Insights From Genomics Into Spatial and Temporal Variation in *Batrachochytrium dendrobatidis*

A.Q. Byrne*, J. Voyles†, G. Rios-Sotelo†, E.B. Rosenblum*,1
* Department of Environmental Science Policy and Management, University of California, Berkeley, CA, United States
† Department of Biology, University of Nevada, Reno, NV, United States
1 Corresponding author. E-mail address: rosenblum@berkeley.edu

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Abstract

Advances in genetics and genomics have provided new tools for the study of emerging infectious diseases. Researchers can now move quickly from simple hypotheses to complex explanations for pathogen origin, spread, and mechanisms of virulence. Here we focus on the application of genomics to understanding the biology of the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), a novel and deadly pathogen of amphibians. We provide a brief history of the system, then focus on key insights into Bd variation garnered from genomics approaches, and finally, highlight new frontiers for future discoveries. Genomic tools have revealed unexpected complexity and variation in the Bd system suggesting that the history and biology of emerging pathogens may not be as simple as they initially seem.

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Batrachochytrium dendrobatidis (Bd) is a fungal pathogen of amphibians that causes the lethal disease chytridiomycosis. Since its initial discovery in 1998, Bd has been inextricably linked to precipitous amphibian declines occurring around the world[1–3] (Fig. 1). To date, no other pathogen is known to have had such a dramatic effect on such a broad range of host species and in so many different environments.[1] As a result, Bd-related declines have been dubbed "the most spectacular loss of biodiversity due to disease in recorded history".[4]

Bd is a member of an ancient lineage of fungi, the Chytidiomycota, which is in the order Rhizophydiales.[5] The Chytridiomycota are relatively diverse, and most chytrids are saprobes and are primarily aquatic or found within wet soils.[6] Bd was the first chytrid recognized to cause disease and mortality in vertebrate hosts.[7,8] A second related chytrid, *Batrachochytrium salamandrivorans* (Bsal), was recently isolated and found to cause lethal disease in salamanders,[9] but is not discussed in detail here.

Chytridiomycosis has garnered considerable interest from the scientific community since the discovery of Bd in 1998.[7,8] Early investigations used

Figure 1 A diseased frog (*Pristimantis cruentus*) found during a chytridiomycosis outbreak in Panama. Bd, the fungal pathogen that causes chytridiomycosis, has been linked to dramatic amphibian declines around the world.[7] Photo by Jamie Voyles.

ecological, physiological, and immunological methods to understand the effect of Bd on amphibians at the organismal, population, and community scales (reviewed in Refs. [10,11]). However, many questions about Bd origin, emergence, spread, and virulence remained unanswered for many years. In the last decade, molecular approaches have provided key insights into the history and virulence of this pathogen. From early phylogenetic studies with few genetic markers to recent whole genome analyses, genetic and genomic tools have revealed that chytridiomycosis is a far more complex disease system than initially appreciated.

Here, we highlight how molecular approaches have contributed to our understanding of Bd by providing a more mechanistic and nuanced understanding of disease ecology and the epidemiology of chytridiomycosis. We also outline some of the most important unanswered questions and highlight new frontiers for discovery. Throughout this review, we focus on how genomic tools can help reveal variation and complexity in the Bd system. A more in-depth understanding of Bd history, variation, and virulence will lead to a richer perspective on fungal pathogenesis, epidemiology and host–pathogen dynamics, and may also point to practical conservation solutions for emerging diseases of wildlife.

1. INSIGHTS FROM GENOMICS INTO BD VARIATION

1.1 A Molecular Toolkit for Bd

The study of Bd genetics and genomics has evolved over the last decade as new technologies and genomic resources have become available. There are several key molecular approaches that have been applied to Bd research. Quantitative PCR is commonly used as a diagnostic assay to detect Bd from amphibian skin swabs.[12,13] The advent of a qPCR assay allowed researchers to document Bd presence and infection intensity from noninvasive skin swab samples collected in laboratory experiments, field studies[12,13] and from museum specimens.[14,15] To date, the use of swabs for population genetics has been limited by their low quality and/or quantity of DNA. Some studies have sequenced genetic markers from swabs, but these have been limited to the ribosomal DNA internal transcribed spacer (ITS)[16,17] or a small number of polymorphic repetitive loci.[18]

Population genetics and genomics studies have more commonly relied on Bd cultures that must be laboriously isolated and maintained. Many studies

have used traditional Sanger sequencing of nuclear markers to characterize Bd genetic diversity.[16,19] However, whole genome resequencing became feasible after the first Bd genomes were sequenced and as sequencing costs decreased.[20,21] Whole genome sequencing provides thousands of markers for population genomics and allows analysis of structural variation in the Bd genome.[20,21] Whole genome resources for Bd have also made gene expression studies possible,[22,23] which have contributed to our understanding of the pathogenesis of chytridiomycosis. Insights from these molecular approaches into Bd variation at the phylogenetic and geographic levels and at the structural and functional levels are discussed later in the chapter.

