CHAPTER 4

Batrachochytrium dendrobatidis, the amphibian chytrid

4.1 Introduction and literature review

4.1.1 Biology of the Chytridiomycota

The Chytridiomycota is one of the four phyla of true fungi and contains about 1000 species. The name is based on the flask shaped appearance of sporangia as "chytr" means earthen pot in Greek. The phylum has one class, Chytridiomycetes, which is divided into five orders - Chytridiales, Blastocladiales, Monoblepharidales, Spizellomycetales and Neocallimastigales (Barr, 1990; Barr, 2000). DNA sequencing suggests that the orders are monophyletic but that the Blastocladiales may be more closely allied to the phylum Zygomycota (James et al., 2000). Early classifications regarded chytrids as separate from the higher, true fungi due to their distinctly different appearance and their zoosporic lifecycle. Chytridiomycetes are typified by the presence of chitin in the cell wall and the production of motile zoospores with a single posteriorly directed flagellum. Ultrastructure of the zoospore is useful for classification as it is more conserved among phylogenetic groups than the morphology of the thallus (i.e. the entire organism), which can vary even within a species (Barr, 1990).

Chytridiomycetes have been found in almost every type of environment, including rainforests, deserts, arctic tundra and in the sea (Barr, 1990). Some chytrids have a thick walled, resistant resting spore stage that can survive for decades in extreme conditions (Powell, 1993) and can then become reanimated for rapid growth at times of sporadic rainfall. Chytrids are frequently found in soil and water where they digest complex substrates such as chitin from insect cadavers, cellulose from vegetable matter, keratin from hair and skin, or pollen. These species function as important primary bio-degraders and are possibly vital to the ecosystem. Others are parasites of protists, fungi, algae, plants and invertebrates, and a few of these cause significant diseases (Barr, 1990; Powell, 1993). As chytrids are not recovered using routine mycological cultures they have received little attention despite being ubiquitous, and only a handful of people in

the world specialise in studying them. It is likely that many species are yet to be discovered.

Powell (1993) discusses the significance and inherent value of chytridiomycetes and reviews the ability of parasitic species to cause disease. The onset of chytridiomycete parasitism of phytoplankton is often correlated with a rapid decline in host population and so has a major impact on the ecology of the host. *Synchytrium endobioticum* causes black wart disease of potatoes in Europe and Canada, and was introduced to the USA in the early 1900's but has since been eradicated. *Coelomomyces* has been considered for use in biological control of mosquitoes as heavy infections kill larvae through exhausting their fat reserves. An amazing dispersal mechanism exists where larvae with light infections are able to become adults but fungi replace the ovary in infected females and are deposited instead of eggs. Some chytrids transmit pathogenic plant viruses. Chytrids have not been reported to cause disease in vertebrates. However, there are cellulose-digesting species that occur among the normal rumen flora of ruminants (Barr, 1990), and an *Allomyces* sp. was isolated from gills, fins and skin of diseased *Tilapia* fish that may have been ill due to infection with other fungal species (El-Sharouny and Badran, 1995).

Sparrow (1960) describes the evanescent nature of chytrid epidemics in algae, with their sudden appearance, brief period of rapid multiplication and then decline and disappearance. This pattern is related to their virulence, ability for rapid reproduction, and the loss of optimal environmental conditions. Factors affecting the epidemiology of chytrid blooms include seasonal temperature changes, water pH, light, nutriton and dissolved oxygen (Sparrow, 1968). Temperature affects the growth rate of fungi and also the period of zoospore motility (Powell, 1993).

Most chytrids occur in aquatic habitats and the unwalled, flagellated zoospores require water for dispersal. Zoospores often display chemotaxis towards their particular substrate enabling them to reach hosts or nutrients in the vicinity that are not abundant, although water flow is probably the main method of dissemination (Sparrow, 1968). Zoospores may respond to nutrients, temperature, light, electrical fields, gravity, streams and to one another (Fuller, 1996). Changes in the stratification of temperature and oxygen in water bodies was correlated with distribution of zoospores (Sparrow,

1968). Zoospores probably do not require an exogenous energy source and their metabolism is directed towards producing energy for flagellar movement and maintaining homeostasis (Fuller, 1996). Zoospores of many fungi produce an adhesive as they encyst on their host (Bartnicki-Garcia and Sing, 1986). Encysted zoospores grow into single or colonial sporangia that vary in size with nutrient quality and quantity. Size of zoospores is relatively constant within a species so the number of zoospores per sporangium varies with the size of the sporangium (Sparrow, 1960). Zoospores that develop within a stationary sporangium are released through discharge tubes. In some species the opening may be covered by a cap (operculum) that lifts off. Some sporangia may remain mature but do not release zoospores until stimulated by some external factor. The first zoospores to escape appear to be ejected under pressure but the later ones swim or creep out (Sparrow, 1960).

Chytrid species vary greatly in morphology and growth habits. Some species have an endogenous growth form where the nucleus remains inside the zoospore cyst, as opposed to exogenous growth where the nucleus moves into a germ tube and the sporangia develop there. Monocentric chytrids develop one sporangium from each zoospore whereas with colonial development the nucleus undergoes mitotic divisions to produce separate sporangia. Sporangia may have a branched anucleate rhizoidal system for absorption of nutrients, and anchorage or penetration of substrates and nutrients. Filamentous or polycentric chytrids form large masses of rhizomycelia with nuclei throughout. Sexual reproduction has not been observed for most species but diverse methods are known. Sexual reproduction may occur by zoospores fusing with each other, zoospores fusing with sporangia, rhizoids fusing, or production of motile gametes of unequal size (Barr, 1990).

4.1.2 Epidemics caused by the introduction of zoosporic fungi

Many species of aquatic zoosporic fungi are found worldwide and there are no reported examples of endemicity (Sparrow, 1960), although there are examples of fungi that have restricted distributions. The most obvious introductions of fungi are of pathogenic organisms whose effects on larger organisms are noticeable. Examples include the introduction of the chytrid *Synchytrium endobioticum* (black wart disease of potatoes),

and the oomycetes *Phytophthora cinnamomi* (Jarrah die-back) and *Aphanomyces astaci* (crayfish plague) (Dawson and Weste, 1985; Powell, 1993; Lilley et al., 1998).

4.1.3 Discovery and identification of *B. dendrobatidis*

Although an unidentified protist had been seen on the skin of some of the last individuals of *T. acutirostris* that died in the wild and in captivity (Speare, 1995), no studies had been done to investigate its significance and identity. During the survey of ill frogs in Australia (Chap 8; Chap 10), it became apparent that this organism was associated with mass mortality, and its pathogenicity was confirmed by the initial transmission experiment using infected skin scrapings (Chap 7; Berger et al., 1998). Experts were consulted for identification of the organism. Skin samples were sent to Peter Daszak for transmission electron microscopy and to Louise Goggin for PCR, while I examined skin smears and histological sections by light microscopy. Alex Hyatt and I also studied infected skin using the scanning electron microscope. We eventually identified the organism as a new genus in the order Chytridiales (Berger al., 1998). For more complex strain identification, I then sent samples to Jess Morgan for sequencing (Morgan, 1999). Unknown to us, investigations were being conducted on the same parasite causing mortality of captive poison dart frogs in the USA (Pessier et al., 1999). This group was able to culture the fungus and later described the new genus and species (Longcore et al, 1999).

4.1.4 Taxonomy of *B. dendrobatidis*

The amphibian chytrid was placed in a new genus, *Batrachochytrium* (Phylum Chytridiomycota, Class Chytridiomycetes, Order Chytridiales) and an isolate from a captive blue poison dart frog (*Dendrobates azureus*) that died at the National Zoological Park in Washington was named *B. dendrobatidis* (Longcore et al., 1999). The zoospore ultrastructural morphology, amphibian host and ssu-rDNA sequence of *B. dendrobatidis* show that it is distinctly different from other chytrid fungi and is isolated on phylogenetic trees (Berger et al., 1998; Longcore et al., 1999; James et al., 2000).

