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Chytrid fungi and global amphibian declines

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ABSTRACT:

Discovering that chytrid fungi cause chytridiomycosis in amphibians represented a paradigm shift in our understanding of how emerging infectious diseases contribute to global patterns of biodiversity loss. In this Review, we describe how the use of multidisciplinary biological approaches has been essential to pinpoint the origins of amphibian-parasitising chytrid fungi, including *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans*, to time their emergence, to track their cycles of expansion and to identify the core mechanisms that underpin their pathogenicity. We discuss the development of experimental methods and bioinformatics toolkits that provide a fuller understanding of batrachochytrid biology and inform policy and control measures.

[H1] Introduction

The reasons why modern-day amphibians are suffering rates of extinction that far exceed those of any other class of vertebrates long mystified conservation biologists. The discovery of the disease chytridiomycosis and its aetiological agents, chytrid fungi in the genus *Batrachochytrium*, provided the link between emerging infections and global amphibian declines. Historically underappreciated and infrequently studied, these ancient, aquatic, flagellate fungi have earned notoriety as the leading infectious disease threat to biodiversity. Following the concurrent detection of chytridiomycosis in Central America and Australia in the late 1990's [1] and identification of the cause [2], *Batrachochytrium dendrobatidis* (*Bd*) has been found to infect species across all continents where suitable hosts occur [3,4]. Although *Batrachochytrium* was initially thought to contain only one species the local extinction of fire salamanders in the Netherlands by chytridiomycosis in 2010 led to the discovery of another pathogenic species in the genus, *Batrachochytrium salamandrivorans* sp. nov. (*Bsal* [5]). Both pathogens (here called 'batrachochytrids' for brevity) infect the skin of amphibians. This leads to ulceration due to infection of epidermal cells by *Bsal* whereas *Bd* infects and develops in subcutaneous epidermal cells. Because amphibians need to osmoregulate and respire through their water-permeable skin, skin disruption impairs its essential homeostatic functions and leads to the death of heavily-infected individuals.

Despite over 1,000 studies published since the discovery of *Bd* the original questions regarding the extent of this panzootic **[G]** are still relevant today: where did these pathogenic chytrids come from, when did they emerge, how do they cause disease in amphibians and what can we do to prevent their impact? In this Review, we describe how the adoption of new techniques and methods from across biology and informatics has recently led to a radical change in our understanding of batrachochytrids and chytridiomycosis. To achieve these advances, we explain how a multidisciplinary scientific community built global networks for sharing data, combined field research with modern biological techniques to dissect complex biological systems, and improved the integration of resulting epidemiological data into policy and law with the aim to limit the further spread of these pathogens.

[H1] Mapping the chytrid panzootic

By their very definition, panzootics are a global problem and cannot be tackled by individual people or specialities. The realization that similar patterns of amphibian declines occurred on several continents at the same time was a wake-up call and highlights that an interdisciplinary scientific approach is needed to understand and respond to novel conservation threats. In isolation, herpetologists had recorded rapid

and persistent amphibian declines as early as the 1970s; however, these declines were only recognized as a global phenomenon at the landmark first World Congress of Herpetology held in Canterbury in 1989 and quantified over a decade after the Canterbury meeting [6]. Many declines were initially classified as 'enigmatic', occurring in pristine habitats largely untouched by habitat destruction. These observations spurred a search for the underlying cause, which ultimately led to the discovery and description of *Bd* and its life cycle through a multidisciplinary collaboration [1,2,7].

The development of a non-invasive, robust and probe-based quantitative molecular diagnostic for *Bd* [8] enabled several regional surveillance efforts eventually compiled in an online database, the Global *Bd* Mapping Project. This web-based system for collating *Bd* incidence and associated metadata is an early example of a web-accessible database with application programming interfaces (APIs) for data storage, data uploading, summary statistics, and visualisation of spatial data using Google Maps. The Global *Bd* Mapping project is being integrated into the core AmphibiaWeb site (https://amphibiaweb.org) where data compilation will continue in the foreseeable future. Global mapping provided the first overview of the panzootic: as of May 2019, *Bd* was found infecting 1,015 of 1,854 (54%) species and at 3,705 of 9,503 (39%) field sites (personal communication by D. Olson and K. Ronnenberg, US Forest Service). In 2014, *Bd* infected 50% of tested frog species (order Anura), 55% of salamander and newt species (clade Caudata) and 29% of caecilian species (Gymnophiona) [9] testifying to an extraordinary and heretofore unmatched pathogen host range. By comparison, the host range of *Bsal* is largely restricted to Caudata, with only transient infection of anurans reported[10]. Global surveillance and molecular diagnostics enabled rapid outbreak analysis of *Bsal* in the year of its discovery and in doing so identified an Asian origin of the European *Bsal* outbreak [11,12]. As with *Bd*, surveillance of *Bsal* is being managed and coordinated using online databases and informatic tools (http://www.salamanderfungus.org/about-bsal/ and https://amphibiandisease.org).

Reconstructing the impact of the emergence of the batrachochytrids on amphibian biodiversity has proven a complex task. This difficulty owes to declines occurring years before the identification of *Bd* and frequently in remote areas where amphibian surveillance efforts are haphazard at best. Nevertheless, a meta-analysis[4] synthesised data from multiple sources, including peer-reviewed studies, the International Union for Conservation of Nature (IUCN) Red List of Threatened Species and consultations with the scientists investigating the declines as they occurred (e.g. [13]), and retrospectively (e.g. [14,15]). This meta-analysis revealed that chytridiomycosis has contributed to the decline of at least 501 species (6.5% of all amphibian species), leading to 90 presumed extinctions and decreases in abundance exceeding 90% in another 124 species. At the time of writing, this represents the greatest documented

loss of biodiversity attributable to a non-human species. Truly, *Bd* has earned its nom de guerre as the 'doomsday fungus' [16].

Alongside the collation of epidemiological data, a worldwide effort to collect and archive isolates of *Bd* for molecular and phenotypic analyses was initiated by the European Union Project RACE (Risk Assessment of Chytridiomycosis to European amphibian diversity). This project focussed on modifying the original protocols for isolating *Bd* developed by Joyce Longcore[2] and methods for cryopreservation[17]. RACE developed a largely non-destructive procedure for isolating chytrids from amphibians that, during a decade, was successfully used by a broad collective of researchers working across 5 continents, 23 countries and 62 amphibian species. As a result, *Bd* was isolated from all three orders of Amphibia and from all continents on which infection occurs[18]. This project integrated online databases and digital mapping to store project-related data in a way that enabled access from study sites using the GPS-smartphone enabled epidemiological software EpiCollect [19] (https://five.epicollect.net/project/bd-global-isolation-protocol). With these webtools and smartphone-based technology, research groups working on the batrachochytrids communicated and shared data on a scale that had never before been used to track wildlife diseases, which has been essential for subsequent tracing of the evolutionary history of these pathogens.

