Molecular Principles of Fungal Pathogenesis
Edited by Joseph Heitman et al.

Chapter 20

Amphibian Chytridiomycosis as an Emerging Infectious Disease of Wildlife: What Can We Learn from the Earliest Diverging Fungi?

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THE EMERGENCE OF CHYTRIDIOMYCOSIS

Emerging infectious diseases are microbial diseases that are increasing in incidence, host, or geographical range, are newly evolved, or have only recently been discovered. Examples of emerging diseases of humans are many: AIDS, methicillin-resistant *Staphylococcus aureus* infection, West Nile virus infection, and others. Increased incidence rates of human diseases are often attributed to changes in modern lifestyles and demography, such as global travel, pollution, and overpopulation (38). Zoonoses are diseases that infect both wildlife and humans, and roughly half of human diseases are zoonotic (19). Until recently, little attention was paid to the possibility that wildlife populations could also be suffering from emerging diseases and that the emergence of wildlife diseases is also likely to be initiated by anthropogenic disturbance of animal ecosystem and habitat (18). The rapid expansion of such zoonotic diseases in wildlife populations may ultimately cause "spill-over" of pathogens into human populations (e.g., avian flu and Lyme disease [18]).

Amphibian populations have been precipitously declining in many regions worldwide since the 1970s (9, 25, 54). Furthermore, amphibian species are going extinct at a rate higher than that for other fauna, such as birds and mammals (51). Investigation by wildlife biologists into the cause of the declines has revealed that many factors (e.g., pollution, habitat loss, and invasive species) are likely to be involved (15).

Some regions with the greatest amphibian mortality in recent years include protected areas where the impacts of anthropomorphic disturbance are likely to be relatively minor, such as tropical montane forests in Central America and Queensland, Australia (31, 47). Exotic disease was suspected first in these regions because declines showed the temporal and geographic signatures of an epidemic (30) and mass mortalities were observed (32). A combination of microbiological and histological examination of dead and dying frogs from these regions revealed the presence of numerous sporangia of a Chytridiomycete fungus (chytrid) on the epidermis (7) (Fig. 1). Berger et al. (7) hypothesized that declines in both Central American and Australian frog populations were caused by the same fungal disease, chytridiomycosis. In total, 19 frog species from areas of dieoffs in Australia and Panama were positive by histological examination for the chytrid; in contrast, museum samples collected before the dieoffs were negative for signs of chytrid infection. Longcore et al. (34) have recently described the agent of chytridiomycosis as a new genus and species of chytrid, *Batrachochytrium dendrobatidis*. Since the discovery of chytridiomycosis in 1998, the pathogen has been found associated with population declines in western North America (13) and Europe (11). It has also been detected in Africa (45), New Zealand (4), and South America (35). This was the first case ever reported of vertebrate mortality caused by an infectious chytrid.

THE CHYTRIDIOMYCETES AS UNIQUE BASAL FUNGI

The Chytridiomycetes are a relatively poorly known and understudied phylum, in part due to their microscopic size and minimal effects on humans. There are currently five orders (Blastocladiales, Chytridiales, Monoblepharidales, Neocallimastigales, and Spizellomycetales) containing approximately 100 genera and 1,000 species. It is uncertain if the chytrids are a monophyletic group or rather an "evolutionary