4.1.4.1 Morphologic features of taxonomic importance

B. dendrobatidis is inoperculate and exhibits monocentric or colonial growth (Longcore et al., 1999). Important ultrastructural features of the zoospore are that the nucleus and kinetosome are not associated, ribosomes are aggregated, the microbody partially surrounds numerous small lipid globules, and the nonflagellated centriole (NFC) is parallel and connected to the kinetosome by overlapping fibres, as well as other details of the kinetosomal root (Berger et al., 1998; Longcore et al., 1999). Most members of the Chytridiales have a rumposome along the edge of the lipids and many have a transition zone plug, but these are not present in *B. dendrobatidis* (Berger et al., 1998; Longcore et al., 1999). *B. dendrobatidis* is also unusual for a member of the Chytridales in having numerous (i.e., nine) lipid globules, as most species have just one or two globules (Longcore, 1993; Longcore et al., 1999).

Significant ultrastructural morphological differences were not observed between isolates from Australia, the USA and Central America (Berger et al., 1998; Longcore et al., 1999).

4.1.4.2 DNA sequencing studies

The first sequence obtained was of ssu-rDNA from an infected *Litoria caerulea* from Queensland, which demonstrated the fungus was a chytrid related to *Chytridium confervae* (Berger et al., 1998). Jess Morgan sequenced the rDNA ITS (internal transcribed spacer) regions of 29 samples of infected skin or cultured zoospores from Australia, and 5 samples from the Americas. Sequences varied up to 6%. Phylogenetic analysis based on low variation identified seven strains (Morgan, 1999) but these do not make epidemiological sense, and isolates that were geographically related often did not show genetic similarities. *B. dendrobatidis* may randomly mutate in this region, and until the changes that occur in isolates over time are characterised, interpretation is difficult.

Sequence of ssu-rDNA from culture from a captive American *D. azureus* was compared to our original sequence from an Australian *L. caerulea* (Timothy James, David Porter and Joyce Longcore, unpub). Only five base pairs were different out of about 1700 bp sequenced, and four of these differences were deletions that may be due to error. All

isolates may be closely related and further DNA sequencing is underway to determine the relationship among strains.

4.1.5 Biology of *B. dendrobatidis*

Some basic biological characteristics of *B. dendrobatidis* have been determined. Sporangia grow within cornifying epidermal cells of amphibians, but as they can be grown in culture media (containing tryptone, gelatin hydrolysate and lactose, or just 2% tryptone) and grew on boiled snake-skin (keratin), they may also be able to exist and proliferate as saprobes in the environment (Longcore et al., 1999). The distribution of sporangia in tadpoles during development followed the changes in the distribution of keratin (Chap 5), confirming the keratinophilic nature of this fungus when occurring as a parasite (Berger et al., 1998).

In culture *B. dendrobatidis* grew slowly at 6°C, developed most rapidly at 23°C but died if kept at temperatures above 29°C (Longcore, 2000; Chap 9). Longcore et al. (1999) reports that the lifecycle takes about 4 - 5 days at room temperature, and this also was seen with Australian isolates.

Zoospores of *B. dendrobatidis* are infective to frogs and tadpoles (Chap 7; Berger et al., 1999a). They can remain motile for over 24 hours, with longer survival times at 4°C than at 22°C (Chap 7). Zoospores are unwalled and require water for dispersal. Although the zoospores tolerate a range of osmotic pressures, they die if transferred directly from distilled water to broth (L. Berger, unpub). In culture *B. dendrobatidis* tolerates some degree of salinity and zoospores will encyst and grow in 6.25 mg/ml NaCl but not in 12.5 mg/ml (Chap 9). Resistant resting spores have not been found (Longcore et al., 1999) and a culture of zoospores and zoosporangia was killed by drying (Chap 9).

Longcore et al. (1999) comprehensively described the taxonomic features of *B. dendrobatidis* and gave detailed morphology based on light microscopic observations of cultures and ultrastructure of serially sectioned zoospores. In this Chapter an overview of the morphology is presented using a large range of methods to examine all life cycle

stages of Australian *B. dendrobatidis* in culture and in frog skin. Methods include light microscopy, transmission electron microscopy (TEM) using conventional methods as well as high pressure freezing and freeze substitution, and scanning electron microscopy (SEM) using critical point drying to prepare samples as well as examination of bulk-frozen and freeze-fractured material.

4.2 Methods

For observations of cultured fungus, light microscopic examinations were conducted with an isolate obtained from a captive *L. dumerilii* (98 1469/10) and ultrastructural studies were done with the first wild isolate, from a *N. dayi* from Tully (98 1810/3). Cultures were maintained on TGhL agar or broth. Infected skin samples were obtained from a wild *L. lesueuri* from Goomburra (97 574/1) and captive *L. gracilenta* and *B. marinus*.

Different methods were used to enable whole and sectioned samples to be imaged with minimal artefacts. Details of methods are included in Chapter 3. TEM was performed on infected skin and culture using conventional methods as well as high pressure freezing followed by freeze substitution (HPF/FS). SEM was done on culture and skin prepared by critical point drying as well as examination of bulk-frozen hydrated samples and freeze-fractured material. For SEM of culture we initially used cultures on agar. As these cultures are relatively dry, we obtained clean images but the agar obscured the view of the base of the sporangia. We then used culture grown in broth on plastic coverslips (Thermanox) and clean preparations were achieved with extended drying, showing fine details of the rhizoids. Samples were processed and examined at AAHL using a JEOL JSM-840 scanning electron microscope and Hitachi H7000 or Philips CM 120 transmission electron microscopes, except for samples in figures 16 and 18 that were processed and photographed at Latrobe University using a JEOL 6340F field emission scanning electron microscope, and samples in figures 10 and 14 that were processed and photographed at Adelaide University using a Philips XL30 FEG microscope.

4.3 Results and review of morphology

4.3.1 Zoospore

Zoospores are the waterborne, motile, flagellated stage (Fig. 4.1). Zoospores of *B. dendrobatidis* are mostly spherical but can be elongate and amoeboid when first released from the zoosporangium (Longcore et al., 1999). They are about 3-5 μm in diameter with a posteriorly directed flagellum (19-20 μm) (Longcore et al., 1999).

Zoospore ultrastructure is used to differentiate orders and genera and many important taxonomic features are in the flagellar apparatus. The ultrastructure of the flagellar region is difficult to describe and is best explained by diagrams (see Fuller, 1996). The features of the zoospore of *B. dendrobatidis* that are common to the order Chytridiales are that the nucleus and kinetosome are not associated, ribosomes are aggregated into a core surrounded by endoplasmic reticulum, the microbody partially surrounds the lipid globules, and the NFC is parallel and connected to the kinetosome (Longcore, 1993; Longcore et al., 1999). The key features of *B. dendrobatidis* (Longcore et al., 1999) are that there are numerous small lipid droplets with the microbodies that are associated with the edge of the ribosomal mass (Fig. 4.2). The kinetosomal root is comprised of a group of microtubules that run parallel to the kinetosome as they extend into the ribosomal mass (Fig. 4.3). Additional key taxonomic structures not shown in the figures included here are that the microtubule root arises near triplets 9-1 of the kinetosome, and that overlapping fibres connect the NFC with the kinetosome. The nucleus is partially nested in the ribosomal mass, mitochondria are adjacent to the ribosomal mass and zoospores contain a single Golgi apparatus (Fig. 4.4).

After a period of motility and dispersal, the zoospore encysts. The flagellum is rapidly resorbed and a cell wall forms (Fig. 4.5).

4.3.2 Germling

After the zoospore has encysted, fine branching rhizoids grow from one or more areas and the young sporangium is known as a germling (Fig. 4.6). Occasional germlings were seen parasitising an adjacent sporangium (Fig. 4.7).

