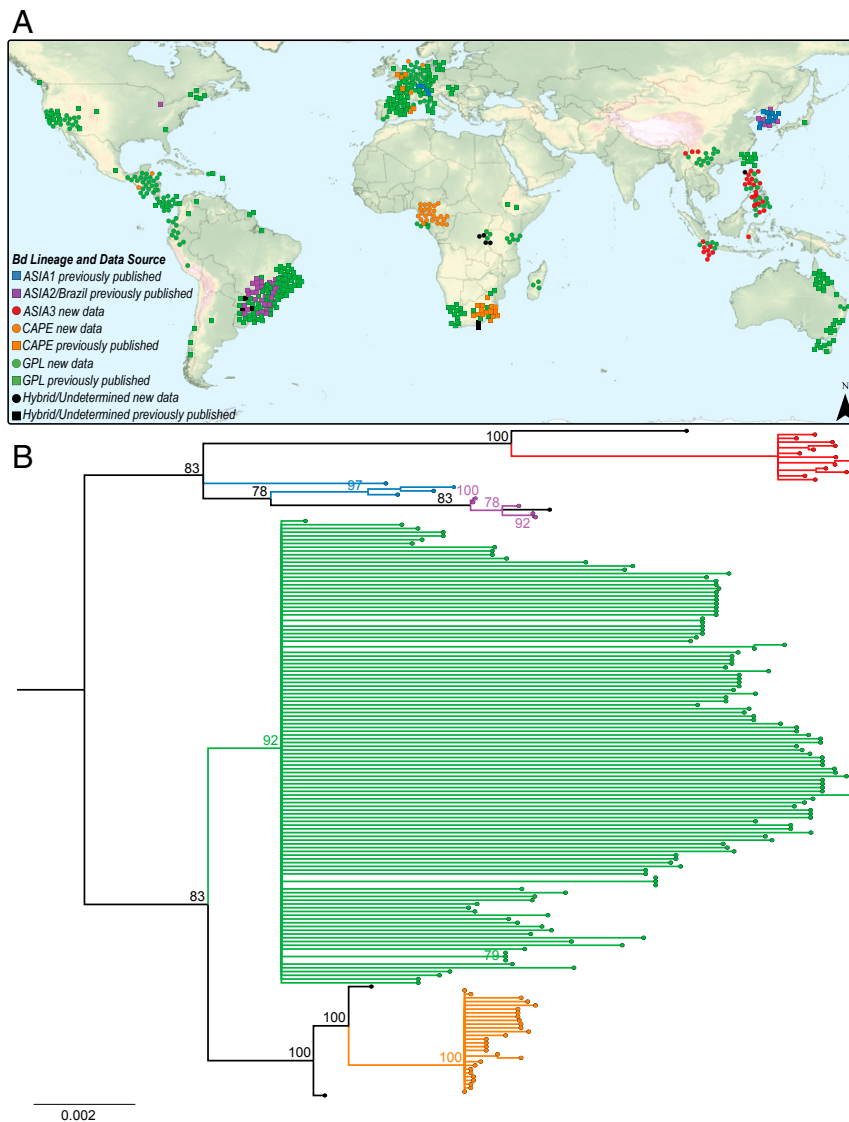


Fig. 1. (A) Global map of *Bd* genotypes. Points within 100 m are dispersed to decrease overlap and demonstrate sampling effort; therefore, map point locations are approximate. Colors indicate major *Bd* lineage, circles are newly genotyped samples ($n = 222$), and squares are previously published *Bd* genotype data ($n = 334$; data from refs. 14–17 and 27). (B) Best scoring unrooted maximum-likelihood tree estimated from 172 concatenated nuclear loci (23,651 bp) and 100 bootstrap replicates performed in RAxML. Branches on phylogeny are colored by major *Bd* lineage. This tree includes newly sequenced samples with at least 84 loci ($n = 131$) and whole-genome data ($n = 47$). Nodes with bootstrap support <50 have been collapsed and nodes >70 bootstrap support are labeled. Phylogeny with tip labels is available in *SI Appendix*, Fig. S1.

documented in a prior study (18). Remarkably, we found that 4 swabs collected from bullfrogs (*R. catesbeiana*) in The Netherlands carried the *Bd*CAPE genotype. This expands the known range of *Bd*CAPE in Europe.