1.2 Phylogenetic and Geographic Variation

One of the earliest applications of molecular tools to Bd research focused on resolving an early debate about the emergence and spread of Bd. Specifically, researchers weighed the evidence in support of the "Novel Pathogen Hypothesis" versus the "Endemic Pathogen Hypothesis" (NPH vs EPH[24]). The NPH postulates that Bd is a novel pathogen that recently spread around the world, possibly via carrier hosts, and caused epizootic events in naive amphibian communities.[24,25] In contrast, the EPH suggests that Bd has been historically endemic in amphibian populations, but could have recently become a lethal pathogen due to an environmental perturbation (eg, climate change[26]). At the outset, the answer to the NPH versus EPH question seemed relatively simple because chytridiomycosis appeared to fit a typical emerging disease model.[24] Specifically, Bd was not ubiquitous in amphibian populations, and researchers identified "fronts" or "waves" of disease in locations where mass mortality events occurred following the arrival of Bd (eg, Central America,[27] South America,[28] California[2,29]). In addition, researchers identified amphibian species that appeared to be tolerant of Bd,[30,31] suggesting that they could act as reservoir hosts and facilitate pathogen transmission in wild communities and in the global commercial trade.[25,32]

The first study to evaluate the emergence and spread of Bd with molecular tools used a relatively small number of sequenced loci to assess genetic diversity among 35 isolates.[33] Morehouse et al.[33] recovered only five variable sites in 10 loci surveyed. Based on the finding of low genetic variation among isolates and lack of discrimination among isolates from different parts of the world, Morehouse et al.[33] suggested that Bd was a recently emerged clone. Despite limited resolution due to few genetic markers, this study was formative for a wide range of subsequent research on Bd emergence and spread, specifically because it seemed to support the NPH.[24]

Subsequent molecular studies have revealed a more complex picture: recent emergence and weak geographic structuring for some Bd clades contrasts with deeply divergent, more endemic patterns for other clades.[20,21] These more recent studies have refined our understanding of phylogenetic diversity in Bd by sampling a wider panel of Bd isolates (ie, collected from diverse geographic locations and host species) and a wider panel of markers. Some of these studies have used traditional Sanger sequencing,[16,19] whereas others have relied on whole-genome sequencing.[20,21] Genome-scale approaches have been particularly fruitful and were made possible by two full Bd genomes sequenced by genome centers (JEL423 isolated from *Phyllomedusa lemur* in Panama and sequenced by the Broad Institute; and JAM81 isolated from *Rana muscosa* in California, United States and sequenced by the Joint Genome Institute). The studies that leveraged these genomes used resequencing approaches to obtain genomic data for global panels of Bd isolates collected from a variety of host species.[20,21] These studies used tens of thousands of variable sites, termed single nucleotide polymorphisms (SNPs), to obtain more resolution on the structure of the Bd phylogenetic tree.[20,21]

Taken together, recent studies have revealed previously unappreciated complexity within the Bd phylogeny. Bd contains a geographically widespread lineage that was named the "Global Panzootic Lineage" (or "BdGPL") that appeared to be associated with areas where chytridiomycosis epizootic events occurred.[20] Although BdGPL appears to be a globally distributed Bd clade, some weak geographic substructure has been reported. Schloegel et al.[34] sequenced 45 strains at 36 loci and named two subclades within the GPL. These authors suggested that there is a North American clade (GPLI) and a less genetically diverse globally dispersed clade (GPLII), although subsequent studies have indicated that GPLI is not restricted to North America.[21] Furthermore, Schloegel et al.[34] named a clade of highly divergent isolates from Brazil (Bd-Brazil). The occurrence of a fairly basal Bd-Brazil clade was corroborated by later sequencing studies,[21,35] and this clade may have been present in frog populations in Brazil as far back as the late 1800s.[36] Bd also contains at least two other geographically restricted clades: one from South Africa that may have been transported to Mallorca, Spain (Bd-CAPE) and one found only in Switzerland (Bd-CH).[21] In addition, molecular studies have revealed a number of isolates that appear to be hybrids,[20,21,34,35] raising important questions about the potential for interaction among strains.