4.3.3 Developing sporangia and zoosporangia

As the sporangia grow, their contents become more complex (Figs. 4.2, 4.8 - 4.18). The sporangia become multinucleate by mitotic divisions. The entire contents then cleave and mature into rounded, flagellated zoospores. During sporangial growth, one or more discharge papillae form. Some young thalli become divided by thin septa and each compartment grows into a separate sporangium with its own discharge tube (up to six were seen), which is referred to as "colonial growth". Larger thalli contain more divisions. Thalli that contain one sporangium have no divisions and develop monocentrically (Longcore et al., 1999). Mature zoosporangia contain fully formed flagellated zoospores. Actively motile zoospores were observed within sporangia before they exited. After the plug blocking the discharge tube has dissolved, zoospores are released. The length of discharge tube is highly variable even within an isolate and depends on type of media and density of culture. Longer tubes (up to 10 μm) were seen in skin and agar cultures than in broth.

4.3.4 Effete sporangia

After zoospores have been released, the sporangia have clear contents (Fig. 4.19). The walls of the sporangia remain and may collapse. Occasional zoospores do not escape and grow within sporangia (Fig. 4.20). In frog skin bacteria may enter through the open discharge tubes and replicate inside (4.20, 4.29, 4.30).

4.3.5 Colonies in culture

On agar *B. dendrobatidis* grows as dry, granular, cream coloured clusters (Fig. 4.21). The zoospores swarm in a film of surface water surrounding each colony. They encyst at the base of the colony and push the older sporangia upwards. Sporangia grow better in clusters and isolated zoospores placed on agar usually die. This "group effect" (Longcore et al., 1999) is unusual in fungi. Rhizoids spread over the surface of adjacent sporangia and tightly intermingle with rhizoids from other sporangia, strongly adhering them together (Figs. 4.22- 4.25). Zoospores appear to be attracted to colonies, and clusters of growth occur whether the culture is growing on plastic, on agar plates or floating in broth. Sporangia adhere well to plastic. Growth of colonies at room

temperature slows dramatically after a few weeks, even though nutrients and space exist for further growth. Inhibition of growth may be due to chemical factors such as by-products of metabolism. The pH of broth cultures did not alter over time.

4.3.6 *Batrachochytrium* in skin

The same stages of the lifecycle occur within epidermal cells in skin infections as in culture, although it has not been determined whether there are differences in the rate of development (Figs. 4.26-4.28). Immature stages occur in the deeper viable cells. Mature zoosporangia and old empty stages occur in the sloughing *stratum corneum*. By the time sporangia have completed their development, they have been carried to the skin surface with the differentiating epidermal cells. Discharge tubes usually point towards the skin surface, whereas in culture they may grow laterally as well as upwards. Discharge tubes usually protrude to the surface through a hole in the epidermal cell membrane. The edge of the keratinised skin adheres closely to the discharge tube and is not easily discerned except by TEM (Figs. 4.28, 4.29). Some zoosporangia mature while still covered by cornified cell layers that have not been sloughed, and appear to discharge zoospores into the spaces between cells in the skin.

Colonial development can occur in skin and sporangia with internal septa can be seen in histological sections, although most thalli in skin are not colonial. Bacteria on the skin multiply on the layers of shedding keratin and commonly grow in empty sporangia and form colonies (Figs. 4.29, 4.30). Sporangia in the skin (5-13 μm in diameter) are smaller than in culture (<40 μm) (Longcore et al., 1999), suggesting that being intracellular restricts their growth. Rhizoids were rarely seen in skin sections examined by electron microscopy. They could not be seen in H&E stained histology sections, but were occasionally discernible adjacent to sporangia when stained with the immunoperoxidase stain (see Chap 6). The fungi are often found in clusters, except in heavy infections where all ventral skin may be diffusely infected.

4.3.7 Lifecycle

The lifecycle of *B. dendrobatidis* is represented in figure 4.31. Sexual reproduction has not been observed.

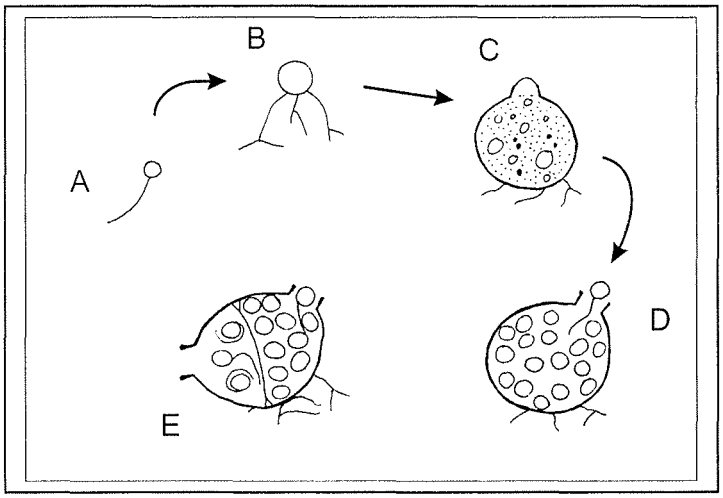


Figure 4.31 Diagram of the lifecycle of *B. dendrobatidis* in culture. After a period of motility, zoospores encyst, resorb their flagella and form germlings. Rhizoids appear from one or more areas. Sporangia grow larger and mature over 4 - 5 days. The sporangia become multinucleate by mitotic divisions and the entire contents cleave into zoospores while the discharge tubes form. The discharge tube is closed by a plug that absorbs water and deliquesces when zoospores are ready to released. Some thalli develop colonially with thin septa dividing the contents into multiple sporangia each with their own discharge tube. A= zoospore, B = germling, C = immature sporangium, D = monocentric zoosporangium, E = colonial thallus.