Africa. In Africa, we found that the *Bd*CAPE lineage is ubiquitous in Cameroon, while *Bd*GPL dominates nearby parts of West Africa and previously uncharacterized parts of Central Africa (Fig. 2C). All 25 *Bd* samples collected from Cameroon are members of the *Bd*CAPE lineage, indicating *Bd*CAPE is the dominant, and perhaps exclusive, *Bd* lineage in Cameroon. Furthermore, we found additional support for previous studies documenting the presence of *Bd*GPL in Madagascar (22) and provide a report of *Bd*GPL in Burundi and Kenya. In Burundi, 43% (3/7) of samples were in the *Bd*GPL lineage and 57% (4/7) of samples were of an undetermined lineage. To further understand why these ambiguous samples did not group with a major lineage, we plotted the

average number of alleles sequenced per locus (Fig. 3). We found that the ambiguous samples from Burundi had a significantly higher average allele per locus than *Bd*CAPE and *Bd*GPL samples (Mann–Whitney *U* test: $P < 0.01$). These samples were most similar in average number of alleles per locus to an experimental mixture of 2 divergent *Bd* isolates and so may be instances of coinfection or hybridization.

Americas. *Bd*GPL is the dominant lineage in the Americas (excluding Brazil, where both *Bd*GPL and *Bd*ASIA2/Brazil are found). However, we report *Bd*CAPE—a lineage that previous studies have found only in Africa and Europe—in Latin America (Fig. 2D). We found that 11% (2/19) of *Bd* samples collected from Cusuco National Park in Honduras in 2014 were *Bd*CAPE, whereas 89% (17/19) of samples were *Bd*GPL. *Bd*CAPE may be newly introduced (or detected) in the Americas and occurs in very close proximity to *Bd*GPL in Honduras. All

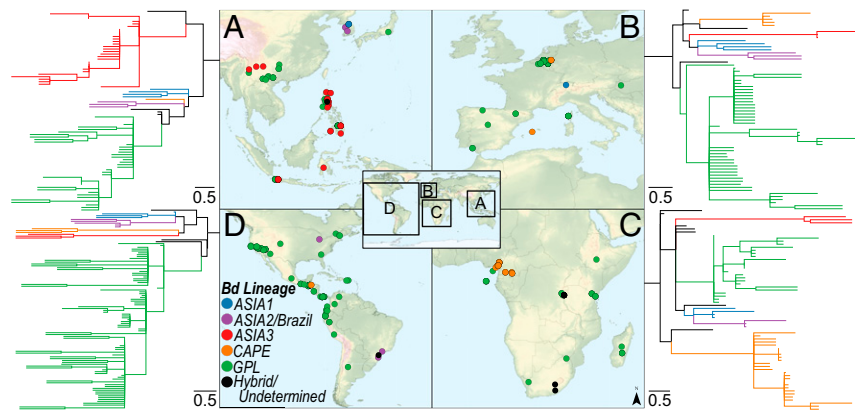


Fig. 2. Maps and regional phylogenies showing *Bd* sampling locations and lineages in Asia ($n = 78$) (A), Europe ($n = 66$) (B), Africa ($n = 66$) (C), and the Americas ($n = 108$) (D). Points and clades are colored as in Fig. 1. Sample sets include representatives of each major clade in addition to all newly genotyped samples collected in that region. Overlapping points on the map are offset by 1° longitude for display purposes. Phylogenies are species tree consensus topologies calculated in Astral (v.5.6.2) from maximum-likelihood gene trees, individually estimated in RAxML for each locus. Full-size versions of the phylogenies with tip and node labels are available in *SI Appendix, Figs. S3–S6*.

other genotyped samples from the Americas were members of the *Bd*GPL clade.