Phylogenetic studies of Bd at a global scale have been complemented by molecular studies that focus on questions at finer spatial scales. For

example, a study by Velo-Anton et al.[37] investigated the question of regional genetic diversity in Bd as it spread from North to South in the Americas. Velo-Anton et al.[37] found that Bd isolates showed a pattern of reduced heterozygosity and increased allele fixation along the established North to South invasion path, providing valuable information on the genetic signature of Bd invasion at a continental scale. Other regional population genetics studies have found signatures of long-term endemism of basal Bd lineages. Studies from Japan, China, and Korea have reported several divergent Bd haplotypes, suggesting a longer-term association of Bd with Asian amphibians.[16,17,37,38] However, studies from introduced bullfrogs in China and Korea also reported the presence of Bd-Brazil and BdGPL lineages.[17,37,38] Nevertheless, these results should be interpreted with caution given the difficulty interpreting ITS data (single Bd strains often contain many ITS haplotypes[34]) and because different molecular markers (ITS vs MLST) have given conflicting results.

At finer spatial scales, molecular approaches have elucidated patterns of regional spread and local disease dynamics. Evidence that a single genotype was introduced and spread throughout the Iberian Peninsula points to NPH dynamics in that system.[39] A population genetics study using microsatellite loci conducted in the alpine lakes of the Sierra Nevada Mountains showed that two Bd introductions may have occurred there,[19] although the reliance on relatively few markers make it challenging to draw firm conclusions. Findings such as these have shown that Bd may be introduced and spread rapidly in some regions, but Bd can also show signs of local endemism in other regions.

1.3 Structural and Functional Genomic Variation

Genome resequencing studies have been used to understand not only phylogenetic relationships among Bd isolates but also the evolutionary dynamics of the Bd genome itself. Overall, these studies reveal that Bd has a surprisingly dynamic genome and point to possible molecular mechanisms of virulence in this pathogen. One of the most dramatic patterns observed is that of chromosomal copy number variation (CNV)[21]; specifically, the number of copies of chromosomal segments varies both within and across isolates (from 1 to 5 copies[21]). Therefore, rather than being a strictly diploid species, Bd exhibits a high degree of variation in copy number, and this CNV may be one mechanism facilitating rapid genomic change.[20] For example, a recent study found variation in copy number even over extremely short time scales: a single isolate maintained in the lab exhibited changes in copy

number at more than half of the largest chromosomal segments over only 30 generations.[40] Another large-scale pattern commonly observed in the Bd genome is loss of heterozygosity (LOH). James et al.[41] provided early evidence for LOH that can occur through mitotic recombination. Since then, other genetic and genomic studies have shown that LOH events can occur quickly and affect large regions of the genome.[20,21,37]

The signature of mitotic—or asexual—recombination is pervasive in the Bd genome, but there is also evidence that Bd may occasionally exhibit meiotic—or sexual—recombination. Originally, Morehouse et al.[33] suggested that Bd reproduces primarily asexually. However, even early molecular work recognized that the diversity among Bd isolates could not be fully explained by clonal reproduction.[19] It was hypothesized that sexual reproduction must occur at least occasionally in Bd.[41] Although sexual reproduction has never been directly observed, there is increasing evidence that hybridization may play an important role in dynamics of genome evolution in Bd. Several studies have found evidence that some Bd isolates resulted from recent hybridization.[20,21,35] In fact, some authors suggest that hybridization may have been important to the initial emergence of Bd,[20] although other studies have not explicitly supported this hypothesis.[42]

Genomic patterns such as CNV, LOH, and hybridization have not yet unequivocally been linked to variation in Bd virulence. However, there is mounting evidence that these processes may ultimately have important functional effects. For example, both Refsnider et al.[40] and Piovia-Scott et al.[43] showed that more virulent isolates exhibited elevated chromosomal copy numbers relative to less virulent isolates. These findings are consistent with research that has found functional effects of CNV in other fungal pathogens.[44,45]