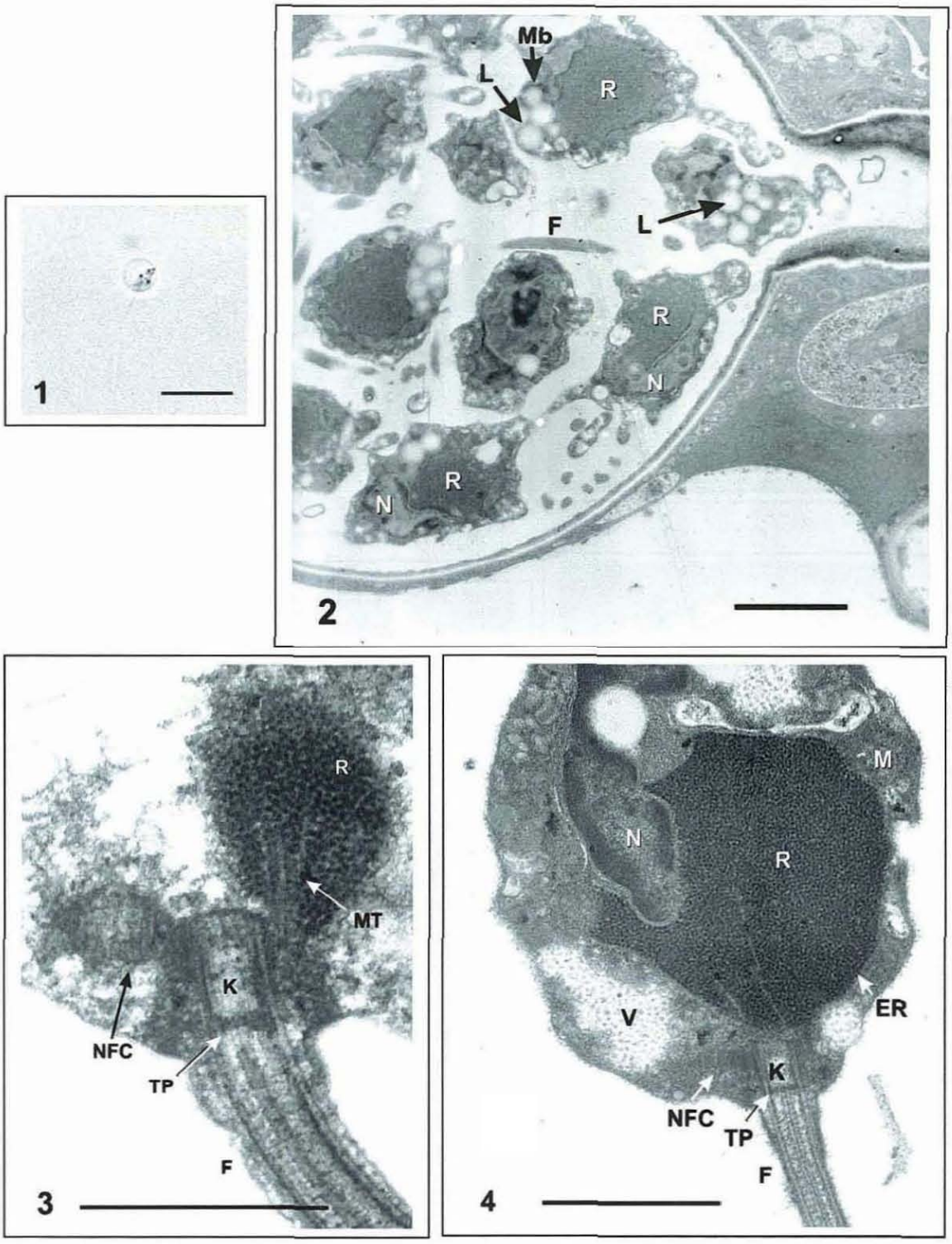


Figure 4.1 Live cultured zoospore of *Batrachochytrium dendrobatidis*. The dark droplets are probably lipid globules. Bar = 6 μm.

Figures 4.2-4 Transmission electron micrographs of zoospores of *B. dendrobatidis*. F = flagellum, N = nucleus, R = ribosomes, Mb = microbody, L = lipid droplet, NFC = nonflagellated centriole, K = kinetosome, M = mitochondria, TP = terminal plate, V = vacuole, ER = endoplasmic reticulum, MT = microtubules. **4.2** Formalin-fixed zoospores within a zoosporangium in the skin of *Bufo marinus*. Zoospores are being released and contain numerous lipid globules that are partially surrounded by the microbody and occur at the edge of the ribosomal mass. Bar = 2 μm. **4.3** Glutaraldehyde-fixed cultured zoospore. The NFC is parallel to the kinetosome. Microtubule root runs parallel to the kinetosome and is embedded in a cone of ribosomes. Bar = 0.6 μm. **4.4** Glutaraldehyde-fixed cultured zoospore. The nucleus is not associated with the kinetosome and is nested in the ribosomal mass which is surrounded by endoplasmic reticulum. Mitochondria are adjacent to the ribosomal mass. Bar = 1 μm.

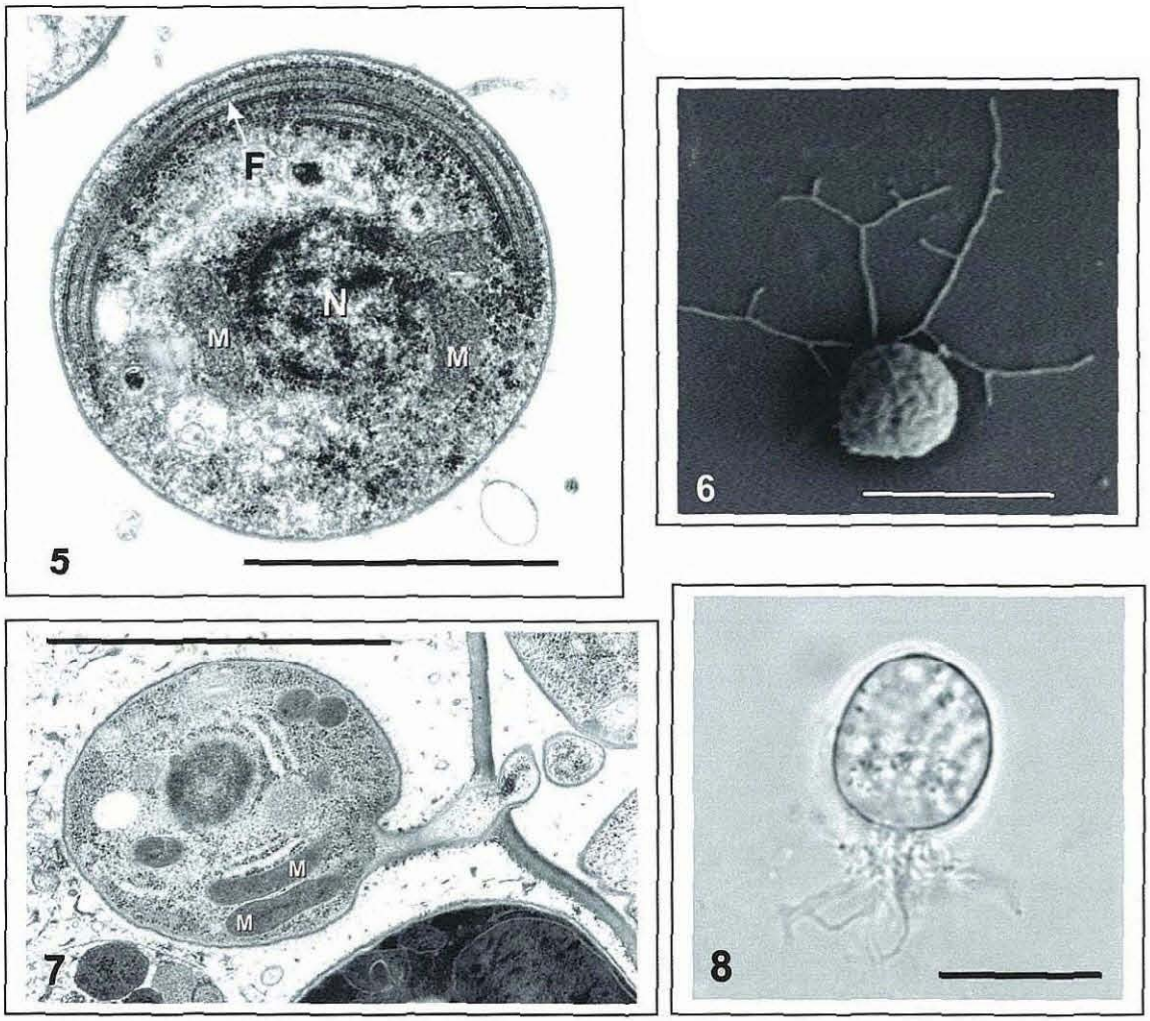


Figure 4.5 Glutaraldehyde-fixed encysted zoospore. The resorbed flagellum is visible and a cell wall has formed. Ribosomes are distributed throughout the cytoplasm. F = flagellum, N = nucleus, M = mitochondria. Bar = 2 μm.

Figure 4.6 Scanning electron micrograph of a germling showing fine rhizoids spreading out along the substrate. The culture was grown on a plastic coverslip and prepared by freeze-drying. The crumpled surface is an artifact of freeze-drying. Bar = 10 μm.

Figure 4.7 TEM of a young sporangium parasitising an adjacent sporangium. A rhizoid is penetrating the cell wall. Prepared by high pressure freezing and freeze substitution (HPF/FS). M = mitochondria. Bar = 5 μm.

Figure 4.8 Live immature sporangium with rhizoids spreading out. Bar = 10 μm.

