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Molecular phylogeny of animal pathogen *Lacazia loboi* inferred from rDNA and DNA coding sequences

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ARTICLE INFO

Article history:
Received 9 September 2008
Received in revised form
9 February 2009
Accepted 21 April 2009
Published online 9 May 2009
Corresponding Editor:
Leonel Mendoza

Keywords:
Ajellomycetaceae
Lacazia loboi
Lacaziosis
Lobomycosis
Onygenales
Phylogenetics

ABSTRACT

Lacazia loboi is a geographically restricted, uncultivated fungal pathogen of humans and dolphins. Previous investigations using 18S small unit rDNA, *chitin synthase 2* and *gp43* DNA sequences positioned *L. loboi* as a close relative of *Paracoccidioides brasiliensis*. However, given the few individuals of *L. loboi* studied and the high degree of genetic variation observed in *P. brasiliensis*, the existence of *L. loboi* as an independent species has been questioned. To investigate the phylogenetic position of this species, we conducted a phylogenetic analysis using 20 *L. loboi* collections (*L. loboi* was obtained from proven cases of lacaziosis and 14 collections were maintained in mice, the others were analyzed from DNA taken directly from infected human tissue.). *L. loboi* DNA sequence was compared to that from 17 *P. brasiliensis* strains that represented the known variation in this species, and outgroup taxa in the Onygenales (*Ajellomyces* and *Coccidioides* species). Our analyses used DNA sequence from *ITS* rRNA, and partial coding sequences of *chitin synthase 4*, *ADP-ribosylation factor*, and *gp43*. Nucleotide variation among strains of *L. loboi* was minor but numerous nucleotide mismatches and multiple gaps were found for these gene regions among members in the Ajellomycetaceae, including *P. brasiliensis*. Phylogenies inferred using neighbor-joining, maximum parsimony and Bayesian analyses showed no significant conflict and depicted *L. loboi* as a well-supported, monophyletic group that was sister to the *Paracoccidioides* clade. These results argue for maintaining *L. loboi* as a taxon independent from *Paracoccidioides* within the Ajellomycetaceae.

Introduction

There are three known, uncultivated, fungal pathogens of humans and other mammals: *Pneumocystis jirovecii* (Cushion 2007), a strain of *Ajellomyces capsulatum* affecting Peruvian monkeys (Miller *et al.* 1998), and *Lacazia loboi* (Lacaz *et al.* 1986; Taborda *et al.* 1999; Mendoza 2007). *P. jirovecii* has been the most extensively studied species because it affects immunocompromised humans and has a worldwide distribution. In contrast, the unusual genotype of *A. capsulatum* from Peruvian monkeys has been reported only twice (Miller *et al.* 1998; Miller & Owens 1999), and *L. loboi* is known only as a pathogen of

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0953-7562/$ – see front matter
doi:10.1016/j.mycres.2009.04.007

a small number of apparently healthy humans in Latin America and of dolphins inhabiting coastal areas of Florida and other parts of the Gulf of Mexico (Lacaz *et al.* 1986; Reif *et al.* 2006). The infections caused by *L. loboi* in humans (lacaziosis) were first described in Brazil in 1930 by Jorge de Oliveira Lôbo (Lôbo 1930). Early investigators reported that yeast-like cells of *L. loboi* possess *in vivo* morphological and antigenic attributes in common with the parasitic yeast cells of another geographically restricted South American pathogen, *Paracoccidioides brasiliensis* (Silva *et al.* 1968; Fonseca & Lacaz 1971; Lacaz *et al.* 1986; Lacaz *et al.* 2001). Based on these characteristics, the etiologic agent of lacaziosis has been largely misunderstood for more than 70 y, and as a result its taxonomic position has remained uncertain (Mendoza & Silva 2004). Herr *et al.* (2001) and later Vilela *et al.* (2005) used DNA extracted from three collections of *L. loboi* yeast-like cells, and phylogenetic analyses with rDNA and protein coding genes, to show that this anomalous pathogen was the sister taxon to *P. brasiliensis* and was closely related to the other onygenalean pathogens, *A. capsulatum*, *Ajellomyces dermatitidis*, and species of *Coccidioides*. Subsequent to these studies, significant variation was found in *P. brasiliensis* (Matute *et al.* 2006; Carrero *et al.* 2008), raising the possibility that *L. loboi* could be accommodated within *P. brasiliensis* (Mendoza & Silva 2004; Mendoza *et al.* 2005).

Investigating *L. loboi*

standard polymerase chain reaction methodologies in 25 μl volume reactions. The samples were initially heated at 95 °C for 10 min and then entailed to 40 cycles consisting of 1 min at 95 °C, 2 min at 60 °C and 3 min at 72 °C, with a final extension at 72 °C for 10 min. All PCR samples were cleaned using Centri-Sep Columns (Princeton Separations, Adelphia, NJ). Primers used in the study were as follows: *L. loboi* endoproteinase Lys/Arg-Arg (*kex*) *Llkex*-1 5′TGCTTCYGGTTTGGGGTTG3′ and *Llkex*-2 5′CACTGGARCCGTCAGCTA3′; *L. loboi chitin synthase 4 LlCHS4*-1 5′CACCACCTGTCTAAAGCT3′ and *LlCHS4*-2 5′CGATTTCAATGTCAGAATA3′; *L. loboi ADP-ribosylation factor (ADP-rf) LlRibosyl*-1 5′GYCTCGATGCTGCCGGAA3′ and *LlRibosyl*-2 5′ACGACACGGTCA CGATCG3′; the existing primers NL2 and NL4 targeting *L. loboi gp43*-like gene (Vilela *et al.* 2005), and the universal primers ITS1 and ITS4 (White *et al.* 1990).