Genomic data have also provided hypotheses about specific genomic regions that may play a role in Bd virulence. One method for identifying candidate genes involved in Bd pathogenicity has been to compare the genome of Bd with those of other pathogenic fungi. For example, Abramyan and Stajich[46] identified genes for cell surface proteins in Bd that are orthologs to virulence factors in a rice blast fungus (*Magnaporthe oryzae*). They also identified genes for molecules within a carbohydrate-binding module family that have been linked to pathogenicity in other fungal pathogens.[46]

Another method for identifying functionally relevant genes has been to look for gene family expansions in the Bd genome compared with the genomes of other non-Bd chytrids. For example, Joneson et al.[47] compared the Bd genome to that of the closely related, nonpathogenic species

Homolaphylctis polyrhiza (Hp, a saprobic fungus that is found in leaf litter and does not parasitize amphibians). Joneson et al.[47] found significant expansion in three protease families, which occurred after Bd and Hp diverged from their common ancestor. These protease families include metalloproteases, serine-type proteases, and aspartyl proteases, which have been implicated in pathogenesis in other fungal pathogens (ie, for adherence to, invasion of, and degradation of host cells).[48,49]

Gene expression studies have provided yet another approach for identifying genes that may be important for Bd pathogenesis.[22,23] Genome scale gene expression studies have revealed high degrees of condition-specific variation in Bd gene expression.[22,23] For example, Bd life stages exhibit striking differences in gene expression, providing candidate pathways that may be involved in growth, infection, and pathogenicity across life stages.[22] Bd has two main life stages[8,11,22] (Fig. 2). The dispersal stage is the infectious *zoospore*, which moves with a posterior flagellum. The zoospore encysts,

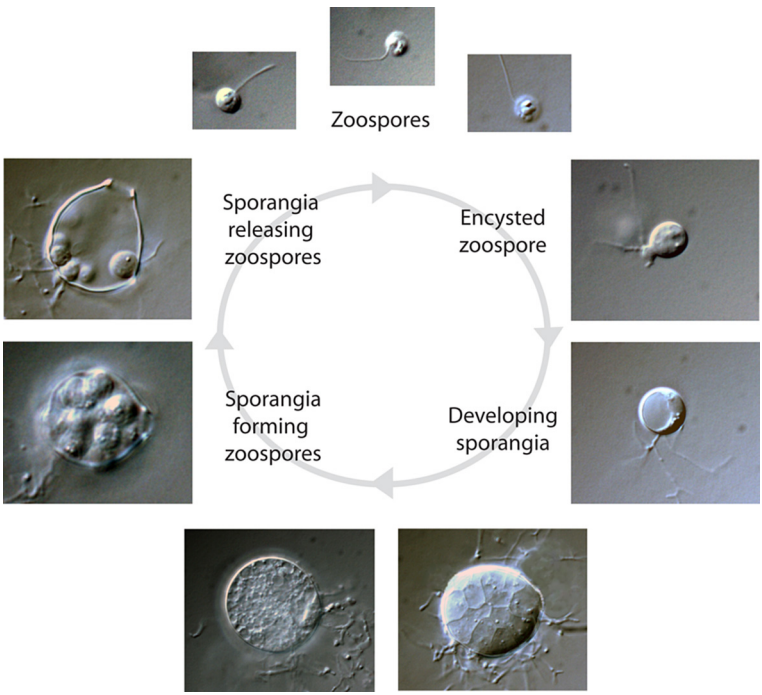

Figure 2 Life cycle of *Batrachochytrium dendrobatidis*. The dispersal stage is the infectious *zoospore*, and the reproductive stage is the *sporangium*. Bd infection of amphibians occurs in the epidermis and disrupts key physiological functions of the skin such as osmoregulation and electrolyte balance.[11]

absorbs the flagellum, and develops rhizoids. The maturing thallus then develops into the reproductive *zoosporangium* (ie, container for zoospores) in which the cytoplasm cleaves and forms flagellated zoospores.[8,11,22] When zoospores are mature, a discharge tube forms and zoospores are released.[8,11,22] The two key life stages (*zoospore* and *sporangia*) have highly differentiated expression profiles with more than half the genes in the genome exhibiting differential expression.[22] Although zoospores are transcriptionally less complex, several transcripts were more abundant in zoospores (including those transcribed from signaling and chitin-binding genes) and may influence Bd colonization.[22] Sporangia, on the other hand, were metabolically and transcriptionally complex, with many genes showing signatures of increased expression during this life stage.[22] Most notably, there was an increase in expression of multiple fungalysin metallopeptidases and serine-type peptidase genes in the sporangia stage, gene families that were previously hypothesized to be candidate virulence factors.[22]