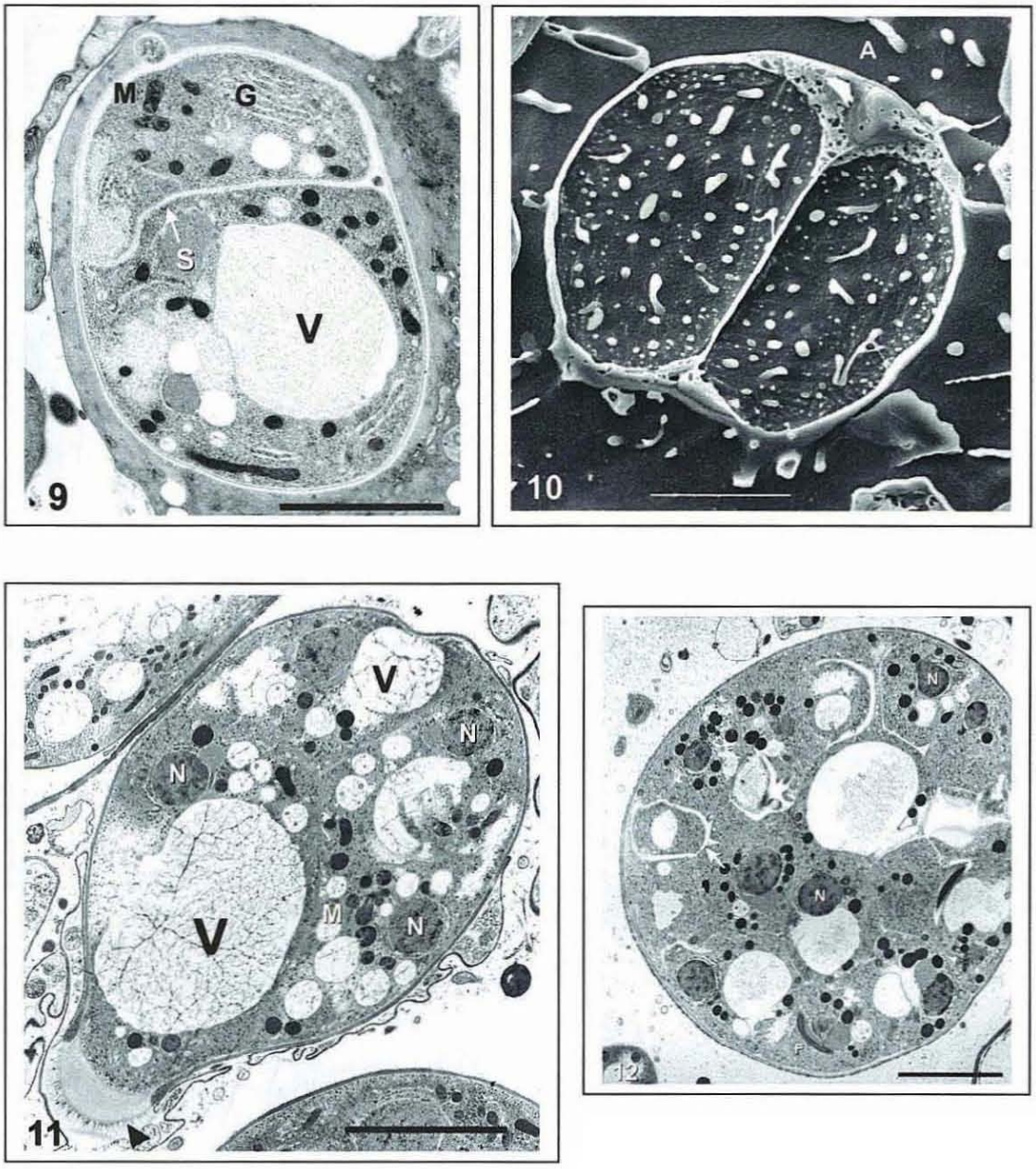
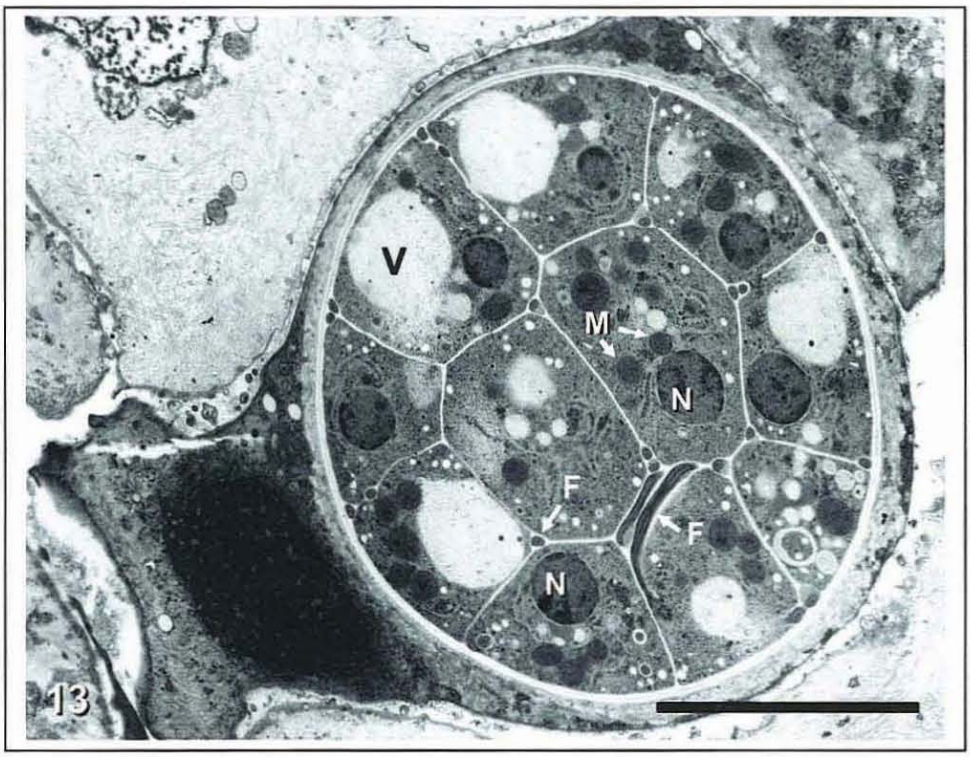


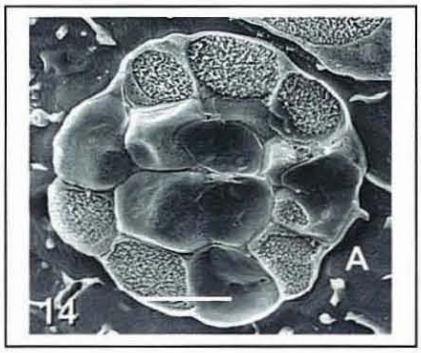
Figure 4.9 TEM (HPF/FS) of an immature colonial sporangium in skin of a *Litoria gracilenta.* A septum (S) divides the thallus into two compartments. V = vacuole, G = golgi, M = mitochondria. Bar = 5 μm.

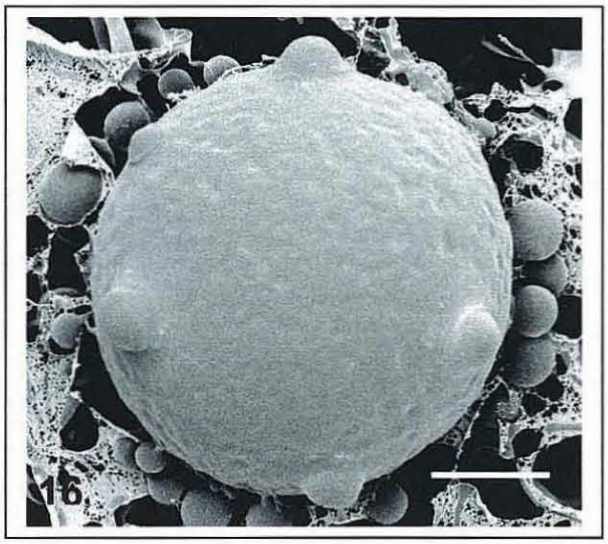
Figure 4.10 SEM of bulk-frozen hydrated culture that has been freeze-fractured. The image shows a colonial thallus divided by a septum. A = agar. Bar = 5 μm.

Figure 4.11 TEM (HPF/FS) of an immature sporangium with a discharge papilla. The cell is multinucleate after mitotic divisions, but the cytoplasm has not yet divided. The plug blocking the discharge papilla is clearly seen (arrowhead). The wall over the tip of the plug has dissolved, demonstrating that *B. dendrobatidis* is inoperculate. Early stages often have large vacuoles (V). Transverse sections of rhizoids occur in spaces between sporangia. N = nucleus, M = mitochondria. Bar = 5 μm.

Figure 4.12 TEM (HPF/FS) of a multinucleate sporangium that is beginning to cleave into zoospores. The arrow indicates a cleavage line. N = nucleus, F = flagellum. Bar = 4 μm.