[H1] Origin and emergence

[H2] Chytrids 'out-of-Asia'

Debate on how chytridiomycosis emerged as a cause of amphibian declines revolved around two competing arguments. The 'novel pathogen hypothesis' (NPH) stated that chytridiomycosis was emerging locally after it has been seeded by intercontinental trade routes into naïve ecosystems. The counterargument, known as the 'endemic pathogen hypothesis' (EPH), held that *Bd* was a widespread endemic commensal of amphibians that had become more virulent owing to global change forcing imbalanced infection dynamics[20]. Early molecular clues from multilocus sequence typing **[G]** supported the NPH, as the isolates of *Bd* sampled at the time showed no signs of phylogeographic structure from the different regions with amphibian declines due chytridiomycosis [21,22]. This molecular evidence matched the observed patterns of chytridiomycosis observed in the Americas[1], Australia[20] and the Caribbean islands[23] (Figure 1). Later, sequencing of two complete genomes by the Joint Genome Institute (isolate JAM81 from *Rana muscosa* in California) and the Broad Institute (isolate JEL423 from *Phyllomedusa lemur* in Panama) in 2008 [24] and the development of high-throughput shotgun-sequencing enabled genome-scale genetic analysis of *Bd*. Early ABI SOLiD genome resequencing of 20 globally distributed isolates from sites

experiencing chytridiomycosis uncovered striking patterns in comparison to sites without disease. Resequencing identified three deeply diverged lineages: *Bd*GPL (globally distributed), *Bd*CAPE (named owing to its discovery in the Cape region of South Africa) and *Bd*CH (a single deeply branched isolate from Gamlikon in Switzerland [25]). Only *Bd*GPL was found across four continents and associated with epizootics **[G]** in North America, Central America, the Caribbean, Australia, and Europe. The extraordinary global range, limited genomic diversity, relatively high virulence, and origin in the early 20th Century based on the phylogeny of *Bd*GPL supported the NPH over the EPH [25]. Heterozygous and triallelic single nucleotide polymorphisms were 3-4 fold more common than homozygous ones in *Bd*GPL, which was held as evidence that the genesis of *Bd*GPL was the result of a sexual 'hybridization' between two dissimilar parental genotypes [26].

Subsequent analysis of a larger panel of isolates cast doubt upon the findings of this earlier study [25], suggesting both greater genetic diversity and an estimated origin of *Bd*GPL 10,000–40,000 years before present [27]. The authors interpreted these results to support the EPH rather than the NPH. Neither study could resolve the geographic origins of *Bd*, variously proposed to be African[28], Japanese[29], East Asian[30], South American[31], or North American[32].

O'Hanlon *et al* [33] resolved much of the debate, publishing new sequence data for 177 *Bd* isolates collected using the RACE protocols [18]. The complete dataset of 234 isolates were collected over nearly two decades and spanned the geographical distribution of *Bd*, events of lethal chytridiomycosis and all three extant orders of the Amphibia. This analysis redefined the evolutionary relationships amongst lineages of *Bd*, aided by the first genome data from Asian isolates. *Bd* from the Korean peninsula comprised a new 4th lineage, *Bd*ASIA-1, and this lineage showed signs of an ancestral relationship with the other lineages. Bayesian-based haplotype clustering **[G]** revealed that the hyperdiverse *Bd*ASIA-1 lineage shared more diversity with the global population of *Bd* than any other lineage and branched at the base of the *Bsal*-rooted *Bd* phylogeny. Tellingly, *Bd*ASIA-1 was the only lineage in mutation-drift equilibrium **[G]**, a characteristic of endemism. All other lineages showed pronounced departures from equilibrium values of Tajima's *D* statistic **[G]** [34], which are indicative of outbreak dynamics. Molecular screening of museum specimens of amphibians from Korea showed infection has been present in the region for over 100 years[35] and contemporary surveillance has demonstrated a widespread yet patchy and rare distribution of batrachochytrids throughout East Asia[12,36,37], further suggesting endemism of *Bd* in this region. Multilocus genotyping confirmed the results of O'Hanlon et al. [33] and discovered a novel 5th lineage, *Bd*ASIA-3, also found in East Asia (the Philippines, Indonesia and China) [38]. This 'chytrid-out-of-Asia' hypothesis supporting the NPH was strengthened by the finding that, following discovery of

chytridiomycosis caused by *Bsal* in Europe, this chytrid could only be detected elsewhere in south-east Asia (Vietnam)[12]. The comprehensive lack of lethal outbreaks or population declines caused by chytridiomycosis in Asia, despite the widespread occurrence of *Bd* and *Bsal* [4,11], are evidence for endemic host-pathogen interactions[39]. Batrachochytrids appear to have been infecting amphibians in the region for over 50 million years, leaving ample time for fungal speciation events and relatively stable host-pathogen dynamics to establish[11]. Accordingly, there is a need for more intensive pathogen discovery across south-east Asia as unmapped batrachochytrid diversity will likely yield further insights into the past emergence and present distribution of these pathogens.

[H2] Timing the panzootic

Final proof of the NPH required congruency between the timing of introductions of *Bd* and the onset of declines caused by chytridiomycosis. Chytridiomycosis-declines peaked globally in the 1980's [4], in keeping with the timing of regional wave-like dynamics suggesting epizootic spread from point sources [40,41]. To time introductions, O'Hanlon et al. [33] used two quasi-independent genomic regions to generate time-calibrated phylogenies and a Bayesian framework to estimate their time to most recent common ancestor (TMRCA, Box 1)**.** These analyses estimated a substitution rate for *Bd*, one that was broadly similar to that estimated for other unicellular fungi. The updated TMRCA for the ancestor of *Bd*GPL ranged between 120 and 50 years ago (1890's–1960's), which broadly agrees with the first inferred chytridiomycosis-related declines in regions that are currently dominated by *Bd*GPL (Australia[20,42], the Mesoamerican peninsula[13] and South America[14,40]). Molecular dating also suggests that the widespread, and still largely unattributed, amphibian declines reported in Europe and North America in the 1950's and 1960's were driven by *Bd*GPL, which has now achieved widespread endemicity across these regions[6,43].

