Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage

Rhys A. Farrerab,1, Lucy A. Weinerta, Jon Bielbyb, Trenton W. J. Garnerb, Francois Ballouxb, Frances Clareab,2
Jaime Boschb, Andrew A. Cunninghamb, Che Weldonb, Louis H. du Preezb, Lucy Andersonb, Sergei L. Kosakovsky Pondab, Revital Shahar-Golanab, Daniel A. Henkb, and Matthew C. Fisherb

*Department Infectious Disease Epidemiology, Imperial College, London W2 1PG, United Kingdom; bInstitute of Zoology, Zoological Society of London, London NW1 4RY, United Kingdom; cMuseo Nacional de Ciencias Naturales, 28006 Madrid, Spain; dUnit for Environmental Sciences and Management, North-West University, Potchefstroom 2520, South Africa; and eDepartment of Medicine, University of California at San Diego, La Jolla, CA 92093

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Batrachochytrium dendrobatidis (Bd) is a globally ubiquitous fungal infection that has emerged to become a primary driver of amphibian biodiversity loss. Despite widespread effort to understand the emergence of this panzooic, the origins of the infection, its patterns of global spread, and principle mode of evolution remain largely unknown. Using comparative population genomics, we discovered three deeply diverged lineages of Bd associated with amphibians. Two of these lineages were found in multiple continents and are associated with known introductions by the amphibian trade. We found that isolates belonging to one clade, the global panzooic lineage (BdGPL) have emerged across at least five continents during the 20th century and are associated with the onset of epizootics in North America, Central America, the Caribbean, Australia, and Europe. The two newly identified divergent lineages, Cape lineage (BdCAPE) and Swiss lineage (BdCH), were found to differ in morphological traits when compared against one another and BdGPL, and we show that BdGPL is hypervirulent. BdGPL uniquely bears the hallmarks of genomic recombination, manifested as extensive intergenomic phylogenetic conflict and patchily distributed heterozygosity. We postulate that contact between previously genetically isolated allopatric populations of Bd may have allowed recombination to occur, resulting in the generation, spread, and invasion of the hypervirulent BdGPL leading to contemporary disease-driven losses in amphibian biodiversity.

chrytridiomycosis | infectious disease | extinction | epidemiology

Emerging fungal diseases present a growing threat to the biodiversity of free-ranging animal species (1, 2). In recent years, a single species within a basal clade of fungi little recognized for their pathogenicity, the Chytridiomycota, has gained substantial notoriety owing to its impact on global amphibian biodiversity (1). Batrachochytrium dendrobatidis (Bd) is known to have driven the local extinction (extirpation) of up to 40% of species in affected communities (3), and to have spread rapidly through diverse environments (1). Despite widespread research efforts, the geographic origin of this emerging infection and its subsequent patterns of global spread remain largely unknown (2, 4, 5). For example, the hypothesis that Bd originated in Africa and spread via the global trade in Xenopus spp. during the first half of the 20th century (6) is disputed by the detection of lower genetic diversity in African isolates of Bd compared with isolates from North America (4). This observation, however, was based on only five African isolates collected from two sites in the South African Cape, compared with 29 US isolates collected from multiple sites across the United States. The genetically depauperate nature of African Bd has been further challenged by the discovery of isolates of African descent exhibiting pronounced differences in morphology and virulence (5, 7).

A separate marker-based study conducted by Morehouse et al. (8) using 10 loci from 35 isolates found very low levels of polymorphism (five variable positions) and fixed heterozygous sites, suggesting a primarily clonal mode of reproduction, although with some evidence for spatially localized genetic recombination (9). These results, and other marker-based studies (4, 6), support the novel pathogen hypothesis, by suggesting that Bd is a recently emerged pathogen (10). Although these results describe the population structure of Bd at a coarse scale, patchily sampled genomes combined with a chronic lack of genetic diversity at the sequenced loci have prevented a reliable inference of Bd’s evolutionary history. Recently, new whole-genome typing methods have greatly increased our ability to decipher genealogies by enabling unbiased sampling of the entire genome, thus increasing our power to date the coalescence of lineages and to identify recombination events. Here, we compare the whole genomes of 20 isolates of Bd to examine the recent evolutionary history of Bd and its patterns of global genome diversity.