Many Bd genes also show expression changes under different growth conditions. To date, studies on environmentally induced gene expression have focused primarily on Bd genes induced when Bd is colonizing amphibian host tissue.[23] Such studies can identify possible virulence factors that are upregulated during host-cell colonization, a key step in pathogenesis.[11,23] For example, one genome wide study of Bd gene expression on laboratory growth media and host amphibian skin showed that when Bd is exposed to amphibian skin, a number of putative Bd pathogenicity factors were upregulated.[23] In particular, metallo-, serine-, and aspartyl-proteases showed increased expression when Bd was cultured on amphibian skin, supporting findings from previous studies[23] and contributing to the search for putative virulence genes. Other Bd genes of interests that were upregulated on amphibian skin included crinkler and crinkler-like effectors, adhesin genes, and triglyceride lipases, again providing targets for future more focused studies on Bd pathogenesis.[22,23]

2. NEW FRONTIERS IN BD GENOMICS

2.1 An Evolving Genomic Toolkit

Emerging molecular methodologies are revolutionizing the fields of disease ecology and evolutionary biology. Genomic studies are expanding in scale and power while decreasing in cost. By adopting emerging technologies,

researchers can now address previously intractable questions in the Bd system. The application of new molecular tools to the Bd system will allow for a fine-scale understanding of genetic diversity across landscapes and through time and a more refined understanding of the functional role of Bd genes. Whole genome sequencing will continue to be an important approach, especially as sequencing costs continue to decrease. However, there are other promising applications of genomics to the Bd system.

Genome sequences from globally distributed Bd isolates provide a resource for identifying polymorphic regions of the Bd genome. Resequencing data can be used to selectively target diagnostic SNPs for the major and minor clade divisions in the Bd phylogeny or segregating SNPs within geographic regions.[20,21] Researchers have used genomic data from ascertainment panels to locate target regions for SNP genotyping in many different study systems.[50–52] However, SNP genotyping approaches can suffer from ascertainment bias.[53] New (microfluidic) multiplex PCR techniques can circumvent some of these issues and expand the scale of Bd studies. Hundreds of genomic regions (several hundred base pair regions rather than SNPs) can now be sequenced simultaneously and cost-effectively. Sample sizes can be increased dramatically because samples can be barcoded and pooled for sequencing, and hundreds or thousands of samples can be sequenced simultaneously. Therefore, researchers no longer need to use relatively costly whole genome sequencing to obtain population genomic data for Bd.

In the past, genomic approaches for Bd generally required pure Bd cultures. However, new multiplex PCR approaches allow for the amplification of many regions of the Bd genome with low quantities of starting DNA. These multiplex genotyping approaches are especially amenable to Bd DNA collected from skin swabs, eDNA samples, or from museum specimens (where DNA quality and quantity are suboptimal). A number of studies have reported successful genotyping of highly fragmented DNA with this approach using bird feathers;[54] historical fish scales;[55] and noninvasively collected mammal fur, feces, and urine.[56] Therefore, new technologies promise to unlock the genetic information stored in the vast libraries of swab samples that have been collected around the world over many decades.

Another powerful approach for the Bd system is RNA sequencing (RNA-seq), which has been used to characterize functional genomic responses in many model and nonmodel systems.[57,58] RNA-seq offers a number of benefits over the microarray techniques that have been used to study global Bd gene expression in the past. First, researchers no longer need

to invest sequencing and bioinformatic resources toward microarray development (although having a transcriptome sequence is helpful for aligning reads from RNA-seq). Second, RNA-seq data can be used not only for gene expression, but also for population genomics inferences. Third, dual RNA-seq can be used to characterize multiple species responses in parallel.[59] In silico techniques can be used with RNA-seq data to separate host and pathogen transcripts and thus provide a powerful tool to investigate disease dynamics from both host and pathogen perspectives simultaneously.[59,60]

Finally, more manipulative molecular approaches may find application in the Bd system. For example, gene knockdown techniques such as posttranscriptional gene silencing via RNA interference[61] and/or genome editing experiments using techniques such as CRISPR/Cas9[62] can be used to test hypotheses about Bd gene function. Some Bd traits raise challenges for implementing these techniques. Relative to the model fungal organisms in which many of these techniques have been developed and applied (eg, filamentous fungi[63,64]), Bd has an unusual life cycle and complex genetic architecture.[11,20–22] Thus far, it has not been straightforward to develop a transformation system for Bd or to express Bd genes in other expression vectors. However, manipulative genetic approaches hold great promise for functional studies in Bd.