What has fuelled the global expansion of *Bd*? That all known lineages of *Bd* are circulating in globally-traded amphibians proves that trade is disseminating amphibian vectors of batrachochytrids worldwide [44] today [33] (Figure 2). For example, 'African' *Bd*CAPE invaded the island of Mallorca through the reintroduction of captive reared Mallorcan midwive toads infected in captivity by African endemic amphibians (*Xenopus gilli*) [45]. More widely, infection-tolerant species such as the African clawed frogs *Xenopus laevis* [28] and north American bullfrogs *Lithobates catesbeiana* [44] are internationally traded in their millions and have been since early the 20th century. Other infection-tolerant species, such as the cane toad *Rhinella marinus*, have established feral populations from their origins in South and Central America. It is likely that these species had an important role in amplifying the worldwide emergence of *Bd* and indeed, molecular methods have identified transcontinental links involving

these species [46]. The evidence therefore suggests that the original out-of-Asia vectors of batrachochytrids were likely amphibians exported either for food, research or collections, or perhaps passively hiding in traded goods. However, identifying these original panzootic 'sparks' will likely prove a challenging task.

[H2] Cycles and circles of expansion

Occurrence of the divergent *Bd*CAPE in Africa, Central America and Europe [33,38], *Bd*ASIA-2/BRAZIL in the Brazilian Atlantic forests [31] and Korea [33], and the ASIA-1-like *Bd*CH in Switzerland show that the evolutionary history of *Bd* is complex and characterized by at least three out-of-Asia emergences of lineages other than *Bd*GPL. With too few isolates to confidently derive measurable evolutionary rates, the TMRCA for these lineages have thus far not been estimated. Notwithstanding, levels of diversity exceed those seen in *Bd*GPL suggesting that their out-of-Asia dispersal predates that of *Bd*GPL [33]. The detection of molecular signatures of *Bd* in Brazilian museum collections of amphibians indicates that Brazil was invaded by *Bd* as far back as 1894 [31]. While awaiting molecular confirmation, it appears that the early invasion was by *Bd*ASIA-2/BRAZIL, followed by a secondary introduction of *Bd*GPL into Brazil in the 1970s. The result was a peak of declines in the 1970s owing to the higher virulence of this lineage [14] and the founding of a region of contact between the two lineages in the Brazilian Atlantic forest [47-49]. To complicate matters further, *Bd*ASIA-2/BRAZIL is itself found in Korean populations of introduced North American bullfrogs, suggesting that these widely-traded frogs have been vectors for this lineage, re-establishing it in its ancestral Asian homeland [33].

Surveillance across Africa shows that this continent also has a complex history of *Bd* introductions[50]. The pathogen is widely present, occurring in Cameroon from at least 1933, Kenya in 1934, Uganda in 1935, South Africa in 1938, the Democratic Republic of Congo in 1950 and Bioko island in 1966 [28,51-54]. The infection status of the amphibians of Madagascar remains unclear [18,55,56]. The extent that Africa has suffered amphibian declines as a consequence of chytridiomycosis is largely undetermined. However, at least one extinction in the wild has occurred (the Tanzanian Kihansi Spray Toad, *Nectophrynoides asperginis* [15]) and the presence of *Bd* has been correlated with declines of amphibian species in Cameroon [57] and South Africa [58]. Genome sequencing [33] and multilocus genotyping [38] has shown the widespread occurrence of both *Bd*CAPE and *Bd*GPL, the former widely distributed in Cameroon, including in caecilians [59], and the latter occurring in both Ethiopia and Uganda. Both lineages occur in Southern Africa where, similar as in Brazil, lineages are in spatial contact. The patchy distribution of *Bd*CAPE in central America and Europe suggests that secondary waves of expansion for this lineage have occurred.

[H2] Recombinants, not hybrids

Genotyping has identified recombinants of *Bd*ASIA-2/BRAZIL and *Bd*GPL in the Brazilian Atlantic forest [48], and genetic mosaics of *Bd*CAPE and *Bd*GPL in South Africa [33]. Within lineages, alleles segregate [47,60], intrachromosomal recombination breakpoints have been detected [25] and when single nucleotide polymorphisms are phased **[G]** , crossovers **[G]** are observed in all lineages that have been tested [26]. Clearly, the extreme genetic bottlenecks that characterise the out-of-Asia evolutionary history of *Bd* have not impaired the ability of this species to recombine. Whereas chytrids such as *Allomyces* and *Rhizophydium* undergo meiosis **[G]**, recombinant mating structures have not been described for *Bd* or *Bsal* nor canonical fungal mating-type alleles **[G]** identified, suggesting that recombination in batrachochytrids may not be meiotic. In support of this, some 'meiotic toolbox' genes defined in yeast are missing in the genome of *Bd* and signatures of sex-associated, repeat-induced point mutations in transposable elements are also absent [61]. Further, widespread chromosomal copy number variation **[G]** [26] is also evidence that recombination may not owe to meiosis. Accordingly, it has been proposed [25,62] that non-meiotic recombination (called 'parasexual' recombination) may be generating the polyploid heterozygous mosaics that characterise *Bd*. However, the cell biology that underpins the widespread recombination, either meiotic or non-meiotic, in *Bd* remains wholly unexplored.

That the global population of *Bd* stems from a genetically diverse Asian population in mutation or drift equilibria and recombines when the opportunity arises, shows that the global *Bd* population is currently behaving as a cohesive biological species. Prior to the discovery of its Asian origin, inter-lineage recombination events were termed 'hybridisations', and the origin of *Bd*GPL was suggested to result from a hybridisation event amongst two related chytrid species [25]. However, the simplest description of the global population genetic structure of *Bd* is that each lineage represents separate genealogical 'draws' from a recombining parental population that is most likely Asian. As multiple founding events do not appear to have appreciably blunted the ability of *Bd* to shuffle its genome if given the opportunity, it is premature to give these lineages species status and to name recombinants 'hybrids'. Accordingly, the most biologically accurate description of the genomic mosaics that are increasingly being described are 'recombinants'.

Finding that *Bd* is a recombining species is not only academically interesting. The process of recombination through secondary contact is likely important in an epidemiological context. Outcrossing can purge deleterious alleles and generate variation that may facilitate host exploitation, exacerbating epizootics. Theory and experimentation have shown that interactions between diverse genotypes can lead to

competitive interactions that result in increased transmission and may exacerbate infection dynamics [63,64]. Coinfections of *Bd* lineages have been observed in South Africa where *Bd*GPL and *Bd*CAPE co-occur[65], and in absence of a defined environmental developmental stage, coinfection is when recombination events will occur. That recombination can affect the virulence of *Bd* was demonstrated in a study[49] that showed that *Bd*GPL and *Bd*ASIA-2/BRAZIL recombinant genotypes were more aggressive than those of both parents in two amphibian species. Their result suggests that outcrossing in *Bd* results in genetic dominance and enhanced fitness. Whereas these hybrids were inferred to be F1, an F2 backcross in Brazil has been observed suggesting that recombinants can survive beyond their immediate F1 genesis [48].