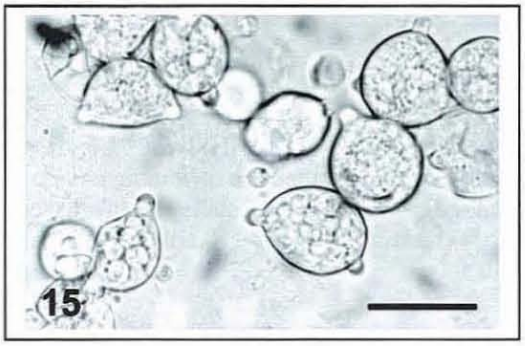
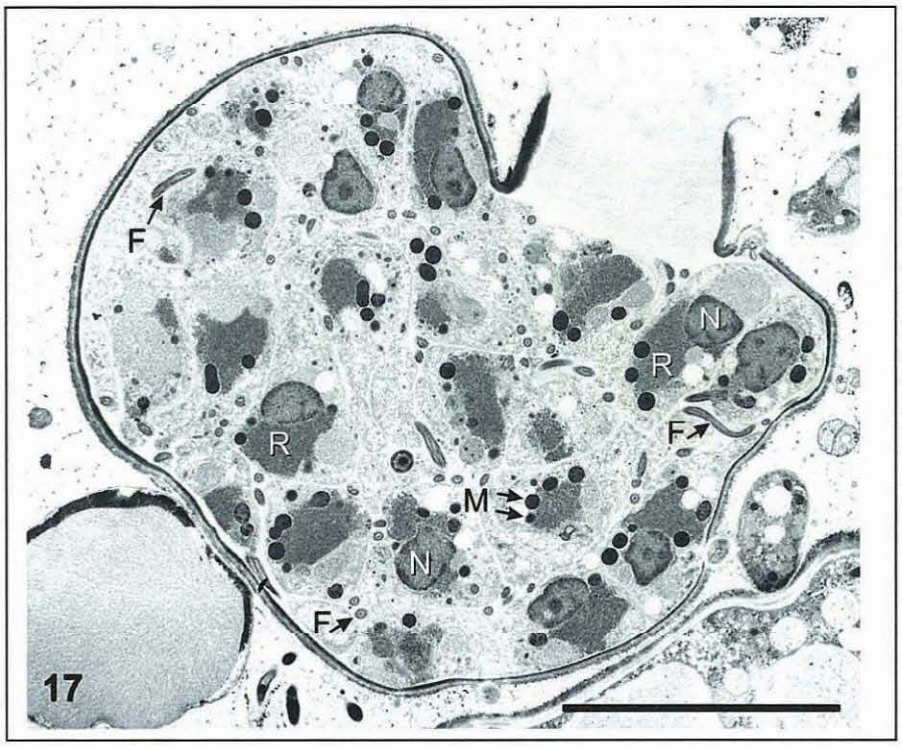


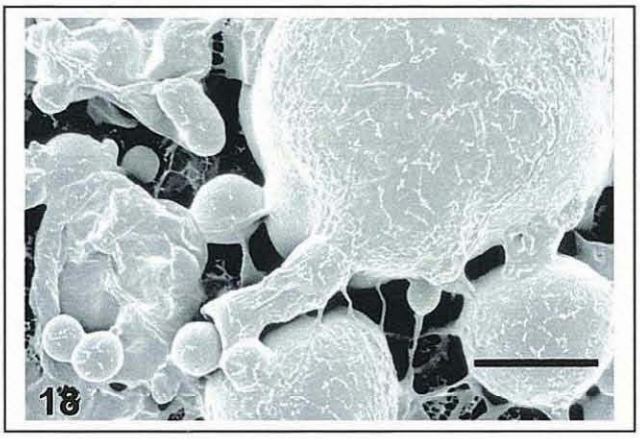
Figure 4.13 TEM (HPF/FS) of a sporangium in skin of a *Litoria gracilenta* with a cytoplasm that has divided into incompletely formed flagellated zoospores. N = nucleus, M = mitochondria, F = flagellum, V = vacuole. Bar = 5 μm.

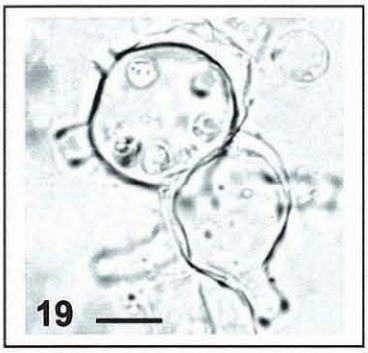
Figure 4.14 SEM of a bulk-frozen hydrated sporangium that has been freeze-fractured. The image is a three-dimensional representation of the similar staged sporangium in figure 4.13. A = agar. Bar = 5 μm.

Figure 4.15 Live sporangia with discharge papillae. Internal structures of the sporangia are at various stages of zoospore development. Bar = 20 μm.

Figure 4.16 SEM of a large zoosporangium on agar with five papillae visible. Zoospores are congregating and encysting around the base. Prepared by bulk-freezing hydrated culture. Bar = 10 μm.







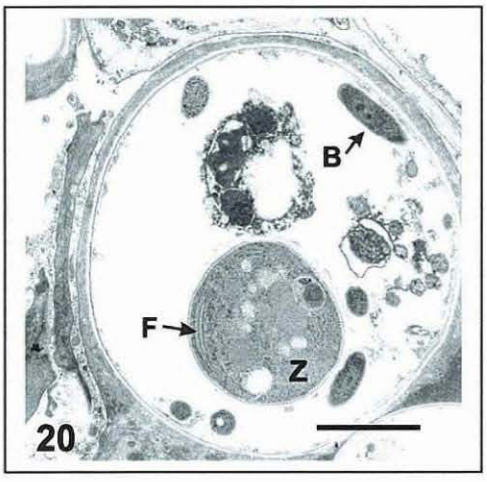
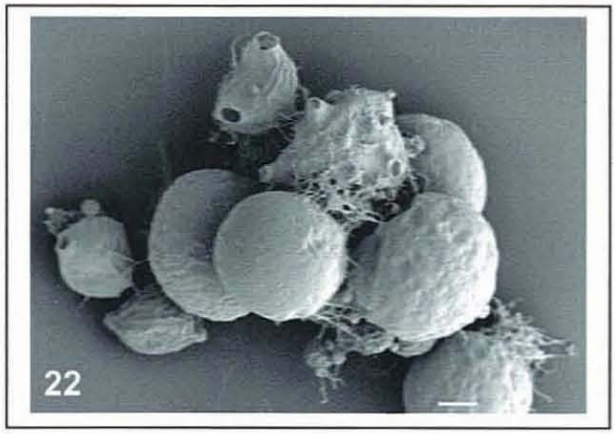


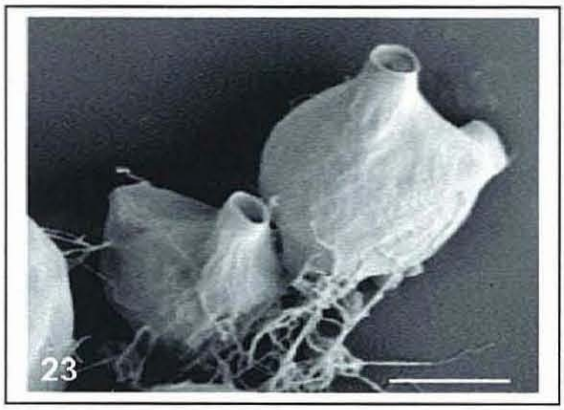
Figure 4.17 TEM (HPF/FS) of a mature zoosporangium with discharge papilla and plug. It is packed with flagellated zoospores. F = flagellum, M = mitochondria, R = ribosomal mass, N = nucleus. Bar = 10 μm.