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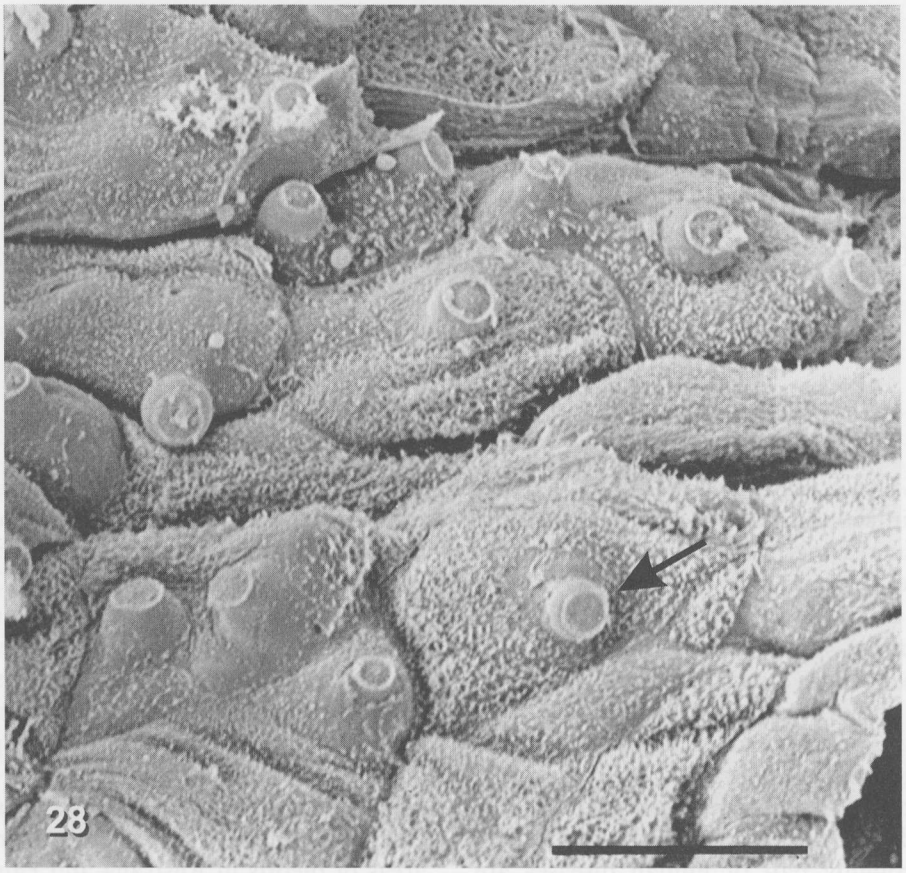

Figure 1. Scanning electron micrograph of the digital skin of the frog *Litoria lesueuri* infected with *B. dendrobatidis*. Shown is the superficial layer of skin, displaying marked roughening and numerous cells containing sporangia. The arrow indicates discharge tubes of the sporangia from which zoospores are released. Bar, 10 μm. Reprinted from reference 6 with permission.

grade" of early-diverging fungal lineages (27). Chytrids reproduce asexually through sac-like sporangia, which are typically produced on either a brief or extensive system of thread-like rhizoids that aid in anchoring to the substrate and absorption of nutrients. At maturity, the sporangia undergo cleavage into zoospores. When fully developed, the unicellular zoospores swim away to find a new host or substrate. The zoospore is the feature that makes chytrids unique among fungi. Zoospores are bound only by a plasmalemma and possess a whiplash, posterior flagellum that drives the cell forward. Zoospores often contain large lipid globules which are presumably used in supplying the zoospore with energy during the motile phase (21). Systematics of the group is based primarily on transmission electron microscopy of the zoospore. Most members of the Chytridiales have a single large lipid globule. *B. dendrobatidis* (Chytridiales) has several lipid globules and other traits that are atypical for this order (Fig. 2).

Most chytrids have a zygotic life cycle in which karyogamy is followed directly by meiosis. However, in *Allomyces* and some other Blastocladiales, the life cycle alternates between diploid and haploid thalli. Sexuality is unknown in the majority of chytrid species. Molecular population genetics suggests that many of these apparently asexual species may actually be vegetatively diploid (33, 37).

The earliest occurrence of chytrids in the fossil record is approximately 400 million years ago (52). It is unclear whether these earliest ancestors of fungi were saprophytic or parasitic (14). Modern chytrids can be saprophytic, parasitic, or both. Chytrids are generally thought to be ubiquitous but not abundant. They are easily isolated from aquatic ecosystems and soil throughout the world, using proper baiting techniques (3), and they are often found attached to moribund and living green algae. The diversity of possible hosts for chytrids is very broad: plants, algae, fungi, and small metazoans. The ecological