[H1] Batrachochytrid virulence

Infection of amphibians by *Bd* and *Bsal* is a remarkably complex process that can have markedly different outcomes, ranging from mild or no symptoms to death (Figure 3). Here we discuss the genetic factors that underpin the expression of the batrachochytrids intrinsic ability to infect the amphibian dermis, alongside the biotic and abiotic factors that modify the outcome of these host-pathogen interactions.

[H2] Genetic factors

The identification of significant variation in virulence both within and amongst lineages has raised more questions than have been answered. We and others have shed some light on which intrinsic genetic factors underpin virulence in batrachochytrids (Figure 4).

Comparisons with the genomes of free-living saprobic chytrids have shown greater secreted protein repertoires and extensive gene-family radiations in the pathogenic batrachochytrids [66,67]. Metalloproteases in the M36 metalloprotease fungalysin family are important pathogenicity determinants in a number of skin-infecting fungi, and are strikingly expanded in both *Bsal* and *Bd* with 110 and 35 of theses proteases, respectively, compared to the free-living saprobic chytrids *Spizellomyces punctatus* and *Homolaphlyctis polyrhiza*, which have 2 and 3, respectively [24 66]. That the M36 metalloproteases are highly expressed *in vivo* and *in vitro* is in line with their role as virulence factors, however differences in the number of copies and timing of their expression between *Bsal* and *Bd* suggest different roles in pathogenicity [66]. Carbohydrate-binding modules (CBM) are markedly expanded in both batrachochytrids compared to free-living Chytridiomycota and may be important in host recognition and adhesion [66,68]. There is pronounced divergence in other gene-families that could explain the substantial variation in the host range and

362 epidemiology of *Bd* and *Bsal*. *Bd*'s significantly smaller genome (23.7 Mb versus 32.6
363 Mb for *Bsal*) contains regions of low gene density characterised by a proliferation of
364 crinkler-and-necrosis (CRN-like) genes, which are expressed during the early stages of
365 infection, whereas the *Bsal* genome contains two expanded secreted tribes of genes
366 of unknown function, which are highly expressed during infection. Clearly, although
367 mining the genomes of the batrachochytrids has identified features that are linked to
368 infection, further exploration is needed to understand the role of these diverse
369 expanded gene-families in infection. The development of new models of infection is
370 needed to increase understanding of batrachochytrids biology. Recent advances, such
371 as amphibian cell culture and skin-explant models [69], and *in vivo* zebrafish *Bd* infection
372 models [70], are exciting developments.
373
374 The observation that different genotypes and lineages show some variation in plastic
375 morphological traits such as the number and size of infectious zoospores suggests that
376 virulence traits may be to some extent governed by simple parameters such as growth
377 rate and fecundity [71,72]. Genetic factors that modify growth rate and investment in
378 zoospores may be found in the large number of genes that are upregulated during
379 infection. Additionally, putative, secreted virulence factors affect host colonization
380 rates, the first step in the pathogen life cycle. Despite its recent evolutionary history,
381 the virulence of *Bd*GPL genotypes is highly variable under controlled experimental
382 settings and virulence is to a large extent determined by how rapidly *Bd* establishes
383 infection [25,33]. Moreover, within the laboratory, passaged isolates show high
384 evolvability [26], attenuation [73,74] and phenotypic variation [75]. As described above,
385 genome architecture is highly plastic across short time-scales, involving large scale
386 rearrangements that should affect traits involved in host damage [26,76]. The plasticity
387 in virulence observed in *Bd*GPL seems to be mirrored by other lineages, with
388 substantial lethality observed in experimental exposures (eg. [39,49]). Although less is
389 known about variation of virulence in *Bsal* owing to all isolates currently stemming
390 from a single epizootic clone, the discovery of an environmentally-persistent encysted
391 zoospore suggests that this species also may manifest phenotypically-plastic life-
392 history traits that affect virulence [10].
393
394 **[H2] Abiotic factors**
395
396 Batrachochytrids may carry a diverse and variable array of genetic traits that influence
397 virulence, but the global emergence of chytridiomycosis is a radically novel
398 epidemiological event, affecting hundreds of host species near-simultaneously and
399 interacting intimately with the diverse environments that they occupy (Figure 3).
400 Despite overwhelming evidence that batrachochytrids are invasive outside of East
401 Asia, once established, environmental factors have an important role for disease
402 outcomes and infection dynamics may map more closely with the predictions of the

EPH. Indeed, ecological factors have been identified as important determinants of disease outcomes, such as climate and altitude [77,78], seasonality [79-82], ultraviolet exposure [83,84] and agrochemicals [85]. Combining field observations with experiments has illustrated the processes through which the environment affects infection and disease. These processes include the importance of reservoirs of transmission [86,87], how the environment affects the survival and abundance of infectious zoospores [81,88-90] and how increasing zoospore density drives host mortality through increasing burdens of infection [91,92]. Trophic interactions can also affect the density of the infectious zoosporic stages in the environment [10,93]. A note of caution here, laboratory measurements of virulence that disregard ecological variation identified in field studies can have limited predictive utility. For instance, repeated experimental observations that virulence of *Bd*GPL exceeds that of *Bd*CAPE [25,33] do not explain why the two lineages are equally likely to be associated with chytridiomycosis and amphibian declines in nature [33]. Even the endemic Korean *Bd*ASIA-1 has been shown to be virulent in non-Korean amphibians [39], showing that its long coevolutionary history has not blunted this lineages virulence.

Extrapolating environmentally-driven processes to global change has been predominantly a macroecological exercise [94] and changing climates have been shown to force patterns of chytridiomycosis. For instance, although early analyses suggesting climate change drove patterns of chytridiomycosis in Costa Rica [95] were only weakly statistically supported [94], associations between El Niño events and chytridiomycosis have been demonstrated [96]. Increasingly, studies are attempting to incorporate environmental factors into epidemiological models that attempt to predict the outcome of infection at the population level, with a focus on single, highly susceptible, host species such as midwive toads [97 37,80]. In these studies the host species that were infected during seasonal 'outlier' events experienced mass mortality events not occurring after colder, longer winters, and included a species previously predicted to be qt low risk of disease by a macroecological analysis [98]. Less disconcerting, a 16-year time-series[99] disentangled the impacts of *Bd* and climate warming on nine montane species in Iberia. Surprisingly, only a small subset of the host community appeared affected by chytridiomycosis, and regional warming promoted range expansions of some species into the region where disease had decimated one host species decades previously; only a single species showed reasonably tight links between temperature fluctuations and infection dynamics [99].