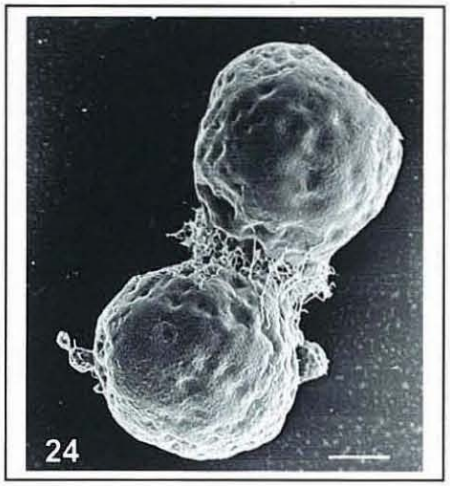
Figure 4.18 SEM of a zoosporangium on agar releasing a zoospore through a long discharge tube. Prepared by bulk-freezing hydrated culture. Bar = 10 μm.

Figure 4.19 Sporangia that have released most of their zoospores. Bar = 10 μm.

Figure 4.20 TEM (HPF/FS) of an old sporangium in the keratinised skin of a *Litoria gracilenta*. A zoospore (Z) that was not released has encysted inside; note thickened wall and resorbed flagellum (F). A degenerate zoospore and bacteria (B) are also inside. Bar = 2 μm.







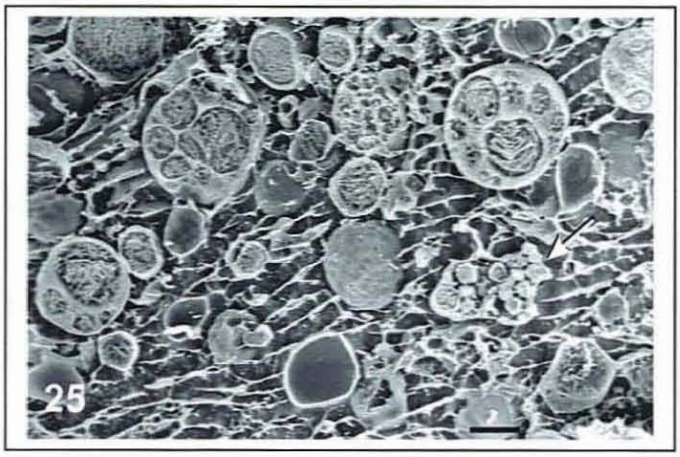


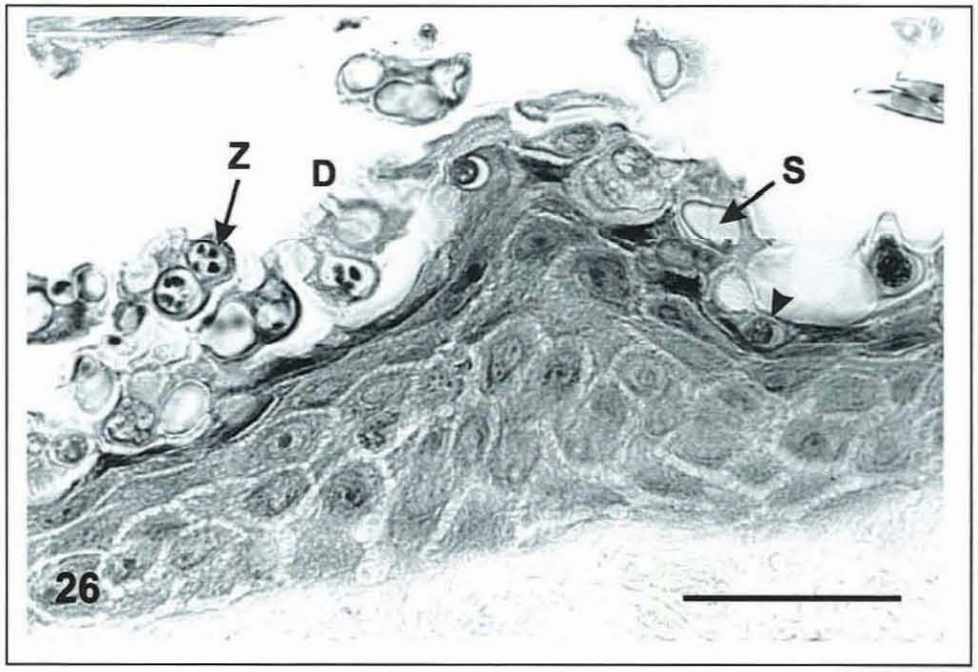
Figure 4.21 Culture on TGhL agar plate. Colonies appear as granular, cream coloured mounds.

Figure 4.22 SEM of a cluster of sporangia grown on a plastic coverslip and freeze-dried. Some sporangia have two or more open discharge tubes. The threadlike rhizoids hold sporangia together. Bar = 10 μm.

Figure 4.23 SEM of thalli with two discharge tubes demonstrating the aptness of the name "chytrid" (i.e. earthen pot). Rhizoids from adjacent sporangia are growing over the surface. Culture was grown on a plastic coverslip and freeze-dried. Bar = 10 μm.

Figure 4.24 SEM of two sporangia showing the attraction between their rhizoids. Culture was grown on a plastic coverslip and freeze-dried. Bar = 10 μm.

Figure 4.25 SEM of freeze-fractured preparation of a bulk-frozen hydrated culture in agar. Most sporangia are immature. One sporangium contains mature zoospores (arrow). Bar = 10 μm.