Results

Using ABI’s SOLiD system (sequencing by oligonucleotide ligation and detection), we achieved high-density coverage (mean 9.5× deep) of the 24-Mb genome for 20 globally distributed (Europe, North and Central America, South Africa, Australia) isolates of Bd from 11 amphibian host species. Eight isolates were from regions where epizootics have been documented (Table 1). We aligned the reads to the genome sequence of isolate JEL423 (http://www.broadinstitute.org/; 11) and searched for discrepancies using a depth-dependent binomial method (SI Appendix, Figs. S1 and S2), finding in total 51,915 nonredundant homologous SNPs and 87,121 nonredundant heterozygous positions (SI Appendix, Figs. S3 and S4). Of these, 21% of the homologous SNP positions and 19% of the heterozygous positions (22 Kb total) were covered ≥4 reads in all 20 samples and were used for phylogenetic analysis. Sixteen of the 20 samples, including the reference strain JEL423, were >99.9% genetically identical and fell within a single highly supported clade (Fig. 1 and SI Appendix, Fig. S5 and Table S1). This “global panzooic lineage” (BdGPL) includes all previously genotyped isolates of Bd and all of the isolates in our panel that are associated with regional epizootics, recovered from five continents (4).

The remaining four newly sampled isolates form two novel, deeply divergent highly supported lineages. The “Cape lineage”


The authors declare no conflict of interest.

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1To whom correspondence should be addressed. E-mail: r.farrer09@imperial.ac.uk.

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Table 1. The samples used and details of alignments

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<th>Collection site</th>
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<th>Collector</th>
<th>Culture reference</th>
<th>Reads aligned (millions)</th>
<th>Depth (X)</th>
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_Bd_ isolates and locations that were resequenced. The first four columns provide information for the recommended naming scheme outlined by Berger et al. (18). The number of reads (millions) aligning to the _Bd_ JEL423 genome assembly and the corresponding depth of coverage. Amphibian hosts include _Afrana fuscigula_ (Cape river frog), _Alytes muletensis_ (Mallorcan midwife toad), _Alytes obstetricans_ (common midwife toad), _Bufo boreas_ (Western toad), _Hadromophryne natalensis_ (natal ghost frog), _Litoria caerulea_ (smooth newt), _Litoria fallax_ (Eastern dwarf tree frog), _Peltophryne lemur_ (Puerto Rican toad), _Rana catesbeiana_ (American bullfrog), _Rana perezi_ (Iberian green frog), _JEL_, Joyce Longcore; _LB_, Lee Berger; _MF_, Matthew Fisher; _TG_, Trent Garner; and _CW_, Che Weldon.

Fig. 1. Phylogenetic analysis of the 20 resequenced _Bd_ mitochondrial genomes demonstrates three divergent lineages. The locations of the isolates belonging to the different lineages are shown using the same colors as on the JEL423 genome assembly and the corresponding depth of coverage. Amphibian hosts include _Afrana fuscigula_ (Cape river frog), _Alytes muletensis_ (Mallorcan midwife toad), _Alytes obstetricans_ (common midwife toad), _Bufo boreas_ (Western toad), _Hadromophryne natalensis_ (natal ghost frog), _Litoria caerulea_ (smooth newt), _Litoria fallax_ (Eastern dwarf tree frog), _Peltophryne lemur_ (Puerto Rican toad), _Rana catesbeiana_ (American bullfrog), _Rana perezi_ (Iberian green frog), _JEL_, Joyce Longcore; _LB_, Lee Berger; _MF_, Matthew Fisher; _TG_, Trent Garner; and _CW_, Che Weldon.

(BdCAPE) includes two isolated from the island of Mallorca, and one from the Cape Province, South Africa. This clade supports the hypothesis of Walker et al. (5) that spillover of _Bd_ from captive Cape clawed frogs (_Xenopus gilli_) into Mallorcan midwife toads (_Alytes muletensis_) led to the introduction of _Bd_ onto the island through a captive breeding and reintroduction program for this endangered species. The discovery of BdCAPE as a separate lineage to previously genotyped isolates demonstrates that multiple emergences of amphibian chytridiomycosis have occurred, as well as confirming that the trade of amphibians is directly responsible for at least one of these emergences. A third novel lineage, “Swiss lineage,” (_BdCH_) is composed of a single isolate derived from a common midwife toad (_Alytes obstetricans_) from a pond near the village of Gamlikon, Switzerland. Further sampling is necessary to establish whether _BdCH_ is a European-endemic isolate or more broadly distributed.