[H2] Biotic factors

Host responses to chytridiomycosis vary on different levels, ranging from individual to population and host community structure (Figure 3). At the individual level, evidence exists for both resistance and tolerance strategies that may involve adaptive and

innate immune responses [100]. *Bd* can evade lymphocyte responses as part of adaptive immunity[101], but evidence exists that hosts can, to some degree, improve repressed immune responses over time [102]. Whether or not chytridiomycosis has exerted selective pressure on these and other components of adaptive immunity is uncertain, but at least for some host species evidence supports this scenario, or alternatively the pre-existence of host genetic variation that preceded the emergence of the global panzootic and facilitated tolerance to infection when the initial outbreak occurred [103]. Equally, or possibly even more, important, is the innate arm of the amphibian immune response, which has been predominantly explored through investigations of secreted antimicrobial peptides (AMPs)[100]. An example of the importance of AMPs is the threatened European salamander genus *Speleomantes*, all species of which secrete skin peptides that decrease zoospore survival and thereby prevent infection [104]. As with adaptive immunity, the innate immunity afforded by AMPs is influenced by exposure to batrachochytrids. Adaptation of AMPs appears to be the primary driver behind the recovery of some anurans that experienced catastrophic declines due to the emergence of chytridiomycosis in Central America [105]. For most amphibians, adaptive and innate immunity vary substantially across host life history stages and age classes, and as a result, so does host susceptibility to infection and disease. This means that, within a single population, one species can simultaneously be a infection-tolerant, often larval, reservoir whilst being at risk of decline due to chytridiomycosis in its mature stages [86 84].

A particularly topical vein of research is exploring how transkingdom interactions between commensal fungi and bacteria of the amphibian skin microbiota may limit batrachochytrid infections [93,106-109]. An extension of this question is to understand how pathogen competition can alter batrachochytrid infection dynamics and virulence. Although at very early stages, experiments have illustrated how intraspecific competition amongst *B. dendrobatidis* lineages may be in part responsible for the emergence of the global pandemic lineage *Bd*GPL [110] and coinfections may be a precursor for the patterns of recombination we have discussed above [33]. Furthermore, batrachochytrids will interact with other amphibian pathogens such as the emerging ranavirus, and field data suggest that host declines to cocirculating pathogens exceed what would be predicted if interactions were additive [111]. Whether this is attributable to shifts in batrachochytrid virulence is uncertain, and a more likely explanation is that sublethal *B. dendrobatidis* exposures are facilitating the invasion of a viral pathogen (TWJ Garner, unpubl. data). In either case, interactions between batrachochytrids and other pathogens can shift epidemiological patterns, either through dynamical processes, natural selection, or both.

[H1] Mitigating batrachochytrid threats

485 Studies [80] [99] showing species-specific and variable responses illustrate how we cannot
486 generalize the impacts of batrachochytrids. The emergence of lethal chytridiomycosis
487 can be persistent or transient and the effects on host communities can in themselves
488 modify the virulence of batrachochytrids [4,105]. Nevertheless, the global increase in
489 incidence of new fungal infections alongside those that have evolved to evade control
490 has led to the recognition that we urgently need to strengthen detection, monitoring
491 and control of fungal disease [112,113]. The identification of East Asia as a hotspot of
492 batrachochytrid diversity alongside its relatively unsurveyed status suggests
493 undiscovered chytrid biodiversity in this region that requires urgent investigation. Our
494 finding that all known lineages of *Bd* are circulating in globally-traded amphibians
495 proved that, despite listing by the World Organisation for Animal Health, trade is still
496 disseminating amphibian vectors [33] (Figure 2). Stage-specific goals and management
497 actions can theoretically be deployed to prevent and/or manage wildlife disease[114].
498 Before the emergence of wildlife pathogens, biosecurity is a first line of defence and
499 therefore needs strengthening through import controls and establishment of an
500 infection-free trade [115]. Motivated by the discovery of *Bsal*, the European Union has
501 implemented health protection measures for the trade of salamanders [116], and similar
502 measures have been adopted by the USA [117] and Canada [118]. These pre-emergence
503 'prezootic' biosecurity-oriented strategies remain the best option for avoiding disease
504 emergence and should be urgently adopted across uninfected regions and countries.
505
506 Combating wildlife diseases after invasion is extremely challenging with only one
507 partially successful example for chytridiomycosis. In this example a chemical-led
508 approach using the antifungal itraconazole and the environmental disinfectant virkon
509 was applied to eradicate *Bd* from Mallorca, which only partially succeeded. However,
510 this approach is not likely applicable to more ecologically complex settings [45,119].
511 Bioaugmentation of amphibian cutaneous microbiota and vaccination have been
512 proposed as methods to strengthen the resilience of amphibians against invasive
513 chytrids. However, despite promising *in vivo* studies (reviewed by [115,120]), this
514 approach has yet to be successfully implemented (but see [121]). In situations in which
515 species are highly threatened by the pathogen, their safeguarding through
516 establishing *ex situ* captive breeding programs currently remains the only active
517 conservation method to avoid species loss after invasion. Amphibian Arks **[G]** maintain
518 the possibility for selective breeding or genetic modification of amphibians for
519 resistance, and it is likely that advances in gene-editing will be used to augment
520 amphibian immune responses to batrachochytrids in the future[115]. Clearly, the factors
521 discussed above do not operate in isolation. Interactions between chytridiomycosis
522 and other threatening processes are well-described, and we are beginning to explore
523 how pathogen genotype, host immunity and environmental conditions generate non-
524 linear patterns of infection and disease. There is every possibility that strategies for
525 mitigating chytridiomycosis in nature will involve largely ignoring the pathogen and

526 focussing on mitigating other threats or modifying environments and host
527 communities so that host responses may operate more effectively. Whatever our
528 responses, the main lesson from the panzootic of chytridiomycosis has been that
529 biodiversity is far less resilient against emerging infections than was previously
530 believed [122]. This has been further confirmed in other systems as microorganisms
531 continue to cross continental barriers—the devastating emergence of bat white nose
532 syndrome is a case in point [123]. The fragility of wildlife health in the face of
533 globalisation eroding geographical constraints to pathogen spread is exemplified by
534 panzootic chytridiomycosis. It is heartening to see that rapid policy measures enacted
535 following scientific advances are on the rise now that the consequences of failing to
536 prevent batrachochytrid introductions are more widely realised. Although we believe
537 that research will eventually yield the means to mitigate the emergence of wildlife
538 diseases, for research to have its impact reinforcing links between science, policy and
539 the public will be key to success.
540
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Competing interests

