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Authors
Byrne, Allison Q
Vredenburg, Vance T
Martel, An
et al.

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

Allison Q. Byrne, Vance T. Vredenburg, An Martel, Frank Pasmans, Rayna C. Bell, David C. Blackburn, Molly C. Bletz, Jaime Bosch, Cheryl J. Briggs, Rafe M. Brown, Alessandro Catenazzi, Mariel Familiar López, Raul Figueroa-Valenzuela, Sonia L. Ghose, Jef R. Jaeger, Andrea J. Janí, Miloslav Jirku, Roland A. Knapp, Antonio Muñoz, Daniel M. Portik, Corinne L. Richards-Zawacki, Heidi Rockney, Sean M. Rovito, Tariq Stark, Hasan Sulaeman, Nguyen Thien Tao, Jamie Voyles, Anthony W. Waddle, Zhiyong Yuan, and Erica Bree Rosenblum

“Department of Environmental Science, Policy, and Management, University of California, Berkeley, CA 94720; “Museum of Vertebrate Zoology, University of California, Berkeley, CA 94720; “Department of Biology, San Francisco State University, San Francisco, CA 94132; “Department of Pathology, Bacteriology and Avian Diseases, Ghent University, 9820 Merelbeke, Belgium; “Department of Vertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington DC 20560; “Department of Herpetology, California Academy of Sciences, San Francisco, CA 94118; “Florida Museum of Natural History, University of Florida, Gainesville, FL 32601; “Department of Biology, University of Massachusetts Boston, Boston, MA 02125; Museo Nacional de Ciencias Naturales, Consejo Superior de Investigaciones Científicas (CSIC), 28006 Madrid, Spain; “Research Unit of Biodiversity, CSIC–Universidad de Oviedo–Gobierno del Principado de Asturias, E-33600 Mieres, Spain; “Department of Ecology, Evolution, and Marine Biology, University of California, Santa Barbara, CA 93106; “Department of Kansas Biodiversity Institute, University of Kansas, Lawrence, KS 66045; “Department of Ecology and Evolution, University of Kansas, Lawrence, KS 66045; “Department of Biological Sciences, Florida International University, Miami, FL 33199; “School of Environment and Sciences, Griffith University, Gold Coast, QLD 4215, Australia; “Department of Evolution and Ecology, University of California, Davis, CA 95616; “School of Life Sciences, University of Nevada, Las Vegas, NV 89154; “Department of Oceanography, University of Hawai‘i at Manoa, Honolulu, HI 96822; “Institute of Parasitology, Czech Academy of Sciences, 370 05 Ceske Budejovice, Czech Republic; “Sierra Nevada Aquatic Research Laboratory, University of California, Mammoth Lakes, CA 93546; “Department of Biodiversity Conservation, El Colegio de la Frontera Sur, San Cristobal de las Casas, Chiapas 29290, Mexico; “Department of Ecology and Evolution, University of Arizona, Tucson, AZ 85721; “Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260; “Environmental Sciences Graduate Program, Oregon State University, Corvallis, OR 97331; “Unidad de Genómica Avanzada (Langebio), Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Irapuato, Guanajuato CP36824, México; “Reptile, Amphibian and Fish Conservation, 6525 ED Nijmegen, The Netherlands; “Vietnam National Museum of Nature, Vietnam Academy of Science and Technology, Hanoi, Vietnam; “Department of Biology, University of Nevada, Reno, NV 89557; “One Health Research Group, The University of Melbourne, Werribee, VIC 3030, Australia; and “College of Forestry, Southwest Forestry University, Kunming 650224, Yunnan, China.

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Biodiversity loss is one major outcome of human-mediated ecosystems 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 BdASIA3, that appears to be widespread in Southeast Asia. This lineage co-occurs with Bd in multiple localities in multiple countries. Additionally, we shed light on the global distribution of BdGPL and highlight the expanded range of another lineage, BdCAPE. 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 naive 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).

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—BdASIA3. 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/ (Bioproject PRJNA555719).

1To whom correspondence may be addressed. Email: rosenblum@berkeley.edu.

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

**Africa.** In Africa, we found that the *BdCAPE* lineage is ubiquitous in Cameroon, while *BdGPL* 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 *BdCAPE* lineage, indicating *BdCAPE* is the dominant, and perhaps exclusive, *Bd* lineage in Cameroon. Furthermore, we found additional support for previous studies documenting the presence of *BdGPL* in Madagascar (22) and provide a report of *BdGPL* in Burundi and Kenya. In Burundi, 43% (3/7) of samples were in the *BdGPL* 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 *BdCAPE* and *BdGPL* 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.** *BdGPL* is the dominant lineage in the Americas (excluding Brazil, where both *BdGPL* and *BdASIA2/Brazil* are found). However, we report *BdCAPE*—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 *BdCAPE*, whereas 89% (17/19) of samples were *BdGPL*. *BdCAPE* may be newly introduced (or detected) in the Americas and occurs in very close proximity to *BdGPL* in Honduras. All
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. 2A) 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—not 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 lineages, 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
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 Bd/Korea and Bd/Brazil into Bd/ASIA2/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 Bd/CAPE and Bd/GPL clades in the Africa phylogeny (SI Appendix, Fig. S5).

However, they do not appear closely related to previously published Bd/GPL/Bd/CAPE 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 Bd/GPL and Bd/CAPE. Our ongoing work includes sequencing more samples from this region to test these 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 Bd/CAPE and Bd/GPL in Honduras. While previous studies have documented Bd in this area and attributed amphibian declines to the pathogen (26), none have reported Bd/CAPE in the Americas. Our finding of Bd/CAPE 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 Bd/GPL and Bd/ASIA3 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 Bd/GPL and Bd/ASIA3 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 Bd/GPL 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 Bd/CAPE and others had Bd/GPL, 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 Bd/GPL, while the native species from the same locality carried Bd/ASIA3. 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 Bd/GPL 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 naive 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 ampiclon 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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