2.2 Applying Genomics to Understand Temporal Variation in Bd

Temporal dynamics represent an important frontier in Bd research. A focus on temporal dynamics over multiple time scales will provide key insights into epidemiological and evolutionary patterns in this host–pathogen system. Molecular tools can be applied to understand genetic variation in Bd over seasonal time scales, during and following epizootic events, and over evolutionary time scales. Molecular tools can also be applied to Bd in both natural and laboratory settings, providing new insights on temporal disease dynamics.

Bd-related amphibian declines have elicited concerted sampling efforts in many affected regions of the world.[2,27–29] As a result, many research groups have built a collection of Bd skin swabs and Bd isolates from natural systems before, during, and after epizootic events.[2,27–29] These swabs have primarily been used to document the prevalence and intensity of Bd in natural systems using qPCR.[12,13] However, the multiplex genotyping methods described previously, designed for use with low quality and quantity DNA samples,

now provide an opportunity to obtain temporally stratified population genetic data from Bd skin swabs.

Genotyping swabs can provide insights into a number of unanswered temporal questions in the Bd system. For example, genotyping Bd swabs across seasons will provide vital information on possible annual fluctuations in the strain composition of the Bd community. Similarly, genotyping swabs that were collected during waves of colonization and spread will help reveal dynamics of genetic diversity over longer time scales (eg, whether Bd typically loses genetic diversity during colonization waves, as has been suggested in some systems).[37] Comparing genotypes of Bd in epizootic and enzootic environments will also be important to understand the hallmarks of genetic diversity as Bd becomes established in new regions and to determine whether there is strain turnover over time.

Another exciting application of multiplex genotyping techniques is to garner molecular data from preserved museum specimens. Molecular studies of museums specimens are increasingly common (reviewed in Ref. [65]), and recently published protocols offer the possibility of obtaining reliable DNA sequences from specimens preserved in formalin (a common preservation methods for amphibians).[66] Multiple studies have documented the presence of Bd on preserved museum specimens and have provided important information on the historical distribution of this pathogen.[14,15,36] However, these studies focused on historical presence/absence of Bd and did not genotype historical Bd samples. Genotyping assays designed with short target lengths are particularly amenable to the often-fragmented DNA present in historical samples. Population genetics analyses on swab data will provide a powerful investigation into the NPH versus EPH question at local and global scales, will shed light on the possible origin of the BdGPL, and will provide an understanding of changes in Bd genetic diversity in natural systems over longer time scales.

Although swabs represent a tremendous untapped resource, obtaining Bd isolates will continue to be essential for understanding evolution at the molecular level. At least for the near future, whole genome sequencing will still be relatively restricted to pure isolates that have high DNA quantity and quality. When researchers isolated and cryoarchived Bd from natural populations, more mechanistic temporal questions about selection can be addressed. For example, comparing the genomes of historical and contemporary Bd isolates may allow researchers to identify signatures of selection in the genome.

Temporal questions about genome evolution and fundamental evolutionary hypotheses about fungal disease dynamics can also be addressed using controlled laboratory experiments. Experimental evolution studies have provided important insights into microbial evolution and species interactions in many systems.[67,68] Bd is well suited for experimental evolution approaches, being easily cultivable and amenable to cryogenic freezing.[69] Prior studies have also shown that Bd can undergo rapid evolution in the lab, observable at both the genotypic and phenotypic levels.[67,70,71] Factors such as passage timing,[67] passage number,[71] and temperature[70] can potentially be agents of selection in the lab. Future experimental evolution studies can solidify putative links between genomic and phenotypic changes with well-designed replication across strains and treatment groups. Passaging Bd under different environmental conditions or different passage regimes (and periodically cryo-archiving isolates for future infection experiments) can reveal how quickly Bd virulence can evolve. Furthermore, exposing Bd to a wide range of selective environments, including re-isolating from host species,[72] will provide a more mechanistic understanding of how Bd can evolve in response to particular hosts or environments. Pairing experimental evolution studies with whole genome sequencing will allow researchers to identify regions of the genome that are correlated with observed phenotypic shifts and will likely reveal more specific molecular targets for selection and virulence in the Bd genome.