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Figure 1: Global distribution of *Batrachochytrium*. As of 2019, *Batrachochytrium dendrobatidis* (*Bd*) has invaded and caused chytridiomycosis in six regions globally: western Australia, the Mesoamerican peninsula, South America, the western United States, Africa and Europe. Five lineages of *Bd*, as well as recombinants, have been identified. In addition, another species, *Batrachochytrium salamandrivorans* (*Bsal*), was discovered in 2010. Batrachochytrids cause severe amphibian declines. The figure shows declines that match Scheele et al[4] category 3 or above (3, extreme decline with >90% of individuals lost; 4, presumed extinct in the wild (no known extant populations, and no individuals detected at known historical locations, but some reasonable doubt that the last individual has died); 5, confirmed extinct in the wild (as per IUCN listing). Maps adapted from [65]

Figure 2. Global spread of *Batrachochytrium dendrobatidis* and amphibian trade. A | Intercontinental movements of *Batrachochytrium dendrobatidis* (*Bd*) was inferred from geographically separated isolates that form closely related phylogenetic clades with high bootstrap support (≥90%). Numbers show where isolates of *Bd* have been recovered from traded amphibians, with pictures of the species involved shown on the left hand side of the figure. B | The movement of CITES-listed amphibians is listed and the figure shows their global movements owing to trade. Part B adapted from ref [131] (map from https://science.sciencemag.org/content/363/6434/1386 & permission is needed or should be redrawn).

Figure 3: Factors influencing the virulence of batrachochytrids. The host response to batrachochytrid ranges from resistance to lethal infection and several factors have been identified that contribute to this variability. For one, pathogen lineages vary in their genetic repertoire of proven and suspected virulence factors, including proteases, carbohydrate-binding modules, Crinkler-like proteins and other secreted proteins, such as tribes of expanded gene families. The genomic potential for virulence is influenced by the genome plasticity of batrachochytrids, which has contributed to the expansion and radiation of gene families with potential roles in pathogenicity. Host susceptibility also varies greatly, depending on the host immune responses, prior exposure to chytrids and/or other pathogens, the host microbiota and the host life history (for example, developmental stage). Amphibian larva, as well as other alternative hosts such as crayfish, can function as pathogen reservoirs. Finally, abiotic,

environmental variables, such as climate, water system properties, pesticides, fertilizers and others, also influence the outcome of batrachochytrid exposure.

Figure 4. Pathogenic potential of batrachocytrids. (A) Genome alignments show gene-family expansions that discriminate pathogenic batrachochytrids (*Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal)*) from non-pathogenic chytrids (*Homolaphlyctis polyrhiza (Hp)* and *Spizellomyces punctatus (Sp)*) (B) For example, the M36 metalloproteases, a gene family involved in infection, have been amplified in the genomes of pathogenic batrachochytrid lineages, and especially in the genome of *Batrachochytrium salamandrivorans* (*Bsal*). (C) *Bd* growing on explanted amphibian skin secretes proteases, which cause extensive skin digestion (far right), whereas the non-pathogenic *Hp* (middle) leaves the skin intact. (D) *Bd* but not *Bsal* zoospores show high concentrations of proteases prior to infection suggesting that the proteases have a role in the initial establishment of infection for *Bd* but not *Bsal*. Part A adapted from ref [66], part B and D reproduced from ref [66] and part C reproduced from ref [24].

Box 1. Dating the emergence of *Batrachochytrium dendrobatidis.* Sequence data is increasingly being used to time epidemiological events ranging across different infections (for example, the emergence of HIV-1 [124], the spread and diversification of plague [125] and the emergence of *Cryptococcus gattii* in North America [126]). For microbial species with rapidly evolving genomes or short generation times, genetic lineages may measurably diverge over observable timespans, allowing substitution rates to be directly calculated rather than assumed [127]. Calculation is based on known dates of isolation to determine the rate of evolution. For example, the amount of sequence change that has occurred between cultures of *Batrachochytrium dendrobatidis* (*Bd*) isolated from *Xenopus* and *Litoria* frogs together with the date of isolation ($T_{(X)}$ and $T_{(L)}$ in figure, part A) can be used to estimate an evolutionary rate and thus the time at which the pathogen lineages in the two frogs most recently shared a common ancestor (T_{MRCA}). This method is known as tip dating [128] and several computational packages exist to carry out such analyses (reviewed in [129]). Measurable molecular evolution has occurred between $T_{(X)}$ and $T_{(L)}$, which, together with data from other isolates (figure, part B) can be used to estimate the rate of evolution. A core assumption of tip dating is that sequences are not recombining, as this introduces additional divergence that is not linearly related to T_{MRCA}. To avoid this bias, genome sequences can be statistically 'cleaned' of recombining sites using programs such as Gubbins [130], or can focus on recombination-free genomic regions such the mitochondrial genome. Attempts to date the emergence of *Bd* either assumed a rate of molecular evolution extrapolated from other eukaryotic species [27], or used tip dating on nuclear genomes in which major recombination breakpoints had been taken into account [25]. The former method dated the origin of *Bd* in the region of 26,400 years

ago, whereas the latter method estimated a more recent origin 35–257 years ago. At 299,707 bp *Bd* has the largest mitochondrial genome of any fungus [33] and contains substantial diversity. Tip dating based on the mitochondrial DNA of *Bd* estimated a T_{MRCA} for the emergence of *Bd*GPL as 1962 (1859–1988), substantiating earlier estimates based on nuclear DNA and matching the onset of global amphibian declines [4] (figure part C; arrow indicates when *Bd* was discovered; severity of declines is shown as the cumulative number of lost individuals). Part C adapted from ref[4].

Glossary

Panzootic: global outbreak of an infectious disease in animals.

