The taxonomic status of *Lacazia loboi* and *Rhinosporidium seeberi* has been finally resolved with the use of molecular tools

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The etiologic agents of lobomycosis and rhinosporidiosis, *Lacazia loboi* and *Rhinosporidium seeberi* respectively, have long been confined in a taxonomic limbo. Although their invasive cells in the tissues of infected humans and other animals are abundant and readily detectable histologically, they have been intractable to isolation and culture. Thus, it has not been possible to objectively place them in any of the kingdoms in which all living entities are classified. In addition to their uncultivatable status, *L. loboi* and *R. seeberi* shared in common morphological features that resemble those of certain pathogenic fungi and protozoans: viz. essentially similar inflammatory host reactions, unresponsiveness to antifungal drugs and a long history of taxonomic uncertainty.

In general, these two uncultivated enigmatic pathogens cause cutaneous and subcutaneous infections and rarely have been known to cause deepseated systemic infections. *R. seeberi* is known to infect not only humans but, a large variety of domestic and wild animals in all the continents except Australia. In contrast, *L. loboi* has been found to infect humans only in the Americas from Mexico to Argentina and two species of dolphins off the coasts of Surinam and Brazil, both coasts of Florida and the Gulf of Mexico off the coast of Texas.

The phylogenetic hunt for the last two enigmatic pathogens in medical mycology

When Seeber first described rhinosporidiosis in 1900, he believed that its causative agent was a protist close to the coccidia [1]. He did not foresee, however, that *R. seeberi*, often considered to be a fungus, would remain a taxonomic mystery for the next 100 years. A similar situation occurred with the pathogen *L. loboi*. When Jorge Lobo described this pathogen in 1931 [2], he believed it to be a fungus very similar to the Latin American dimorphic fungal pathogen *Paracoccidioides brasiliensis*. More than 70 years later, however, his hypothesis was still awaiting verification.

To resolve this taxonomic standoff, a team of scientists from Sri Lanka, where *R. seeberi* infections are common, and Brazil, where lobomycosis is endemic, and the USA was assembled to study *R. seeberi*'s and *L. loboi*'s phylogenetic links with other eukaryotic organisms. The strategy was to collect fresh tissue samples from cases of lobomycosis and rhinosporidiosis, isolate genomic DNAs and then amplify their 18S small subunit (SSU) rDNA by using standard primers and PCR technology. In turn, the sequenced fragments were used to construct phylogenetic trees utilizing a variety of sequences from other microorganisms available in the database. One interesting aspect of these studies was the fact that both teams were entirely coordinated by e-mail and postal mail. The majority of the group's members did not know each other at all and, with the exception of one of our graduate students who flew to Brazil, none of the team members met during these studies. Nonetheless, both groups achieved their respective objectives. What they reported has already changed the way we approach and study these two unusual pathogens.

Rhinosporidium seeberi is not a fungus!!

Surprisingly, the first result of these studies was to find that *R. seeberi* was not a fungus but a protist belonging to a novel group of fish parasites [3] (Figure 1). Shortly thereafter its protistan nature was also confirmed by Fredericks *et al.* [4]. Originally, this group of pathogens was identified by Ragan *et al.* [5] as the DRIP clade, an acronym derived from *Dermocystidum*, rosette agent, *Ichthyophonus* and *Psorospermium*. With the addition of *R. seeberi* to the group, however, the DRIP acronym was no longer appropriate. Herr *et al.* [3] replaced it with the term Mesomycetozoa (between fungi and animals). The other surprise was the finding that *R. seeberi*'s closest relatives were *Dermocystidium* spp. This finding was reassuring since these microorganisms like *R. seeberi* produce endosporulating cells in their infected hosts, have not been cultured and their morphological similarities had been noted earlier by other investigators (Figure 2).

Most remarkable was the unusual phylogenetic placement of the mesomycetozoans. This group of pathogens was found to be located between the fungal and the animal divergence. The phylogenetic distribution of this novel group of parasites suggests that the features they possibly share with early diverging animals and fungi may