Discussion

Are There Undiscovered *Bd* Lineages in Wild Populations? Our discovery of a divergent lineage of *Bd* endemic to Asia (*Bd*ASIA3) supports the hypothesis that *Bd* originated in Asia and highlights our contention that substantial gaps remain in our understanding of the global genetic diversity in *Bd*. Recent whole-genome studies have proposed an Asian origin for *Bd*, citing the genetic signatures of long-term endemism in the *Bd*ASIA1 lineage and noting the high lineage diversity in Southeast Asia (14). Interestingly, our global phylogeny (Fig. 1A) shows that *Bd*ASIA3 is now the earliest diverging named *Bd* lineage. In addition, *Bd*ASIA3 has the longest interior branch lengths of any described lineage, indicating that it may have persisted in isolation and/or that closely related lineages have not yet been found or have gone extinct. Furthermore, there are additional well-supported nodes within the *Bd*ASIA3 clade, indicating some within-clade genetic structure. This phylogenetic pattern is consistent with constant population-size dynamics for this lineage (23) and supports the hypothesis that *Bd*ASIA3 is an endemic Southeast Asian lineage. In contrast, the *Bd*GPL clade shows long external branch lengths, indicating periods of exponential growth—a pattern consistent with the documented global spread of this lineage. It is likely that additional *Bd* lineages remain to be discovered, which may further alter our understanding of *Bd*'s evolutionary history, including the time and place of its origin.

Another line of evidence suggesting that our current understanding of *Bd* genetic diversity is incomplete comes from samples that could not be confidently assigned to a known major *Bd* clade. For example, one sample (RMB10661 collected from the relatively pristine forests of Luzon Island in the Philippines) was collected in an area where both *Bd*GPL and *Bd*ASIA3 are present (Fig. 24) and was phylogenetically estimated to be sister to the *Bd*ASIA3 clade (Fig. 1B and *SI Appendix, Fig. S3*). Our analyses indicate that this sample is not a mixed infection—nor a hybrid—of 2 different *Bd* lineages. Thus, RMB10661 may represent genetic diversity that is not yet present in our current library of *Bd* genotypes. In fact, this sample may come from yet another undescribed, early diverging Asian *Bd* lineage. However, we refrain from naming this lineage given that there is only one representative sample. It is possible that additional cryptic *Bd* diversity remains undocumented in isolated, unstudied amphibian populations around the world.

What Is the Current Distribution of *Bd* Lineages in Previously Understudied Parts of the World? Our study expands the understanding of *Bd* lineage distributions in many parts of the world where *Bd* diversity was previously uncharacterized. While we are not the first to report *Bd*CAPE in Cameroon (14), we increased the sample size for Cameroon *Bd* genotypes (Fig. 2B). The ubiquity of *Bd*CAPE in Cameroon is unique—we do not currently know of any other country occupied only by this lineage. Another study reported the presence of *Bd*GPL and other unidentified lineages in Cameroon (24) but used the ribosomal ITS region to genotype *Bd*, which is not phylogenetically informative (14). Our findings point to either a long relationship of *Bd*CAPE in Cameroon or a recent complete sweep. A previous study that did not include genotype data reported *Bd* in Cameroon dating back to 1933 (25). Indeed, *Bd*CAPE may have originated in this area and spread to other parts of Africa, Europe, and now Central America, or it may have recently invaded and spread in Cameroon as well. This highlights an important point: *Bd* lineages are often named for the areas where they were first discovered (i.e., *Bd*CAPE was first discovered in Cape Province, South Africa; ref. 17), but these names may

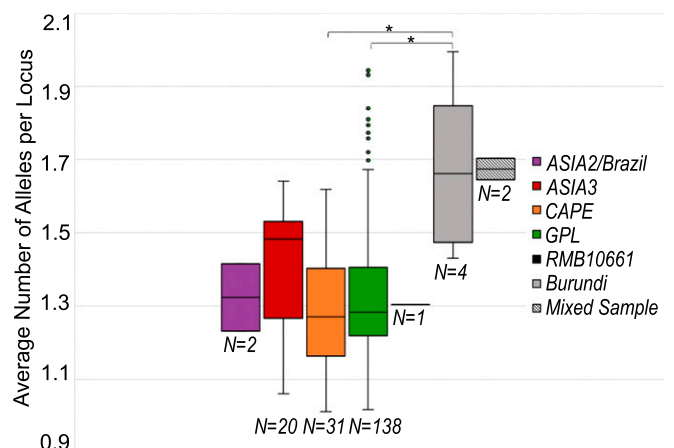


Fig. 3. Average number of alleles for each major *Bd* lineage and ambiguous samples sequenced via the Fluidigm Access Array method. The mixed sample represents an experimental mixture of *Bd*GPL and *Bd*Brazil/ASIA2 isolates. *A significant difference between the Burundi samples and the *Bd*GPL/*Bd*CAPE lineages (Mann–Whitney U test: $P < 0.01$).

become misleading as we discover more about the history and distribution of each lineage. Some lineage names have been changed or combined as more sequence data become available (such as the joining of *BdKorea* and *BdBrazil* into *BdASIA2/Brazil*; ref. 14). We raise this point to recognize that lineage names can sometimes introduce biases that may arise from historical attachments to original lineage designations and to suggest that alternative lineage naming schemes (i.e., numeric) may be worth considering in this system.