Multilocus sequence typing: Matching DNA sequences of fragments of multiple housekeeping genes to assay genetic diversity

Epizootic: local outbreak of an infectious disease in animals

Bayesian-based haplotype clustering: population assignment using large numbers of resequenced genomes

Mutation-drift equilibrium: where the rate at which variation is lost through genetic drift is equal to the rate at which new variation is created by mutation

Tajima's *D* statistic: population genetic test statistic to distinguish between DNA sequences evolving neutrally (at mutation-drift equilibria) to those evolving under a non-random process such as demographic change or natural selection

Phased: assigning alleles to the paternal and maternal chromosomes

Crossovers: segregation of alleles between homologous chromosomes through DNA breaks and reconnections

Meiosis: sexual recombination resulting in crossovers

Mating type-alleles: genes regulating compatability leading to meiosis in fungi, also called mating type 'idiomorphs'

Chromosomal copy number variation: where the number of copies of a haplotype varies between one individual and another, also known as 'aneuploidy'

Amphibian Arks: *ex situ* breeding of threatened species in biocontainment facilities

Related links

AmphibiaWeb https://amphibiaweb.org

North American Bsal Task Force http://www.salamanderfungus.org/about-bsal/

The Amphibian Disease Portal https://amphibiandisease.org

Epicollect https://five.epicollect.net/project/bd-global-isolation-protocol

ToC blurb

Worldwide amphibian declines caused by pathogenic chytrid fungi are emblematic of emerging infectious diseases driven by globalisation. Fisher and Garner discuss how these wildlife pathogens emerged to drive global declines in amphibian biodiversity and the implications for policy and control measures.