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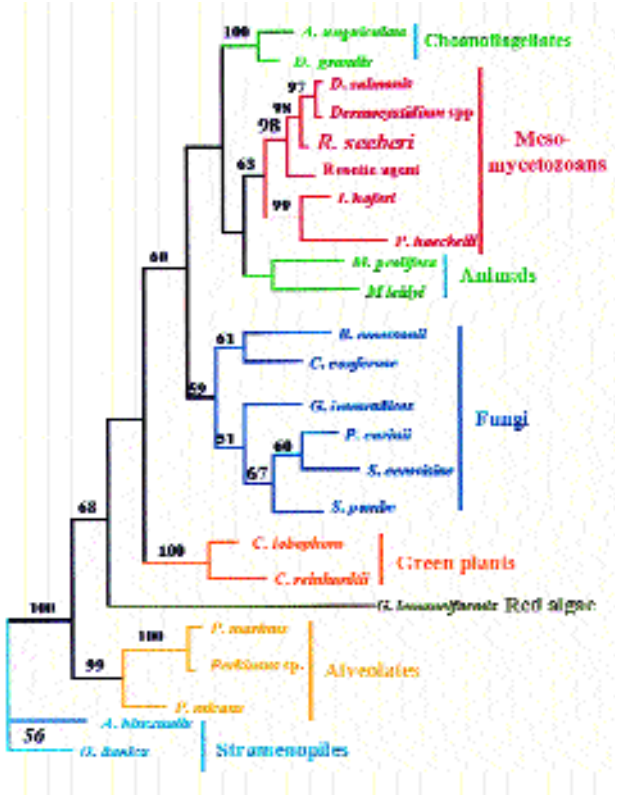

Figure 1. Phylogenetic tree generated with data from the 18S SSU rDNA of *Rhinosporidium seeberi* and 22 other eukaryotic organisms (Herr *et al.*, 1999 [3]). The mesomycetozoans (red) are located between the animals, choanoflagellates and fungi.

offer clues on the appearance of this ancestor. Since nothing was known about their taxonomy, Cavalier-Smith created the class Ichthyosporae to accommodate them. However, the finding that not all members of this group are fish pathogens and that preliminary analysis showed that *R. seeberi* may have chitin synthase genes [6], rendered his epithet inappropriate. Based on these arguments, Ajello and Mendoza [7] recently proposed the class Mesomycetozoae as a more suitable epithet for this group of unique pathogens.

Table 1. Current taxonomic distribution of the class mesomycetozoea including new members.

Table 1 shows the current taxonomy of the mesomycetozoans proposed by Cavalier-Smith [8]. In this nomenclature the mesomycetozoans are classified as part of the subkingdom Neozoa, infrakingdom Neomonada, phylum Neomonada, subphylum Choanozoa, class Mesomycetozoea. The class Mesomycetozoea has two orders: the Dermocystida and the Ichthyophonida. In the order Dermocystida, we have introduced the family Rhinosporideaceae to accommodate *Rhinosporidium seeberi*, *Dermocystidium* spp. and the rosette agent, whereas in the order Ichthyophonida we built the class Ichthyophonae to hold members with phylogenetic features in common with the genus *Ichthyophonus* and *Psorospermium*. Interestingly, all of the new members recently added to the class Mesomycetozoea have been placed within the family Ichthyophonae.

Lacazia loboi may be a dimorphic fungus

In contrast to *R. seeberi*'s genomic DNA isolation, the recovery of genomic DNA from *L. loboi* was not an easy task. During those studies, it was found that *L. loboi* produced powerful proteases and endonucleases that destroy the content of the yeast-like cells of this pathogen within minutes after tissue collection (Figure 3). This fin-

