



Lacazia loboi and *Rhinosporidium seeberi*: a genomic perspective

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Summary

In the past five years, with the use of molecular strategies the phylogenetic affinities of the two more resilient pathogens studied in medical mycology, *Lacazia loboi* and *Rhinosporidium seeberi* were finally deciphered. These studies found that *L. loboi* was the sister taxon to *Paracoccidioides brasiliensis*, and *R. seeberi* was closely related to protistan spherical aquatic fish pathogens, located at the point where animals diverged from the fungi, in the class Mesomycetozoa. These initial studies indicated that a molecular strategy was the ideal approach to further understand these anomalous pathogens. However, the limited amount of information gathered so far from few DNA sequences, although crucial to place these organisms in the tree of life and to take a glance at their ecological preferences, did not provide answers to other important traits. In the following pages we discuss a genomic perspective for both pathogens and the benefit that such information could generate to understand more about these two uncultivated pathogens.

Key words

Lacazia loboi, *Rhinosporidium seeberi*, Phylogenetic affinities, Genome

Lacazia loboi y *Rhinosporidium seeberi*: una perspectiva genómica

Resumen

En los últimos cinco años, las afinidades filogenéticas de los dos patógenos más insidiosos estudiados en Micología Médica, *Lacazia loboi* y *Rhinosporidium seeberi*, han sido finalmente descifradas con la utilización de estrategias moleculares. Estos estudios encontraron que *L. loboi* era un taxón hermano de *Paracoccidioides brasiliensis* y *R. seeberi* estaba muy relacionado con los protistas esféricos patógenos de peces acuáticos, localizados en el punto en el que los animales divergen de los hongos, en la clase Mesomycetozoa. Dichos estudios iniciales indicaron que una estrategia molecular era la vía ideal para entender en profundidad estos patógenos anómalos. Sin embargo, la cantidad limitada de información reunida hasta el momento a partir de unas pocas secuencias de ADN, aunque crucial para situar estos organismos en el árbol de la vida y tener una visión de sus preferencias ecológicas, no ha proporcionado respuestas a otras características importantes. En las páginas siguientes discutimos una perspectiva genómica para ambos patógenos y el beneficio que esta información podría generar para entender mejor a estos dos patógenos que no pueden ser cultivados.

Palabras clave

Lacazia loboi, *Rhinosporidium seeberi*, Afinidades filogenéticas, Genoma

The two anomalous pathogens *Lacazia loboi* and *Rhinosporidium seeberi*, were the last taxonomic mysteries to be solved in medical mycology [7]. Although their phenotypic structures developed in the infected tissues were extensively studied, characteristics frequently used to classify them as a fungal or protistan microbes, their true taxonomic relationship with other microorganisms in the tree of life remained elusive for decades [9,10]. This was in part due to the fact that these hydrophilic pathogens resisted being cultured [10], attribute that steamed a great controversy about their epidemiology, taxonomy, pathogenesis and life cycles. To make things more complex, they shared morphological features with both protistan and fungal microbes and did not respond well to most antifungal drugs. Thus, at one point some investigators

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suggested that these pathogens were not fungi, but some type of unusual protists [10]. The uncultivated nature of *L. loboi* and *R. seeberi* slow down, not only efforts to study their immunological features in the infected hosts and other important characteristics, but also their taxonomic relationship with other organisms. As a response to this frustration, some investigators introduced extreme hypothesis to explain some unknown aspects of these anomalous pathogens [9]. The fact is that using traditional tools these two pathogens were largely misunderstood.

After several years without a decisive breakthrough on the taxonomy and life cycle of these two uncultivated microbes, it was clear that a new strategy was necessary to solve these enigmas. Molecular methodologies to study these and other uncultivated microbes were early suggested by some investigators [7]. However, it was only recently when Herr et al. [5,6], and Fredericks et al. [4] established using molecular tools that *R. seeberi* was a protistan pathogen closely located to the point were the fungi and animals first diverged some 1.7 billions years ago [9], whereas *L. loboi* was found to be the phylogenetic sister taxon to the dimorphic fungal pathogen *Paracoccidioides brasiliensis* nearby the other pathogenic dimorphic Onygenales. These innovative studies confirmed that a molecular approach to address old questions about the phylogeny of *L. loboi* and *R. seeberi* was the right strategy. What we have learned in the past five years using molecular strategies provided insides about these two pathogens not previously anticipated. Although these early studies used only rDNA and conserved protein (chitin synthase) sequences, the collected data indicated that the molecular approach was the appropriate phylogenetic strategy to further characterize these two uncultivated microbes.