In East Africa, our data reveal an interesting pattern in the newly sequenced region of Burundi. Here, we also encountered samples we could not assign to a major *Bd* clade. However, unlike the ambiguous sample from Asia, the unassigned Burundi samples had an average number of alleles per locus that was similar to levels of allelic diversity found in experimental mixtures of 2 divergent *Bd* isolates (Fig. 3). Thus, these ambiguous samples may be a coinfection (on single hosts) of different *Bd* lineages, or a possible hybrid—as they lie between the *BdCAPE* and *BdGPL* clades in the Africa phylogeny (*SI Appendix, Fig. S5*). However, they do not appear closely related to previously published *BdGPL/BdCAPE* hybrids (14). Thus, additional work will be needed to differentiate between coinfection versus hybridization and to test whether these samples represent a separate hybridization event between *BdGPL* and *BdCAPE*. Our ongoing work includes sequencing more samples from this region to test these alternative hypotheses (for example, by comparing mitochondrial and nuclear loci and analyzing patterns of linkage disequilibrium between loci).

Where Are Divergent *Bd* Lineages Coming into Contact? As more data become available, they reveal that divergent *Bd* lineages are overlapping across fine spatial scales. Our study documents multiple instances where 2 different lineages coexist at the same time and place (e.g., sampled meters apart). For example, we find both *BdCAPE* and *BdGPL* in Honduras. While previous studies have documented *Bd* in this area and attributed amphibian declines to the pathogen (26), none have reported *BdCAPE* in the Americas. Our finding of *BdCAPE* outside of its previously reported range is alarming for a number of reasons. First, we know that *Bd* is capable of hybridizing across lineages, as has been documented in multiple parts of the world (14, 16, 18). Second, hybrid lineages can sometimes be more virulent than parental lineages (19). Third, although some amphibian species may have developed resistance and/or tolerance to a particular *Bd* lineage, it remains unclear how they might respond to the introduction of a new lineage or exposure to a hybrid lineage (27). Finally, although some amphibian host communities are beginning to recover from *Bd* outbreaks (e.g., refs. 27 and 28), many populations are persisting only in small numbers, making them especially vulnerable to new disease outbreaks.

We also found co-occurrence of divergent *Bd* lineages in parts of Asia. For example, we found *BdGPL* and *BdASIA3* at almost every sampling locality in the Philippines. Our data indicate that these lineages have been coexisting in this region for at least 7 y. The earliest samples (from Mindanao Island in 2005) and more recent samples (from the same island in 2012) had both *BdGPL* and *BdASIA3* present (*Dataset S1*). Previous studies found *Bd* to be widespread in the Philippines, but the genotype of these samples was unknown (29). Our findings are consistent with either a slow spread of *BdGPL* through the Philippines or a longer, more stable coexistence of divergent lineages. In West Java, Indonesia, we found similar evidence of lineage co-occurrence in high montane amphibian communities. However, we do not yet have time-series samples from this area and so cannot make inferences concerning the timing of arrival of different lineages.

In Europe and Asia, we see additional examples of *Bd* lineages co-occurring at small spatial scales, this time in populations of

invasive bullfrogs (*R. catesbeiana*). In The Netherlands, some samples collected from *R. catesbeiana* had *BdCAPE* and others had *BdGPL*, despite being collected in the same year in close geographic proximity. In the Yunnan province of China, the single *R. catesbeiana* sampled was infected with *BdGPL*, while the native species from the same locality carried *BdASIA3*. These findings support other recent studies suggesting that invasive *R. catesbeiana* are contributing to the spread of *Bd* around the world (14, 18). *R. catesbeiana* are consumed as food by humans globally and are one of the most commonly traded amphibian species. Commercial farms that raise *R. catesbeiana* may create disease spillover in regions with high amphibian-species richness, including Brazil and Asia (30). Thus, our study provides additional evidence that bullfrog trade should be a major concern as it creates potential pathways for short- and long-distance *Bd* dispersal (14).