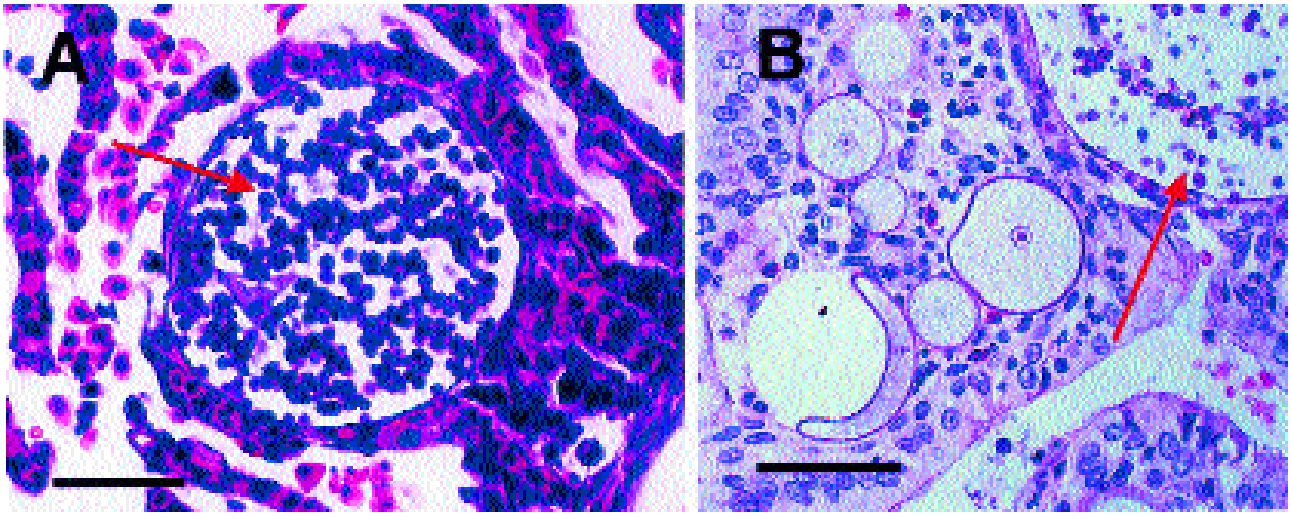

Figure 2. Comparative histopathological features in the infected tissue of the sporangia of *Dermocystidium salmonis* affecting the gills of a fish (Panel A, H&E. Bar= 30 µm) and *Rhinosporidium seeberi* from humans (Panel B, H&E. Bar= 60 µm). Note the spherical shape and the production of endospores in both microorganisms (red arrows) (*D. salmonis* micrograph from Dr. Kristen Arkush).

ding was in agreement with Dr. Paulo Taborda's data (personal communication) that ~60% of the yeast-like cells were already dead within the host's tissues. To circumvent this problem, one of the team's members flew to Brazil to directly isolate *L. loboi*'s genomic DNA within a few seconds after collecting biopsed tissue from humans with lobomycosis. By using this approach he was able, for the first time, to obtain excellent genomic DNA samples from *L. loboi*'s cells.

Using the same approach as in *R. seeberi*, the 18S SSU rDNA obtained from *L. loboi* was used for phylogenetic analysis with a variety of microorganisms. These analyses showed that *L. loboi* was an ascomycetous fungus in the Class Onygenales (Figure 4). This result was also confirmed by using 600 bp of the chitin synthase 2 gene from *L. loboi* and more recently by using its internal transcriber spacers 1 and 2 (ITS1-2) and its 5.8S sequences (unpublished data). Interestingly, the sister taxon of *L. loboi*, on all the trees created during these studies using both DNA molecules, was *P. brasiliensis*. This result was not unexpected, because ever since the first report of the disease the morphological features of *L. loboi* were linked to *P. brasiliensis*. Actually, based on their morphological resemblances, Fonseca and Lacaz [9] at one point named it *P. loboi*. The most exciting finding in this study was the data showing that *L. loboi* was closest to *P. brasiliensis* and also close to the other systemic dimorphic fungal pathogens in the Onygenales (*Ajellomyces dermatitidis*, *Ajellomyces capsulatum*, and *Emmonsia parva*). This finding supports the view that *L. loboi* probably exists as a dimorphic fungus with a hyphal form in nature. To prove this conjecture, the development of DNA probes is presently under way to detect this unique pathogen in environmental samples.

Figure 4. The 18S rDNA phylogenetic tree shows that *Lacazia loboi* is a sister taxon of the dimorphic fungal pathogen *Paracoccidioides brasiliensis*. This clade is linked to the other dimorphic fungal pathogens (*Ajellomyces capsulatum*, *A. dermatitidis* and *Emmonsia parva*). The whole section of the tree is nested among the other ascomycetous Onygenales (Herr *et al.*, 2001 [11])