The main problem with these early molecular approaches [5,6] was the fact that they could only investigate DNA sequences obtained by PCR mainly with the used of universal primers of well-known proteins or rDNA sequences. From a genomic perspective this fact renders this approach of limited value. In an effort to resolve in part this problem, Vilela et al. [14] recently introduced a new molecular strategy to investigate unknown proteins of interest in the immunology and pathogenesis of the infections caused by *L. loboi*. Based on the finding of Herr et al. [6], indicating that *P. brasiliensis* was the phylogenetic sister group to *L. loboi*, Vilela et al. [14] suggested that these two South American pathogens might also share similar coding DNA sequences. They also indicated that the accessibility to several key proteins of *P. brasiliensis* deposited in GenBank could be used to build primers from their conserve regions and target their homologous sequences in *L. loboi*.

Vilela et al. [14] took also advantage of the finding that the gp43 antigenic protein of *P. brasiliensis* strongly cross-reacted with sera of patient lacaziosis. This feature was used to theorize on the possibility that the gp43 DNA coding sequence of *P. brasiliensis* could have a similar homolog in *L. loboi*. They designed primers, based on the conserved regions of several fungal 1-3 β -glucanase (cellulase) including the gp43 cellulase of *P. brasiliensis*, and amplified 483bp of the gene coding the gp43-like sequence of *L. loboi*, confirming their hypothesis. Their study indicated that the 483bp DNA sequences encoding the gp43-like gene in *L. loboi* share several attributes in common with the gp43 sequence of *P. brasiliensis*, a finding that supported previous molecular studies in this pathogen [5,6].

A careful look into the 483bp DNA sequence coding for the gp43-like gene of *L. loboi* deposited by Vilela et al. [14] (AY697436), revealed several interesting features of *L. loboi* DNA sequences not previously described. Commonly, most DNA sequences coding protein motifs possess high amino acid sequences identity, whereas their nucleotide sequences usually has low nucleotide identity when compared with their homologous sequences available in the data-base. However, the deduced amino acid sequence obtained from the 483bp DNA sequence of *L. loboi* possesses only 75% identity at the amino acids level, and an unexpected 85% identity at nucleotide level with the gp43 of *P. brasiliensis* and other fungal cellulases. This finding indicates that *L. loboi* has a nucleotide identity the opposite of the nucleotide sequences exhibited by the coding regions of most pathogens and saprotrophic microbes. The other available DNA sequence in GenBank encoding the chitin synthase 2 gene of *L. loboi*, has also a tendency to low identity at amino acid level versus high identity at nucleotide level. We believe that this could be related to the parasitic life style of this pathogen, which remains in the subcutaneous tissues for years, sometime more than 40 years. The lack of a known sex-cycle and contact with its environmental ecological niche for long periods of time may be an important factor to retain nucleotide motifs in its DNA sequences, and therefore, contributing to its high nucleotide identity. A rapid survey of few sequences coding proteins in other uncultivated microbes such as *Mycobacterium leprae*, *Pneumocystis jiroveci*, and *Treponema pallidum*, indicated that some DNA sequences coding proteins also have the tendency to high nucleotide identity over their coding amino acids sequences, when compared to homologous sequences in other microbes. Assuming that a host is infected with only one strain of *L. loboi*, one could speculate that this adaptation could be a response to a lack of recombination with other *L. loboi* strains during its parasitic stage and a prolonged contact only with the genome of the infected hosts. A similar hypothesis has been proposed for *M. leprae* [3,15].

Of interest was also the finding that the primers designed by Vilela et al. [14], amplified the predicted 486bp amplicon in *P. brasiliensis*, but only 483bp DNA fragment in *L. loboi*. The three missing nucleotides encoded the amino acid threonine at position 121. The absence of this amino acid in the gp43-like sequence of *L. loboi* seems at first glance irrelevant. However, the report of extensive loss of gene function (reductive evolution) in host restricted uncultivated pathogens such as *M. leprae*, *Rickettsia prowazekii*, *Chlamidia* spp., and the endosymbiont *Buchnera* spp., in which entire pathways have been eliminated, suggests a that this could well be an interesting feature of some obligated pathogens [1-3,11,12]. Reductive evolution (gene reduction, genome decay) has been defined as "the stochastic loss of genetic material resulting in decreased fitness and generating little genetic variability (Muller's ratchet hypothesis)" [3]. It has been speculated that reductive evolution could be a feature of highly specialized obligated pathogens with no known sex-cycle to acquire DNA and repair genetic lesions by recombination or acquisition of new genes.