2.3 Applying Genomics to Understand Spatial Variation in Bd

Many of the molecular approaches described previously to study temporal variation in Bd can also be applied to future studies of spatial variation in Bd. Although a number of studies have used genetic and genomic approaches to understand Bd genetic diversity at the global scale,[20,21,34] Bd dynamics across finer spatial scales remain understudied. To date, studies of fine-scale genetic diversity in Bd have relied on relatively few samples and/or relatively few genetic markers.[16,17]

Key questions about spatial variation in Bd can now be addressed with whole genome sequencing from cultures and with multiplex genotyping from swab samples and museum specimens. Specifically, spatial variation in Bd can be assessed at multiple nested scales. For example, researchers can study genetic variation of Bd within a single host individual, within a population of a single host species, within a community of multiple host species, and within geographic regions.

Understanding the spatial population structure of Bd at nested scales will help reveal how different Bd strains are distributed across environments and among species and how different biotic or abiotic factors correlate with Bd genetic variation. Large sample sizes across environmental conditions and host species could provide insight into the selective pressures acting on Bd in natural systems. Studies of spatial genetic variation could also help resolve dynamics of Bd dispersal over both short and long distances. Comparing Bd genotypes in vulnerable species to Bd genotypes found in the environment (from eDNA samples) or potential reservoir species could also help reveal pathways of infection and transmission across species. For example, if Bd genotypes collected from single host species are typically more similar to each other than Bd genotypes collected from other hosts in the same community, it may suggest conspecific contact as a primary means of Bd dispersal. Conversely, if Bd genotypes from all host species in a community tend to be similar to each other and to Bd genotypes collected from the environment via eDNA sampling (as done in Schmidt et al.,[73] Chestnut et al.[74]), it may suggest the primacy of environmental reservoirs for Bd transmission. Ultimately, population genomics assays with large sample sizes will provide answers to outstanding questions about Bd dispersal, transmission, and evolution across spatial scales.

2.4 Applying Genomics to Understand Functional Variation in Bd

Building on the work of previous functional genomics Bd studies that characterized gene expression across life stages[22] and growth substrates,[23] future studies can capture additional functional variation and better link genotype to phenotype in Bd. Through the wider adoption of genomic methods in experimental and natural studies of Bd, functional genomics can help resolve outstanding questions about virulence in Bd.

To date, no published studies have characterized Bd gene expression from live hosts, across different host species, or in natural systems. Expanding the current scope of transcriptomic investigations in these ways will provide insights on the specificity of Bd gene expression in different environments. Studies of Bd in more natural conditions could also benefit from a dual RNA-seq approach,[59] whereby gene expression in the host and the pathogen are measured simultaneously. Integrating host and pathogen perspectives in this way could reveal how different host species respond to infection and how Bd gene expression varies under different host conditions.

Studies of Bd in the laboratory will also remain critical for understanding molecular and functional mechanisms of Bd virulence. A particularly intriguing area for gene expression approaches in the Bd system is the interaction of Bd with the amphibian immune system. Previous research suggests that Bd may be able to suppress the host immune system.[75] Some susceptible host species seem to lack a robust immunogenetic response to Bd,[76] although immunogenetic responses have been shown to vary widely among susceptible species.[77,78] To better understand the mechanisms Bd uses to evade or suppress the amphibian immune system, genomics approaches could be integrated with lymphocyte challenge assays, which are now a common tool for phenotypically profiling the ability of different Bd strains to kill amphibian immune cells.[75,79] Implementing a dual RNA-Seq approach[59] using lymphocytes cultured with different Bd strains could provide valuable information on the interaction of host and pathogen at the cellular level and could potentially identify genes induced during the host–pathogen interaction.

In addition to characterizing how Bd interacts with host immune defenses, understanding the functional response of Bd to antifungal treatments is an interesting avenue for future research. Many anti-Bd treatments have been proposed or experimentally tested, including antifungal chemicals, bacteria that produce antifungal compounds, and heat.[80–82] Characterizing Bd's transcriptional response to these treatments could provide insights into how different treatments affect the pathogen at a mechanistic level and could be important for designing new antifungal treatment strategies.