To ascertain whether genomic features were associated with these lineages, we searched for evolutionary differences by looking for mutation biases, copy number variation (CNV), and genes undergoing diversifying selection in the BdCAPE and BdCH lineages (SI Appendix, Figs. S6–S13) compared with BdGPL. We found that most (89%) of the polymorphisms in complementary determining sequence (CDS) regions caused a transition (A: T ↔ G:C) with a mean _T_v / _S_v of 8.03 with no lineage-specific biases. Synonymous mutations accounted for most of the polymorphisms in the CDS (ratio of 2.21 to nonsynonymous) concordant with the fact that most transitional mutations at twofold-degenerate sites are synonymous. Using the DoS measure of selection proposed by Stoletski and Eyre-Walker (12) and _d_s / _d_d ratios (13), we identified 114 genes in the _BdCAPE_ and _BdCH_ lineages that were significantly different from neutral expectations or had a _d_s / _d_d > 1 (SI Appendix, Fig. S9). Most of these genes had no significant blast matches in the National Center for Biotechnology Information (NCBI) nr database and were otherwise indistinguishable from the rest of the transcriptome. We also detected no evidence for deviations in CNV between lineages using average read depth over each gene as a proxy.

Although no obvious interlineage variation was uncovered by these analyses, clear differences emerged when we examined the locations of the polymorphic sites within the genome. We identified a highly uneven distribution of homozygous SNPs and heterozygous positions across the 16 BdGPL isolates (Fig. 1), which could be observed using a range of window sizes (SI Appendix,
Fig. S14). Furthermore, this pattern was found to be unique to the Bd/GPL (SI Appendix, Fig. S15) and was relatively similar between isolates. For example, we observed a region of low heterozygosity spanning supercontig 2 that was common to all isolates within Bd/GPL. To investigate whether this clustered distribution was due to recombination events, we tested whether the observed pattern was explained by multiple different evolutionary histories using the GARD (genetic algorithm for recombination detection) method (14). We identified 26 recombination breakpoints across the nuclear genome, 14 of which remained significant after Kishino–Hasegawa (KH) testing (SI Appendix, Fig. S16 and Table S3). Seven of these breakpoints were found within chromosomes and seven were found occurring between chromosomes. This suggests that the independent assortment of individual chromosomes expected under a model of frequent meiosis has not eroded the congruent phylogenetic signal between chromosomes, suggesting that meiosis is rare. Taken together, these data support the hypothesis of a single hybrid origin of Bd/GPL via an ancestral meiosis as proposed by James et al. (4).

To further characterize the differences between the 15 significantly different recombination segments, we reconstructed separate topologies for each segment, but sharing the same model of evolution, using Bayesian phylogenetic inference techniques implemented in BEAST v.1.6.1 (15) (SI Appendix, Fig. S16). The three distinct lineages under a model of frequent meiosis has not eroded the congruent phylogenetic signal between chromosomes, suggesting that meiosis is rare. Taken together, these data support the hypothesis of a single hybrid origin of Bd/GPL via an ancestral meiosis as proposed by James et al. (4).

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Fig. 2. Two possible mechanisms of achieving the uneven distribution of heterozygous and homozygous SNPs throughout the Bd/GPL genome. Each black and white bar represents a haplotype identified in a parental isolate, and the charts in the Middle represent the plots illustrating regions where heterozygosity predominates (blue) and where homozygosity predominates (red for homozygous SNPs and empty for homozygous identical to reference). On the Left of the diagram, meiosis generates recombinant haploid genomes that then are united via syngamy into new diploid offspring with patchy heterozygosity. Meiosis involving fusion of diploid gametes could result in similar patterns if chromosomal segregation remains independent. On the Right of the diagram, mitotic gene conversion generates patches of homozygous sites via homologous DNA repair in diploid progeny.

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Fig. 3. Kaplan–Meier survival curves illustrating postmetamorphic survival of animals exposed to BdGPL isolates (red) and BdCAPE lineage isolates (blue). (A) BdGPL isolates were significantly different from both BdCAPE lineage isolates and the negative controls. (B) Significant differences in sporangia size between the three lineages. (C) No significant difference was observed between the three lineages in minimum inhibitory concentration values.

Discussion

One of the more puzzling aspects of the emergence of amphibian chytridiomycosis has been that, whereas epizootics have been widely observed, many susceptible amphibian communities apparently coexist alongside Bd with no evidence of disease. Such coexistence has been attributed to the context-dependent nature of susceptibility to disease, and whereas this is undoubtedly an important factor (20), our data show that Bd genotype is also an important epidemiological determinant. Here, we found that there is a much greater diversity of Bd than was previously recognized, and that multiple lineages are being vectored between continents by the trade of amphibians (BdGPL and BdCAPE). We have characterized hypervirulence in BdGPL, suggesting that the emergence and spread of chytridiomycosis largely owes to the globalization of this recently emerged recombinant lineage (21).