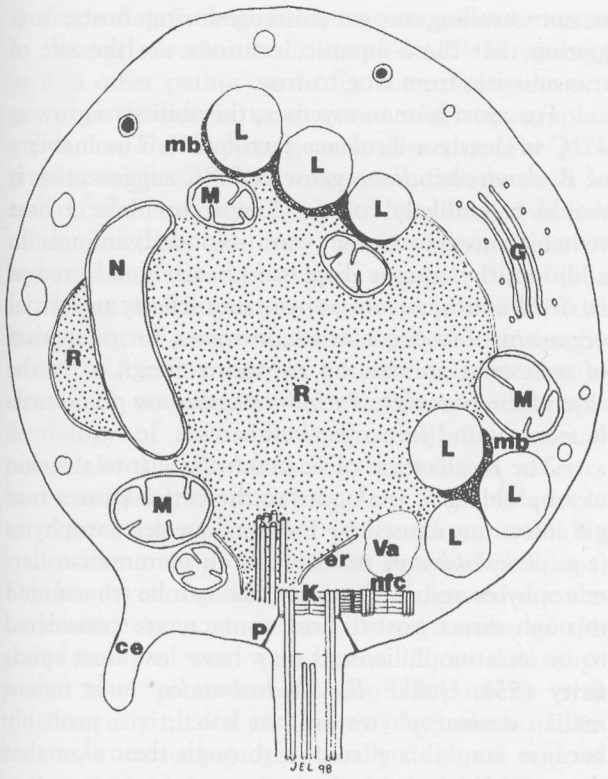

Figure 2. Ultrastructural diagram of the zoospore of *B. dendrobatidis*. ce, cytoplasmic extension; er, endoplasmic reticulum; G, Golgi apparatus; K, kinetosome; L, lipid globule; M, mitochondrion; mb, microbody; nfc, nonflagellated centriole; N, nucleus; P, prop; R, ribosomes; Va, vacuole. Reprinted from reference 34 with permission.

lability of the chytrids is also observed in other early-diverging fungal lineages (e.g., Entomophthorales, Zoopagales, and Microsporidia). The collective group of basal fungal lineages parasitizes the breadth of eukaryotic diversity, whereas more evolutionarily derived fungi (the Ascomycetes and Basidiomycetes) appear to favor associations with green plants over associations with animals or fungi, which occur more rarely. As parasites, chytrids generally "pick on things their own size," parasitizing organisms such as desmids, mosquitoes, and nematodes (44). Therefore, it came as a surprise in 1998 to find that a chytrid might be at the heart of global amphibian dieoffs, doing something that it was not supposed to do—parasitizing vertebrates, with a dramatic and unpredictable outcome.

As basal fungi, Chytridiomycetes have presumably retained many of the characteristics of the last common ancestor with other Fungi millions of years ago. The central characteristic that the chytrids have retained from their protistan ancestors is the flagellated zoospore (Fig. 2). The relationship of the chytrids to the remaining fungi might be considered analogous to the relationship of the choanoflagellates to the animal kingdom (14). Both chytrids and choanoflagellates are highly simplified protist-like organisms that are presumably very similar to the most recent common ancestors of their respective kingdoms. Gene phylogenies have confirmed the basal position of the chytrids in the fungal kingdom (27, 40) and place their time of divergence from the remaining fungi around 660 million years ago (5). Because of their basal position within the fungal kingdom, a greater understanding of characteristics of Chytridiomycetes, e.g., biochemistry and drug resistance, should elucidate fundamental principles that may apply to the whole of the kingdom Fungi.

PATHOLOGY AND ECOLOGY

Frogs with chytridiomycosis display lethargy, loss of righting response, skin sloughing, abnormal posture, and, in severe cases, skin ulceration (17). Sporangia can be seen on histological examination of the skin between digits and from the ventrum. The presence of sporangia is diagnostic for the chytrid. The fungus appears to be restricted to the skin and causes cell loss, erosion, and hyperkeratosis of the stratum corneum. In severe cases there is also hyperplasia of the stratum intermedium (7). The fungus spreads on the skin of the infected individual through the formation and release of zoospores (Fig. 1). The chytrid is thought to be acquiring nutrition from the host in the form of keratin because it infects only keratinized portions of the body (7, 17). The chytrid can be easily cultured on solid and liquid media containing tryptone, but a chemically defined medium is lacking, as is definitive proof of ability to grow solely on keratin (42). The chytrid can also be observed on the keratinized mouthparts of tadpoles but does not appear to cause mortality at this life stage (8).