Expanded Threats for Amphibian Conservation. Our dataset expands our understanding of how *Bd* lineages are distributed around the world; however, there remain unexplored frontiers in this system. First, there are many parts of the world where we know *Bd* exists, but it remains unclear which lineages are present. For example, *Bd* in Asia is widespread but exists at very low prevalence and often at low infection intensities (29). Moreover, one recent study found that the traditional qPCR assay for *Bd* (20) may not accurately quantify endemic Asian *Bd* lineages because of variation at the ribosomal RNA ITS primer-binding sites (31). This not only could lead to underreporting the presence of Asian *Bd* in wild populations but also could generate a sampling bias for studies like ours that select samples for genotyping based on positive qPCR results. If we exclude samples because they ostensibly have too little *Bd* DNA, it could skew our results in favor of reporting more *BdGPL* genotypes. Therefore, our current estimates of *Bd* diversity may still be grossly underestimated, and there may be additional endemic *Bd* lineages that remain undiscovered in Asia and other parts of the world. Additional *Bd* genotyping in under-sampled areas will be critical for fully understanding the evolutionary relationships between *Bd* and amphibian hosts.

Second, we have yet to fully explore temporal variation in *Bd* genotypes to understand the timing of lineage arrival, turnover, and spread. Swabbing museum specimens to record the historic presence/absence of *Bd* over the last century has produced a rich library of DNA samples for which our genotyping method is ideal (e.g., refs. 32–34). Our current dataset includes 18 successfully genotyped museum swabs collected from around the world (*Dataset S1* and *SI Appendix, Fig. S2*), the oldest from a specimen collected in 1984 in Peru. By genotyping museum swabs, we can test hypotheses for factors driving *Bd*-related declines. Understanding the dynamics of historical amphibian declines is key for predicting future risk.

Third, our data indicate that *Bd* lineages are continually spreading and are co-occurring in close proximity. Given that novel *Bd* lineages and hybrid lineages could be a threat to naïve populations (19), it is increasingly important that we continue to monitor *Bd* presence, prevalence, genetic diversity, and host health. In addition to monitoring, best practices for limiting *Bd* spread must be communicated not only to scientists but also to the public traveling to remote areas and commercial farms. Furthermore, steps should be taken to mitigate cross-continental lineage spread such as restrictions on amphibian imports and exports and mandatory testing and treatment protocols. These precautions not only could prevent new *Bd* outbreaks but also could help curb the spread of many other plant and wildlife diseases.

Conclusions

Our study provides a broader understanding of the cryptic variation in one of the deadliest wildlife pathogens ever documented. We can now better track pathways of disease spread in

this system and link specific pathogen lineages to outcomes in wild populations. Our genotyping method, optimized for low-quality DNA samples, can be further implemented across different sample types (e.g., museum specimen swabs, environmental DNA samples) to further understand the ecology and evolution of *Bd* and to inform management and mitigation strategies. Although *Bd* has a global distribution, individual lineages that vary in pathogenicity still occur in geographically limited ranges. Thus, as *Bd* genotypes continue to expand their range, we need to consider broader actions that may be necessary to halt *Bd* lineage spread and secondary contact that could have grave consequences for amphibian hosts.

Materials and Methods

The full description of methods can be found in *SI Appendix, SI Methods*. Briefly, we genotyped 222 *Bd* samples using a custom amplicon sequencing assay (21) targeting 191 regions of the *Bd* genome. We generated consensus sequences for each sample at each locus. We then integrated our data with previously published whole-genome data and produced both global and regional phylogenies. To create the global phylogeny, we concatenated loci for each sample with <50% missing data and used RAxML (v.8.2.11; ref. 35)

to iterate over 100 bootstrap samples. We created the regional phylogenies using a gene-tree to species-tree approach. First, we generated gene trees for each loci using RAxML. Second, we used Astral (v.5.6.2; ref. 36) to estimate an unrooted species tree given the set of input gene trees from each regional sample group. Finally, to estimate heterozygosity in sample groups, we calculated the average number of alleles by summing the number of unique sequence variants for each locus, per sample, and dividing by the number of loci sequenced for that sample.

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