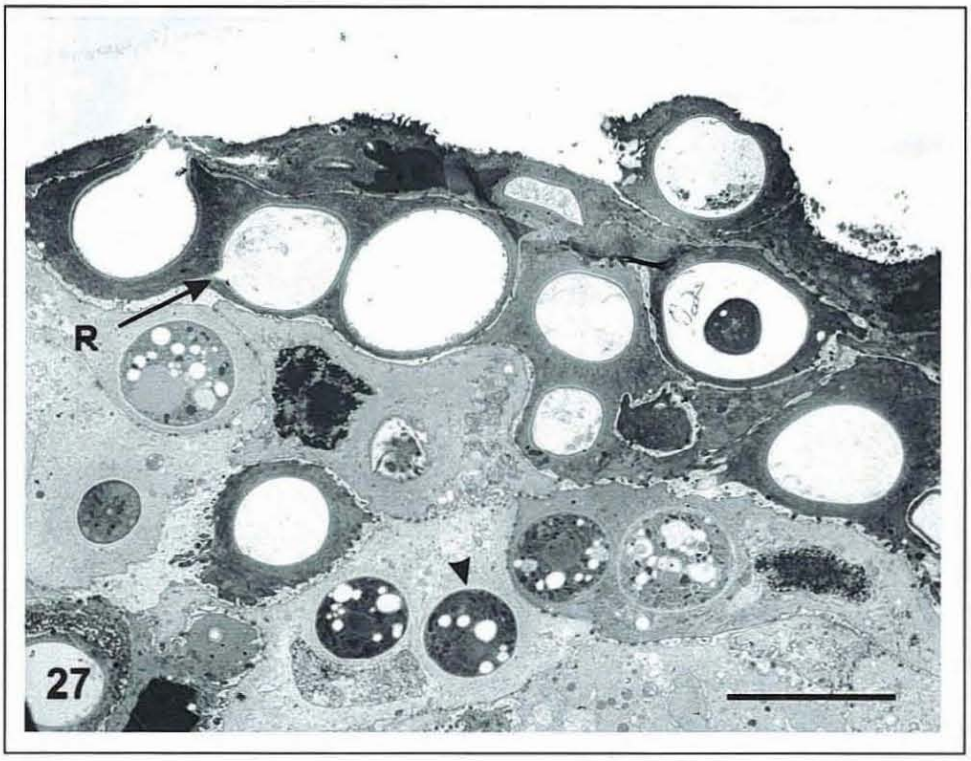
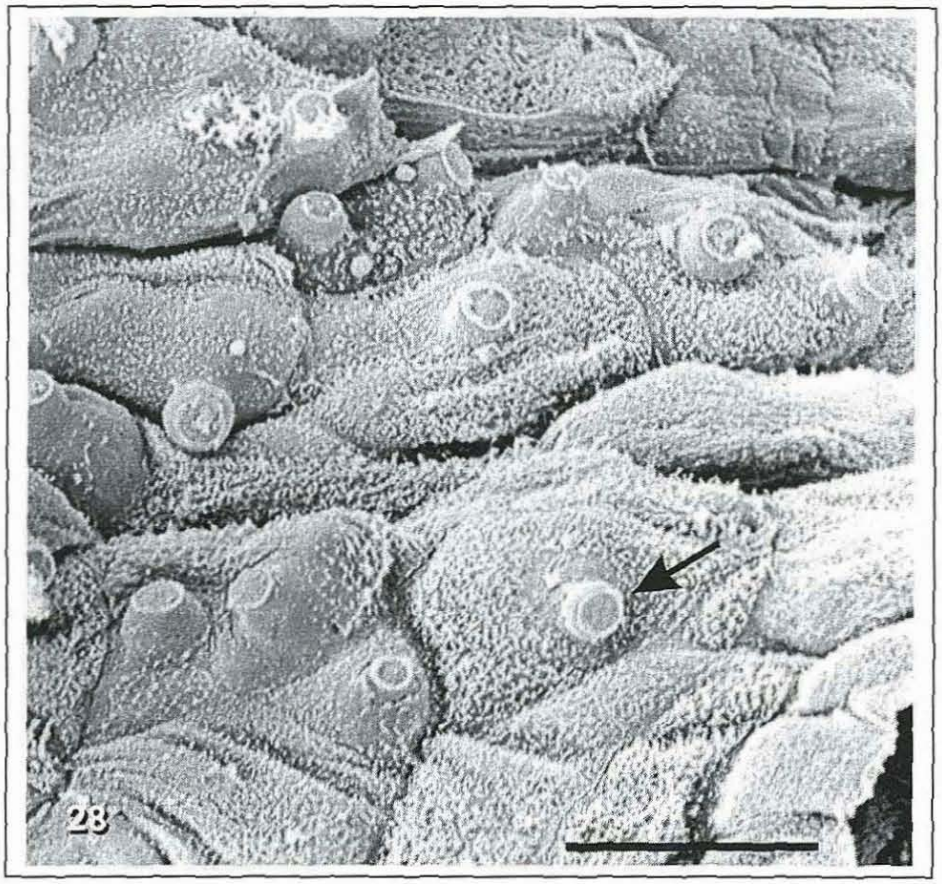
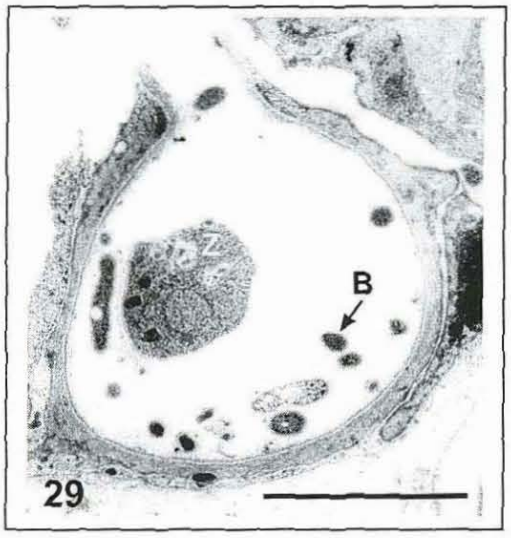


Figure 4.26 Histological section of skin from a *Litoria caerulea*. Dark immature stages occur in the deeper cells (arrowhead). Mature zoosporangia with distinct dark zoospores (*Z*) and old empty stages with open discharge tubes (D) occur in the sloughing *stratum corneum*. Note the colonial thallus with an internal septum (S). Bar = 30 μm.

Figure 4.27 TEM (HPF/FS) of skin from a *Litoria gracilenta* with immature, solid stages deeper in the epidermis (arrowhead), and old empty stages in the flattened, dark, keratinised cells. Infected cells contain between one and three sporangia. R = possible rhizoid. Bar = 10 μm.





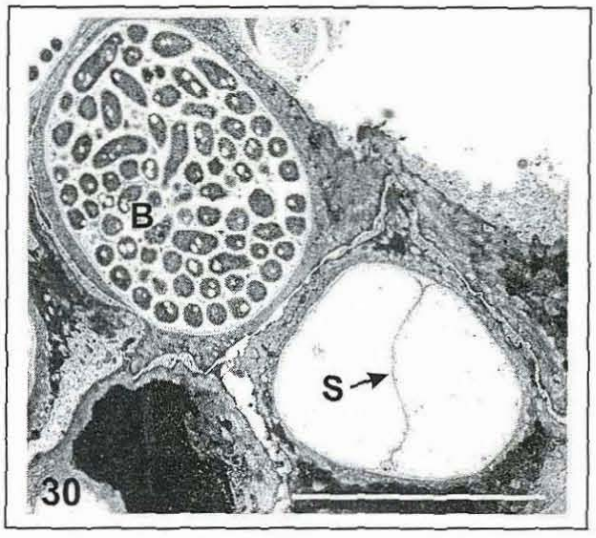


Figure 4.28 SEM of infected toe skin of a *Litoria lesueuri.* Almost all epidermal cells in this field are infected and are bulging. Closed discharge tubes protrude through the skin surface (arrow). Prepared by critical point drying. Bar = 10 μm.

Figure 4.29 TEM (HPF/FS) of an old sporangium containing bacteria (B) and a zoospore (Z). The discharge tube is opening to the skin surface through a hole in the condensed keratinised epidermal cell. The edge of the skin tapers around the tube. Bar = 5 μm.

Figure 4.30 TEM (HPF/FS) of old sporangia in skin of a *Litoria gracilenta.* Bacteria (B) have entered the sporangium through an open discharge tube and replicated into an ordered colony. S = septum in an empty colonial sporangium. Bar = 10 μm.

4.4 Discussion

The lifecycle of *B. dendrobatidis* is relatively simple with the motile, waterborne, infective zoospore for dispersal, and the stationary zoosporangium (intra or extracellular) for amplification. The distinctive ultrastructure of the zoospore and the presence of colonial development allowed a new generic name to be assigned (Longcore et al., 1999).

B. dendrobatidis is well adapted to living in the dynamic tissue of the stratified epidermis. Sporangia live inside epidermal cells that are still able to keratinise and move outwards, and sporangia have a rate of development that coincides with the maturing of the cell. They grow initially in living cells but are able to complete their development in dead keratinised cells without organelles. Discharge tubes have the ability to push through the epidermal cell membranes and open on to the surface of the skin. These specialised adaptations suggest *Batrachochytrium* has long been evolved to live in skin.

Unfortunately we did not catch a zoospore in the act of infecting skin, so the method of penetration remains a mystery. Longcore et al. (1999) suggest the zoospore could encyst on the surface then inject the nucleus and contents through a germ tube. Experimental infection with zoospores and immediate sacrifice of the animal is needed to trace the infection process. Other chytrids have the ability to change from endobiotic to epibiotic growth depending on nutrients and the substrate (Longcore, 1995). The details of the ultrastructural changes that occur within sporangia during development also remain to be studied.

Rhizoids were rarely seen in skin sections; perhaps they do not grow as profusely in skin as in culture, or they were not distinctive in cross-section and could be confused with small vesicles in the cytoplasm. Immuno-labelling on skin sections may answer this question. Rhizoids may not be needed in skin as they are not required for attachment, and nutrition may occur by absorption of enzymatically digested components of the parasitised epidermal cell.

The clustering of *Batrachochytrium* in the skin may be due to zoospores in the water being attracted to foci of infection, or to zoospores that are released from a sporangium

immediately infecting adjacent skin with only a limited period of motility and dispersal. Some zoospores appear to be released into intercellular spaces and may not be able to escape from the site of infection.

As the ultrastructure of different isolates is identical, DNA sequencing is needed to understand relationships between different geographic strains with the aim of determining the origin and spread of *B. dendrobatidis*. The genetic diversity of *B. dendrobatidis* is expected to be greater at its point of origin. Further sequencing of the ITS region is underway in the USA and New Zealand. Discovery of related species could increase our understanding of the origins of *Batrachochytrium.*