For susceptible frog species, chytridiomycosis results in death in typically less than 1 month (7, 34, 39). Using inoculation of poison dart frogs (*Dendrobates* spp.) with zoospores, Koch's postulates have been satisfied (34, 39). How the fungus actually kills the host is unknown, but two primary hypotheses have been put forward (17): hyperplasia of the epidermis may impair the frog's ability to properly respire or osmoregulate, or a fungal toxin may be produced by the fungus and be absorbed by the host. It is clear that *B. dendrobatidis* can act as the sole infectious agent in dying frogs and is not merely creating an environment where an opportunistic infection by another pathogenic microbe can take hold (7).

Chytridiomycosis has a very broad host range, infecting many species of frogs and even salamanders.

Some frog species are highly susceptible and have suffered large population declines, while other, sympatric, species appear to be resistant (32, 46). Some of the species not harmed by the fungus can, however, carry the disease sublethally in small lesions (24). It has been hypothesized that these resistant frogs that can harbor chytridiomycosis could act as disease reservoirs and could have been the mechanism by which the fungus has been globally dispersed (35).

Variation in the susceptibility of different amphibian species suggests some variation in the host's ability to fight the fungus through its immune system. However, it is also possible that environmental or other factors that modulate the immune response may vary among host species and geographic regions (13). Amphibians, like other vertebrates, have innate and adaptive immunity, utilizing macrophages, neutrophils, and lymphocytes to respond to pathogen invasion. They are also capable of synthesizing a broad range of antimicrobial peptides that are secreted from granular glands in the skin. The mode of action of the small 12- to 46-amino-acid peptides is thought to be through disruption of the pathogen's cell membrane (57). There is some evidence that the peptides show some specificity for certain microbes (36), and some of the peptides are active against *B. dendrobatidis* (49, 50). Somehow, *B. dendrobatidis* is able to evade this first line of immune defense; environmental factors may contribute to the weakening of this response (49). On the other hand, variance in susceptibility could actually be related to other intrinsic differences between species, such as the ability of the chytrid to attach to and penetrate the host's epithelial cells or the ability of the chytrid to compete with the other microfauna associated with amphibian skin.

The areas and species affected by chytridiomycosis have a few factors in common. Geographic regions suffering catastrophic declines are typically upland rain forests with cool climates rather than humid lowland forests (17, 30). Also, the disease primarily impacts riparian rather than terrestrial frog species (32). These factors are intrinsically tied to the biology of *B. dendrobatidis* and of chytrids in general. Chytrids are very common in cool freshwater ecosystems. *B. dendrobatidis* has a similar temperature optimum, with maximal growth between 17 and 25°C, and is intolerant of elevated temperatures (42). In fact, exposing sick amphibians at 37°C for 16 h has been shown to be an effective way of treating the disease (56). Another fundamental aspect of chytrid biology is a reliance on water for dispersal to new substrates and hosts. *B. dendrobatidis* favors stream-dwelling or stream-reproducing hosts, suggesting that these aquatic locations are the site of transmission from frog to frog.

For most human mycoses, the ability to grow at 37°C is clearly a virulence factor (26). The inability of *B. dendrobatidis* to grow at 37°C suggests that it would be unlikely for the fungus to make a host switch to homeothermic (warm-blooded) animals. In addition, the aquatic dispersal phase should prevent *B. dendrobatidis* from parasitizing wholly terrestrial organisms. Precedent exists, however, for parasitism of terrestrial animals by zoosporic fungi, as in the case of the oomycete *Pythium insidiosum* on animals in tropical and subtropical climates.