When the entire genome of *M. leprae* was sequenced, it was reported that this obligated pathogen possesses only ~1,655 DNA sequences encoding key proteins and rDNA in its entire genome. This is rather low since *M. tuberculosis* genome has more than ~4,000 gene coding regions [3,15]. To explain this anomalous situation, several authors have proposed that during *M. leprae*

parasitic cycle the function of unused pathways were simply eliminated and their coding regions transformed in pseudogenes, thus reducing the number of active genes to 24% of that in *M. tuberculosis*. This reduction in the number of functional genes and mutations in several active metabolic pathways was recently implicated for the failure of *M. leprae* in vitro growth. It has been reported that gene decay in this prokaryote was more commonly found in genes with degradative functions, than in genes involved in synthetic pathways [15]. Another interesting feature of *M. leprae* was its low G+C content (57.8%) when compare with *M. tuberculosis* (65.5%). Interestingly, the few deposited coding sequences of *L. loboi* have ~55.9% G+C content compared with *P. brasiliensis* (G+C=62.6%), strikingly similar to the percentage of G+C content reported in *M. leprae*.

The obligated nature of *L. loboi* parasitic cell cycle, its prolonged contact only with the genome of its hosts (human and wild life animals), its intractability to culture (mutations in metabolic pathways?), its lower G+C content, and its unusual features encountered so far in the available DNA sequences, strongly suggests that this eukaryotic pathogen has genomic features in common with those prokaryotic obligated parasites with decaying genomes. The features of the available *L. loboi* DNA sequences suggest that this eukaryotic uncultivated pathogen might be an example of a eukaryote parasite with reductive evolution features acquired after years of prolonged parasitic life style. Based on these features it could be predicted that *L. loboi* genome sequence project would yield rewarding results to understand more about the comparative genome complexity of this and other dimorphic pathogenic Onygenales.

Perhaps the most significant finding revealed by the DNA data was the recent report of host specific strains in the genus *Rhinosporidium* [13]. This study suggested that *R. seeberi* is not a monotypic genus but constituted of multiple strains (species?) closely related to their hosts. This finding is very important to further explain the epidemiological features of this enigmatic pathogen. For instance, the idea that the genus *Rhinosporidium* might possess an intermediary host was first postulated more than 80 years ago [9]. With the use of classical mycological tools, however, this hypothesis was never confirmed. With the advent of molecular analysis we have learned that *Rhinosporidium* is related to aquatic microbes (Mesomycetozoa, Ichthyosporea), most of them fish pathogens with no intermediary hosts, but with complex life cycles. For instance, the genus *Dermocystidium* and *Sphaerothecum* (Dermocystida), once outside their hosts, develop unflagellate cells from their released endospores.

These unflagellate cells swim to locate new hosts or encyst for very long periods of time (resistant spore) until the environmental conditions are suitable to developed secondary unflagellate cells. Similar situation occurs in the order Ichthyophonida, but in this group the production of amoeboid infecting units is the main feature of the order. This prompted us to investigate whether *Rhinosporidium* possesses unflagellated cells. Our study showed that *Rhinosporidium* did not develop unflagellate cells using *Dermocystidium* or *Sphaerothecum* protocols [10]. The lack of a unflagellate stage is also a feature in common with the genus *Amphibicystidium*. Thus, based on the information from an epidemiological perspective, most probably *Rhinosporidium* and *Amphibicystidium* do not possess intermediate hosts and their spores are released into the environment (terrestrial or aquatic) becoming resistant units until they contact a susceptible human or animal host. Data supporting this theory come from the reports that, apparently healthy humans from India and Sri Lanka, and other areas, and healthy swans from Florida acquired the infection after bathing their bodies in rivers, waters ponds, or lakes respectively. However, *Rhinosporidium* is not only found in aquatic environments; it has been also reported in the Middle East deserts without water and in the dry regions of Argentina, north India and Sri Lanka. This strongly supports the notion that *Rhinosporidium* possesses resistant spores and that they could also be airborne transmitted in those areas, especially during sand storms [10]. Since no clear evidence of transmission between humans, animals, or vice versa had ever been reported, the presence in the environment of resistant *R. seeberi* infecting units is the most likely scenario.

Although there are more sequences of *R. seeberi* available in GenBank than those in *L. loboi*, all the deposited genes in *R. seeberi* have been from rDNA sequences rather than from coding protein sequences. This, and the fact that nothing is known about the genomic features of the closely related mesomycetozoeans, has greatly limited the accessibility to protein sequences in this newly described class [9]. Nevertheless, with the exception of *Amoebidium* spp., *Ichthyophonus* spp., and *Sphaeroforma arctica*, that can be isolated in culture, the remaining members have resisted culture. Thus, the genome sequences of the cultivated mesomycetozoeans, would be of extremely importance to understand other members of the class and, by extrapolation, the other uncultivated microbes localized at the animal fungal boundaries.

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