Figure 1

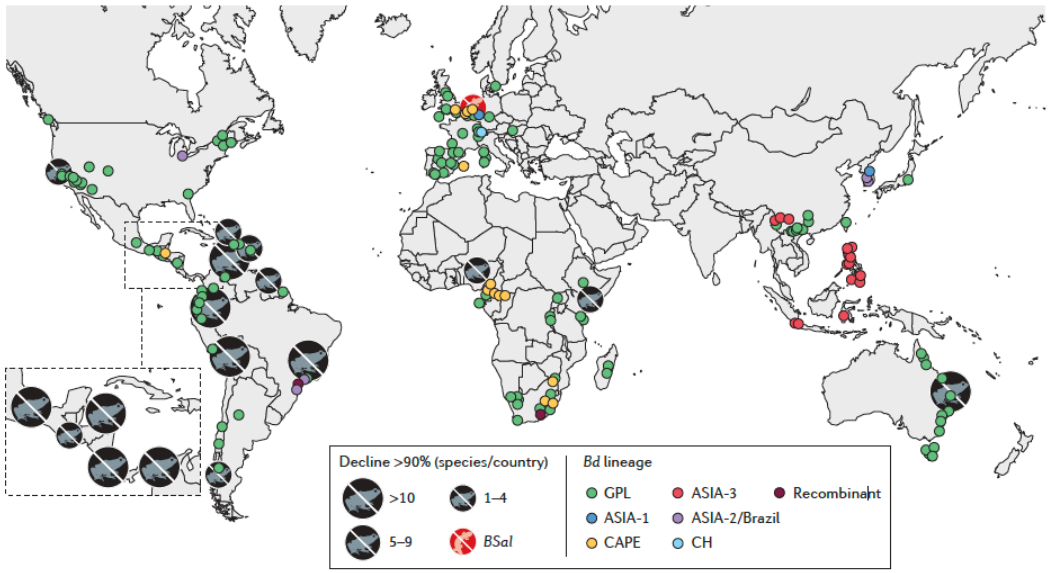

Figure 2

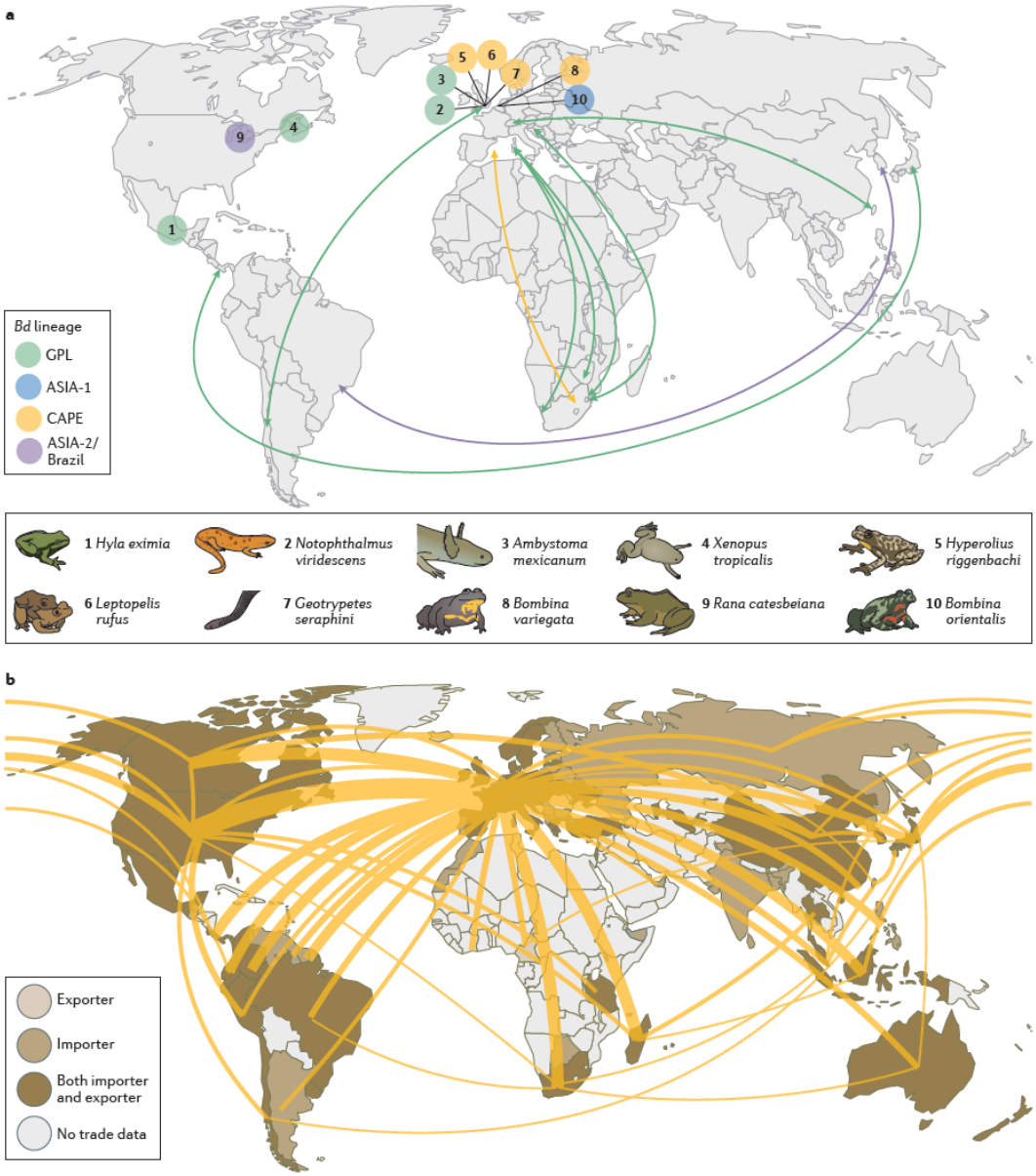

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Figure 3

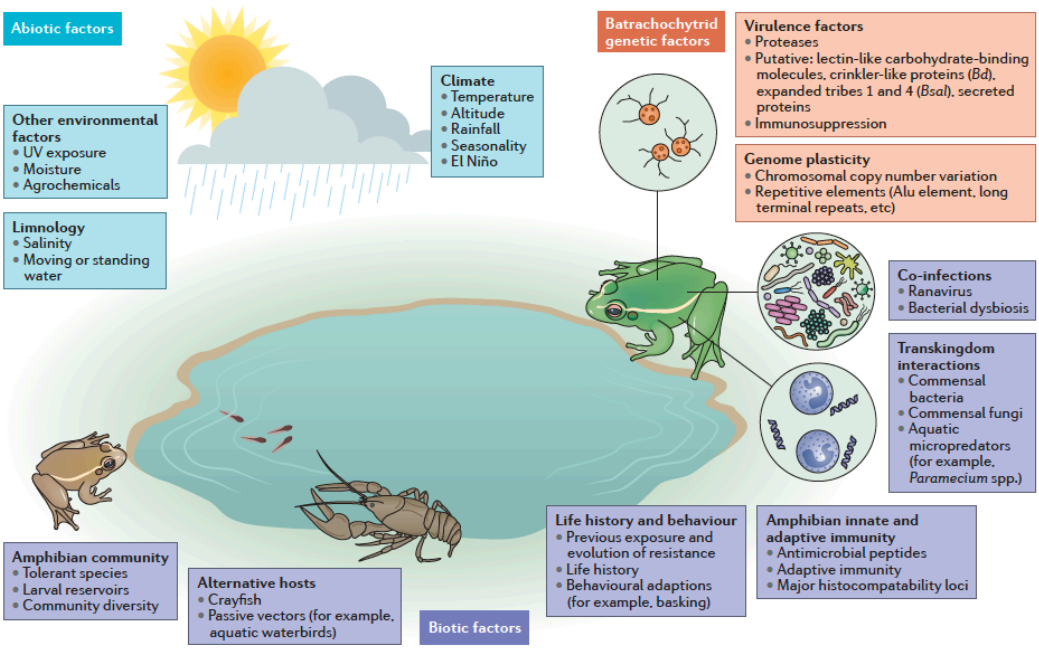

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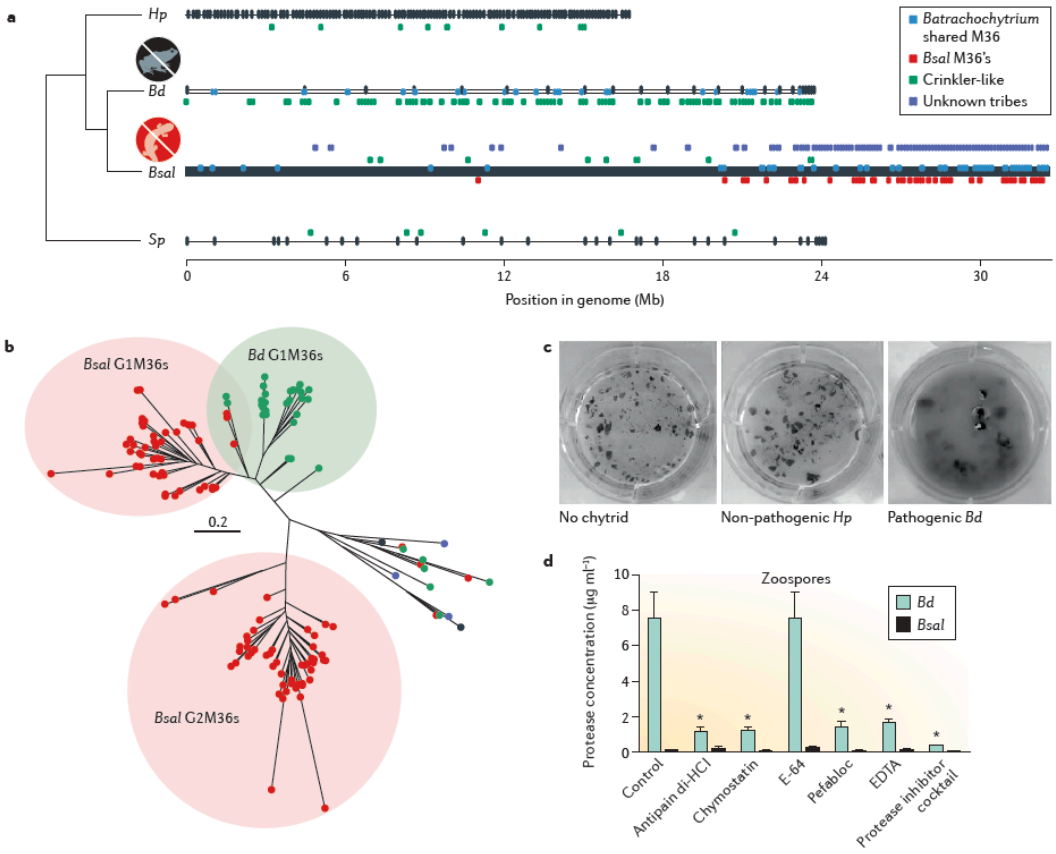
Figure 4



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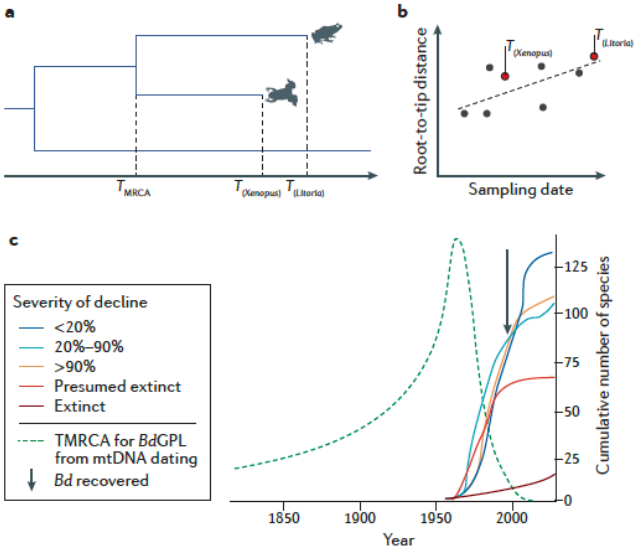
Box 1 Figure



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