The localization of *B. dendrobatidis* to the skin of amphibians is analogous to the better-known fungal infections caused by mammalian dermatophytes (e.g., *Trichophyton rubrum*). Both mammalian dermatophytes and *B. dendrobatidis* can be transmitted through direct host-to-host contact, are considered to be keratinophilic, and may have low host specificity (55). Unlike *B. dendrobatidis*, most mammalian dermatophytes are not lethal; it is probably because amphibians respire through their skin that they are more sensitive to a cutaneous infection.

MOLECULAR EPIDEMIOLOGY

Chytridiomycosis is now considered to be an emerging infectious disease afflicting a wide range of frog species (19). Several hypotheses have been put forward to explain the rapid emergence of chytridiomycosis as a widespread and prevalent disease. One hypothesis suggests that the recent outbreak of chytridiomycosis was spawned by a recent anthropogenic introduction of the pathogen into a naive population, i.e., "pathogen pollution" (17). An alternate hypothesis is that the pathogen may have been endemic to regions of current amphibian declines but was either unnoticed or nonpathogenic until recently. Retrospective studies using museum specimens suggest that the disease was absent until recently and is still on the rise. These studies have determined that chytridiomycosis had emerged by 1974 in the United States and 1978 in Australia (13, 28).

Molecular tools are essential for pathogen surveillance and epidemiology, and amphibian-disease researchers are beginning to develop tools for sensitive detection and population genetics of *B. dendrobatidis* (2, 12, 37). Two goals of population genetic studies are to determine whether the disease has emerged due to recent introduction and to determine where the fungus came from and how has it been dispersed. Molecular phylogenetic studies of the Chytridiomycetes have not provided any clue to the origins of the disease; it is not clear how

B. dendrobatidis originated, because no sister taxon or close relative has been sampled (27). A strategy used in other pathosystems for determining the origin of a disease is to discover which geographic region has the highest genetic diversity. The geographic origins of some notorious diseases such as malaria and late blight of potato have been revealed, using molecular markers, by the observation of higher genetic diversity in these areas (1, 23).

Using multilocus sequence typing (MLST) (the genotyping of single-nucleotide polymorphisms by using DNA sequencing of housekeeping or anonymous loci), we recently addressed the molecular epidemiology of chytridiomycosis (37). Two critical points about *B. dendrobatidis* population genetics have emerged from this study. First, a global sample of 35 isolates revealed only three variable nucleotides among nearly 6,000 surveyed positions. This low level of genetic variation suggested that the coalescence time, and thus the expansion of the global sample, was very recent. These data supported the "recent-introduction" hypothesis over the "recently-turned-pathogenic" hypothesis. Even isolates from such geographically disparate regions as Panama and Australia had identical MLST genotypes. These data implicated the introduction of a single clone to these areas of mass dieoffs. Second, direct sequencing of PCR-amplified gene regions demonstrated multiple sequence types per strain at 4 of the 10 loci examined. These two sequence types were interpreted as two alleles of a diploid genotype. The observation that some loci showed individuals that were all or nearly all heterozygous (fixed heterozygosity) suggested that independent assortment was not occurring and satisfied one of the criteria of clonality (53). This result was in agreement with the absence of sexuality observed in culture or on the host. Taking these results together, we concluded that *B. dendrobatidis* is a recently dispersed clonal organism. However, the MLST dataset included some genotypic variation among the various isolates in the form of combinations of heterozygous and homozygous genotypes among loci. In addition, some heterozygous loci displayed more deviation from Hardy-Weinberg proportions than did others. One explanation for this pattern is that the variation among strains was generated by mitotic recombination, in much the same way as is suggested for *Candida albicans* (16). Location along the chromosome (i.e., distance from centromere) has been correlated with the amount of heterozygosity lost through somatic recombination in *C. albicans* (29); a similar process could be generating differences in heterozygosity among loci in *B. dendrobatidis*.