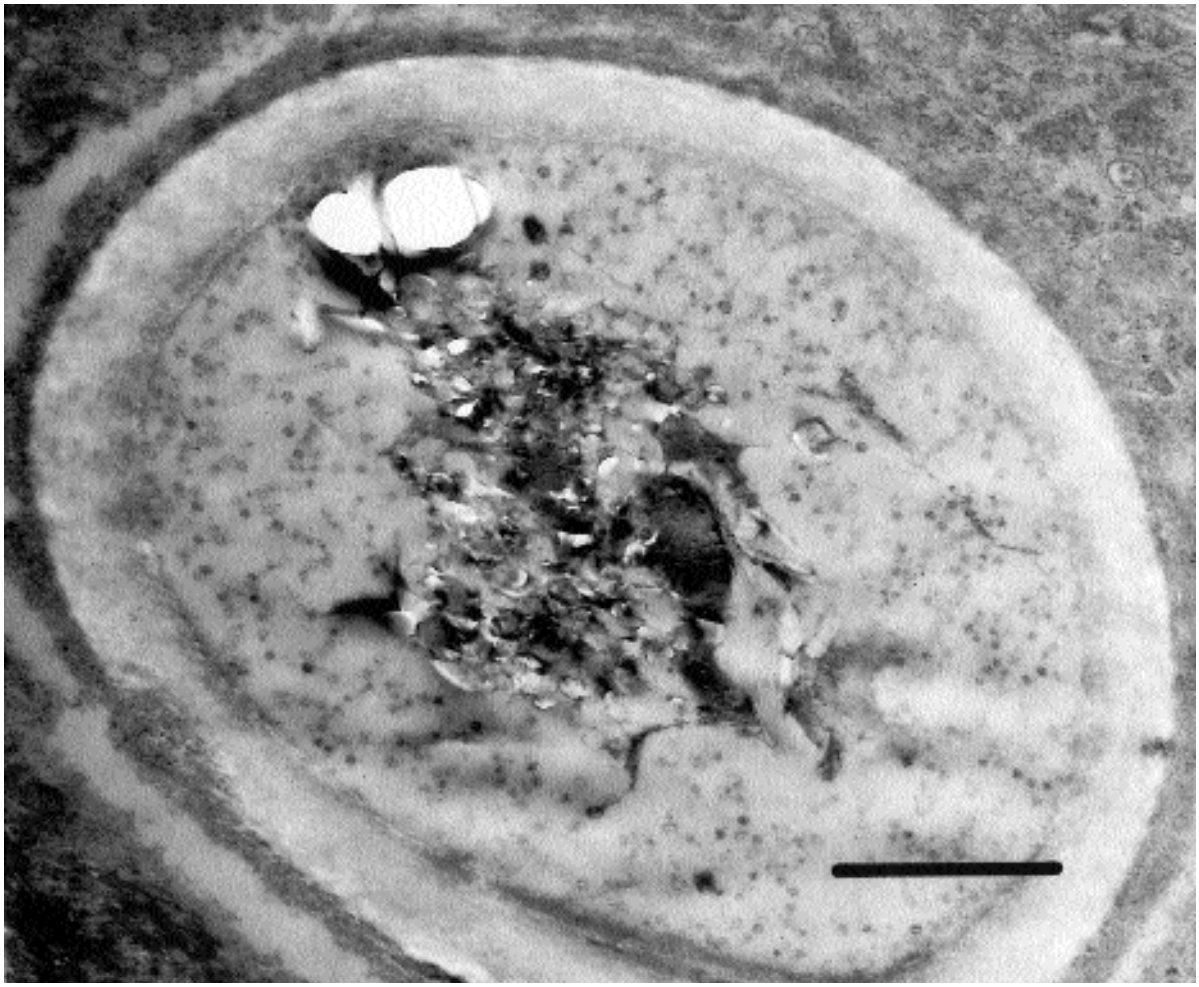

Figure 3. Electron microscopic photograph of a yeast-like cell of *Lacazia loboi* from a patient with lobomycosis. The picture shows lyses of its cytoplasmic content (Bar= 2 µm). We strongly believe that this feature prevented earliest investigators from isolating good DNA from this pathogen (from Eric Tarcha).

Not all microorganisms give up their DNAs easily

Efforts to isolate good DNA from *L. loboi* for phylogenetic purposes were attempted since the early 1990's by several teams, all of them failed. According to our studies the production of lytic enzymes, even during infection, may have contributed to this failure. Data collected during these studies indicated that the 18S SSU rDNA sequences from the target pathogens in tissue were more efficiently amplified by PCR from fresh samples rather than from fixed tissue blocks or from fresh samples stored at –80°C. In fixed samples and in tissue stored at –80°C, *L. loboi* DNA apparently was too degraded to amplify. Without abundant and high quality *L. loboi* DNA, contaminating 18S SSU rDNA, including that of mammals, insects and other fungi, was amplified. In contrast, aseptically collected fresh samples contained abundant target DNA. In these samples PCR amplifications were free of contaminants therefore more reliable.

This finding may explain the results obtained by others [10] on *L. loboi* from infected dolphins. These investigators found that ITS sequences amplified from *L. loboi* fixed tissues were phylogenetically closer to the black fungi. According to our findings, however, we believe that those investigators amplified their ITS sequences from a contaminating black fungus and not from *L. loboi*. Our most recent amplification and sequencing of DNAs taken from other patients with lobomycosis showed that the 18S SSU rDNA and ITS sequences were identical to those found by Herr *et al.* [11], supporting previous results and interpretations.

All in all, these studies have confirmed that molecular approaches are powerful tools for the study of novel and uncultivable organisms. We believe that this approach can be used not only to reveal phylogenetic relationships, but to study other aspects of a pathogen's life cycle in nature that otherwise would be difficult to resolve using classical approaches.

Summary

The taxonomic relationships of the last two enigmatic pathogens studied in medical mycology, *Lacazia loboi* and *Rhinosporidium seeberi*, has always been controversial. Recently, however, using molecular approaches their phylogenetic connections were revealed. It was found that *L. loboi* is more likely a dimorphic fungus phylogenetically closer to *P. brasiliensis* and the other dimorphic fungal pathogens (*A. dermatitidis*, *A. capsulatum* and *E. parva*). Whereas, *R. seeberi* is a protist in the new class Mesomycetozoea, which is situated in the divergence between animals and fungi.

We wish to thank our graduate student Roger Herr for his invaluable contribution to this work, Drs. Paulo Taborda and A.N. Arseculeratne for the tissue samples from patients with lobomycosis and rhinosporidiosis and Eric Tarcha for his involvement with Lacazia loboi.

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