Finally, a critical limitation of gene expression work on Bd to date has been the difficulty of unambiguously linking molecular mechanisms to specific virulence traits. A number of studies have proposed specific gene families, specific patterns of gene expression, or specific genomic processes that could contribute to Bd virulence.[40,43,46,47] However, no studies have robustly linked genotypic and phenotypic variation in Bd. Two approaches offer great promise for the development of a more mechanistic understanding of Bd virulence. First, genome wide association studies could be useful, but only if replicated sets of isolates can be found with and without particular virulence traits. Second, genetic manipulation will ultimately be necessary to conclusively demonstrate the role of particular genes or gene families in Bd virulence. As described previously, there are a number of challenges for applying tools such as RNA interference[61] or CRISPR[62] to the Bd system, but ultimately it will be important to apply manipulative techniques and use insights gained from molecular studies of model fungi to Bd.

3. CONCLUSIONS

Molecular approaches have already provided key insights into the history and virulence of Bd. What was once thought to be a simple story of an emerging pathogen has proven to be a more complex tale. Genomics studies have played a pivotal role in describing phylogenetic and functional variation in Bd and will continue to do so as new molecular approaches are adopted.

The future of genomics research on Bd will now be shaped by how researchers apply emerging molecular techniques to samples collected across space and time (Table 1). It is now possible to couple a wide array of molecular approaches (eg, multiplex PCR genotyping, whole genome sequencing, gene expression analysis, genome manipulation) with a diversity of sampling approaches (eg, swab samples, pure cultures, museum specimens, eDNA samples). For example, multiplex PCR genotyping can be applied to low DNA quantity samples (eg, swabs collected from live animals, museum specimens, eDNA samples) to unlock a wealth of data about Bd diversity across both spatial and temporal gradients. In addition, whole genome DNA and RNA sequencing of cultured Bd isolates collected from the wild and experimentally manipulated in the lab will refine our understanding of evolutionary change in Bd at the molecular level. Furthermore, functional genomics experiments and genomic manipulations can shed light on mechanisms of virulence in this pathogen.

Molecular approaches can advance fundamental knowledge about host–pathogen interactions and can also inform conservation practice. For example, understanding phylogenetic, geographic, and host-associated patterns of Bd variation could help researchers predict virulence of newly invaded Bd isolates. Similarly, identifying regions of the Bd genome under selection and determining the molecular basis of Bd virulence could open new avenues for vaccine development or other chytridiomycosis treatments. Ultimately, developing a mechanistic understanding of spatial and temporal dynamics of Bd in natural systems will be necessary to predict future disease outcomes and mitigate further loss of amphibian biodiversity.

Our focus here has been on application of molecular tools to the study of Bd, but genomics data must be interpreted in light of other complementary datasets to provide a robust understanding of host–pathogen dynamics in chytridiomycosis. Genomics, ecological, evolutionary, epidemiological, physiological, immunological, and mathematical approaches that focus on

Table 1 Common Sampling Methods for Obtaining Genetic Material From Bd and Accompanying Challenges for Genomic Analysis.

Sampling Method	Associated Challenges	Key Genomic Approaches	Potential Novel Contributions
Field-collected swabs	Low quantity/quality DNA	Multiplex PCR genotyping	Genetic variation in Bd across spatial and temporal scales in natural systems
eDNA	Very low quantity/quality DNA	Multiplex PCR genotyping	Bd transmission between hosts and the environment
Museum specimens	Low quantity/quality DNA (highly fragmented in formalin-fixed specimens)	Multiplex PCR genotyping	Bd genetic diversity over historical timescales
Pure cultures isolated from field-collected amphibians	Laborious to isolate Bd from live animals, often requires animal sacrifice	Whole genome sequencing, RNA sequencing	Bd genome evolution and genomic regions under selection in natural systems
Pure cultures manipulated in laboratory experiments	Unintended effects of laboratory maintenance	Whole genome sequencing, RNA sequencing, genomic manipulation	Bd virulence genes and experimental evolution of Bd genomes

Recent advances in molecular techniques have the potential to address these challenges and reveal previously hidden variation in Bd across scales through space and time.

both host and pathogen perspectives must be integrated. When grounded in a natural history perspective, genomics tools can contribute powerfully toward advancing fundamental knowledge and guiding conservation of imperiled amphibians.

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