The striking lack of genetic variation in *B. dendrobatidis* is a major hindrance to determining the geographic origin of the current epidemic. Neither the MLST genotype data nor other molecular markers have provided any evidence for population subdivision based on geography or host (18, 20), nor is there any evidence of areas or hosts harboring higher levels of genetic diversity. The lack of genetic variation in *B. dendrobatidis* could limit its ability to counteract evolved resistance of its host. Recently, it has been reported that some amphibian populations that have suffered from population declines, presumably caused by chytridiomycosis, may be recovering and persisting with a stable demography and rate of infection (46). It is plausible that the hosts have evolved resistance to the pathogen. Perhaps the frog species which can recover from the initial epidemic can win the genetic arms race over their genetically depauperate opponents.

LESSONS LEARNED FROM A MODEL CHYTRID

The investigation of emerging infectious fungal diseases of wildlife can help elucidate principles shared among all pathogenic fungi. For example, it is still unknown if *B. dendrobatidis* possesses homologous mechanisms of pathogenesis with other fungi. Further, monitoring of wildlife diseases will be critical to predicting and mitigating future zoonoses. *B. dendrobatidis* represents a model pathogen of vertebrates from a novel clade of Fungi (Chytridiomycota) on an interesting host (amphibians) acting in a role that is quite familiar to medical mycologists (as a dermatophyte). As a model system, *B. dendrobatidis* informs us about general principles of mycoses, such as invasive growth and evasion of host immunity. *B. dendrobatidis* could also be an important model system in disease ecology, since much of the work on amphibian disease is strongly rooted in community ecology and disease surveillance. Finally, chytridiomycosis has the added advantage that both host and parasite are amenable to experimentation.

In just a few years, research on *B. dendrobatidis* has taught us a few salient points regarding disease ecology, disease management and policy, and fungal biology.

1. Sometimes cures can be very simple if they can take advantage of the basic biology of the parasite. The use of brief exposure to temperatures above those tolerated by *B. dendrobatidis* (37°C) to treat chytridiomycosis exploits the cool-temperature requirement of the organism (56). The reaction of *B. dendrobatidis* to elevated temperatures is probably similar to that of other Chytridiomycetes and

probably reflects their niche in the wild—cool aquatic ecosystems. These results emphasize that it is very important for medical mycologists to understand what fungi that cause opportunistic infections are doing in nature, as is being done with *Cryptococcus neoformans* (26).

2. There is a wealth of information in the specimens archived in museums and herbaria. Retrospective, histological analyses of frog specimens have been able to pinpoint the time at which the disease first appeared (7, 13, 28). In some cases even genetic analyses can be performed on DNA recovered from specimens. For example, analysis of DNA sequences obtained from potato plant samples deposited during the Irish potato blight (1845 to 1847) forced a reappraisal of earlier hypotheses concerning the intercontinental spread of the disease (48).

3. Globalization has caused the rapid spread and increased gene flow of infectious human diseases and may also be occurring for wildlife diseases (18, 19). Intercontinental transport and traffic of animals or plant and animal materials threaten to make pathogenic microbial species one single genetic population through incidental global translocation. Quarantines are needed to protect wildlife resources and to prevent the spread of future zoonoses.

4. Changes in the environment may trigger emerging infectious disease epidemics. Global climate change is thought to play a role in the disappearances of amphibians from Central America (43). Here, global warming is suspected of changing the precipitation patterns and increasing the population density of amphibians, potentially fostering the rapid spread of chytridiomycosis. How a frog species reacts to a disease agent is contingent on a number of things: temperature, pollutants, UV radiation, and the presence of competitors or predators (10, 15, 41). Exposure to a novel pathogen is likely to produce unpredictable results; in the worst case, such as with *B. dendrobatidis*, it may drive species to extinction.

5. Chytrid fungi may be vegetatively diploid and primarily asexual. Identification of asexual lineages can be facilitated by detecting fixed heterozygosity as in other parasitic protozoa and oomycetes (22, 53). However, as in *Candida albicans*, somatic recombination can work to generate novel genotypes by shuffling heterozygous genotypes.

Acknowledgments. We thank Jeri Parrent for a critical reading of this manuscript. Work on *Batrachochytrium* in the laboratory of R.V. is funded in part by NSF-DEB-0213851. We thank Joyce Longcore and Lee Berger for permission to use their figures.

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