Research has recently suggested the existence of a Bd lineage that is associated with the Japanese giant salamander, Andrias japonicus (22). This lineage, defined by sequencing a short fragment of the ribosomal DNA, is dissimilar to the rDNA sequences of BdGPL, which nonnative North American bullfrogs have introduced to Japan. Therefore, it appears that we can now provisionally recognize at least four lineages of Bd, two of which are possibly endemic (BdCH and Japan), one of which may have been previously endemic to South Africa but was then vectored to Mallorca (BdCAPE), and one of which has a pan-global distribution (BdGPL). This diversity was uncovered from sampling only 20 genomes from a cohort biased toward sampling amphibian populations experiencing chytridiomycosis (and hence infected with BdGPL); therefore, our data suggest that a more extensive diversity of amphibian-associated chytrid lineages is pending discovery. Whether these are B. dendrobatidis sensu stricto, or represent cryptic species, remains to be determined. However, observations that populations infected with non-BdGPL lineages do not undergo epizootics suggest that the diversity of Bd represents a patchwork of genetically and phenotypically diverse lineages. In this case, determining the geographical origin(s) of the parental genotypes of BdGPL will likely remain an elusive challenge until a much wider sample of amphibian-associated chytrid lineages has been sampled to identify the full phylogeographic range of diversity held within the order Rhizophydiales.

The origin of novel virulence in fungal species via recombination/hybridization is a well-recognized pathway underpinning disease emergence for increasing numbers of plant and animal pathogens (18, 19); therefore, our dating suggests that the globalization of BdGPL is compatible with its spread within the amphibian trade.

Materials and Methods

Full details are given in SI Appendix. Libraries were constructed according to the protocols provided by Life Technologies (Fragment Library kit). Fragment library sequencing was performed on two flowcells on an Applied Biosystems SOLiD 3 machine. Two pools of libraries were required: pool 1 contained libraries 1–8 with barcodes 1–8, and pool 2 contained libraries 9–20 with barcodes 1–12 (Table 1). Pooled barcoded libraries were sequenced, and ePCR was performed according to Life Technologies’ (“full scale”) specification (templated bead preparation kits). After a run, a total amount of 260–290 million beads was loaded onto the flowcells. The output read length was 50 bp. The genome sequence and feature file for the chytrid fungus Batrachochytrium dendrobatidis (Bd) strain JEL423 was downloaded from http://www.broadinstitute.org (GenBank project accession no. AATT00000000). The feature file for JEL423 had all but the longest splice variants removed for each gene leaving 8,794/8,819 genes. We trimmed the ABI SOLiD reads to 80 nt to remove low-quality bases from the 3′-end and aligned to the nuclear genome and mitochondrial sequence using Burrows-Wheeler Aligner (BWA) v0.5.8 (29) with default parameters.

The method we used for SNP calling was chosen after assessing the false discovery rate of 97 different settings and three separate methods, and was based on three depth-dependent binomial distributions (SI Appendix, Fig. S2B). For each base in the genome, we asked, given the number of bases agreeing with the reference base (k), and the depth of coverage (n), and a
were standardized among isolates for each exposure. Tadpoles in the negative control treatment were exposed on the same schedule to an equivalent volume of sterile media as was used for Bd treatments, but lacking any Bd. All animals surviving to the end of the experiment were humanely killed.

Photography of Bd used in assessing phenotypic differences between lines was conducted using a Canon EOS 350D (3,456 × 2,304 pixel) field. Initial photographs were taken of the floor of the tissue culture flask where sporanidia had settled 3 post culture. We subcultured the isolates into 12-well tissue culture plates (Nunc), with three replicates per isolate at a concentration of ~8,000 zoospores per well (calculated using an improved Neubauer haemocytometer). The bottoms of the wells were photographed at 10, 15, and 20 d postinital culture. Two to three images were obtained from each well for each isolate. Using ImageJ software (33) we measured the diameter of the largest sporangia contained in the field of view. We determined the MDC90 to iritracan for 12 isolates (estimated 5,750 Bd zoospores in 100 μL of culture) using linear extrapolations of iritracan ranging from 0.07 μg/mL to 2.187 μg/mL (7). Each isolate was incubated at 18 °C and replicated three times. Optical densities were read on days 3, 5, 8, 10, and 12 using a BioTek Absorbance microplate reader (ELx808), using an absorbance of 450 nm.

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