The amplicons were independently cloned into TOPO TA plasmid vectors (Invitrogen, Carlsbad, CA, USA) and single clones were selected for sequencing with Big-Dye chemistry (Applied Biosystem, Foster City, CA, USA). Collected reads from a 310 capillary sequencer (Applied Biosystem, Foster City, CA, USA) were base-called using Phred (http://www.phrap.org/phredphrap/phrap.html) and subsequently vector clipped using Lucy (http//compbio.dfci.harvard.edu) with standard parameters. The sequences were compared with the NCBI nucleotide and protein databases using the BlastX algorithm available at the http://www.ncbi.nlm.nih.gov/.

Phylogenetic analyses

The DNA sequences of ITS and partial gene sequences of exons for *ADP-rf*, *CHS4* and *gp43* were aligned using CLUSTAL W, v. 1.81 with default settings (Thomson *et al.* 1994) followed by use of MacClade (MacClade version 4.08), with visual inspection. Aligned, combined sequence was exported for parsimony analysis (heuristic in PAUP* 4.0 Swofford 2003), distance analysis (neighbor-joining in PAUP* Swofford 2003) and Bayesian analysis (MrBayes 3.1.2, Huelsenbeck & Ronquist 2001; Ronquist & Huenselbeck 2003). Large insertions were coded as one event by excluding all but one nucleotide per insertion. Parsimony analysis used a heuristic approach with TBR branch swapping. Neighbor-joining analyses used either uncorrected distances or maximum-likelihood estimates of distances with a general time reversible model (6ST), empirical base frequencies, and either no rate variation among sites or a gamma distribution (shape parameter 0.5) of variation among sites with four rate categories. Bayesian analysis used the GTR + I + gamma model, with two chains (one heated), two runs, sampling every 100th generation for 1×10^6 generations, and exclusion of the first 2.5×10^5 samples (the burn-in) prior to analysis. Support for branches was estimated as the percentage of parsimony trees (1000 resamplings, heuristic, nni branch swapping) or neighbor-joining trees (1000 resamplings, maximum-likelihood distances) containing the branch as well as by determining the Bayesian probability estimated as the percentage of Bayesian trees possessing the branch after discarding the burn-in samples (Huelsenbeck & Ronquist 2001). In addition, the aligned sequences sets of ITS (<409 bp), *CHS4* (216 bp), *ADP-rf* (>408 bp), and *gp43*-like (483 bp), were analyzed separately to visually assess congruence. The incongruence length difference test (Farris *et al.* 1994) or partition homogeneity test (Huelsenbeck *et al.* 1996) was implemented in PAUP 40b010. Gblocks was used to exclude putative problematic motifs within the investigated DNA sequences (Castresana 2000).

Results

Lacazia loboi *DNA sequences*

The *ITS*, *CHS4*, *ADP-rf* and *gp43*-like genes were successfully amplified and sequenced from 20 of the 35 originally investigated strains of *Lacazia loboi* and these strains were chosen for further phylogenetic analyses (Table 1). For the remaining 15 strains, not all four genes could be amplified and, thus, their sequences were not included in this study. We were able to amplify and sequence the *kex* gene from only four *L. loboi* strains (6-SPS, 13-FAV, 35-RCN from Table 1, and 8SFC). There was no variation among the *kex* sequences, so we did not pursue analysis of this gene.

Among the *L. loboi* sequences, there was little nucleotide variation. One nucleotide position varied among the 20 ITS sequences, three among the 20 *CHS4* sequences, four among the 20 *ADP-rf* sequences and 13 among the 20 *gp43*-like sequences. Nucleotide substitutions were far more common than informative indels, by at least 12 fold (334/26). In contrast, numerous nucleotide mismatches (≥50) and several gaps were found between *L. loboi* DNA sequences used in this study and the orthologous DNA sequences of *Ajellomyces* spp., *Coccidioides* spp, and *Paracoccidioides brasiliensis* fetched from the data base (alignment deposited at TreeBASE. Accession # SN4314). High stringency BlastX searches using the sequence of the five *L. loboi* DNA regions revealed similarities mostly with *P. brasiliensis*, *Ajellomyces capsulatum*, *Ajellomyces dermatitidis*, and to the less extent with *Coccidioides immitis* DNA sequences.

Molecular phylogeny of Lacazia loboi

Using species of *Coccidioides*, *Ajellomyces capsulatum* and *Ajellomyces dermatitidis* as the outgroups, the phylogenetic trees resulting from analyses of the combined data by the three phylogenetic methods, parsimony, neighbor-joining and Bayesian, showed no significant topological conflicts. That is, by all three methods, all *Lacazia loboi* isolates formed a well-supported monophyletic clade (100 % parsimony bootstrap, 100 % nj bootstrap, 1.0 Bayesian probability), all *Paracoccidioides brasiliensis* isolates formed a well-supported monophyletic clade (100 % parsimony bootstrap, 100 % nj bootstrap, 1.0 Bayesian probability) and the *L. loboi* and *P. brasiliensis* clades, together, formed a clade that is sister to the clade of *Ajellomyces* spp. (94 % parsimony bootstrap, Bayesian probability 1.0). Within the *P. brasiliensis* clade, two strains of this South American pathogen (*P. brasiliensis*-14 and *P. brasiliensis*-17) formed a well-supported clade (100 % parsimony bootstrap, 100 % nj bootstrap, 1.0 Bayesian probability), with a somewhat less well-supported sister clade comprising all the remaining *P. brasiliensis* isolates (79 % parsimony

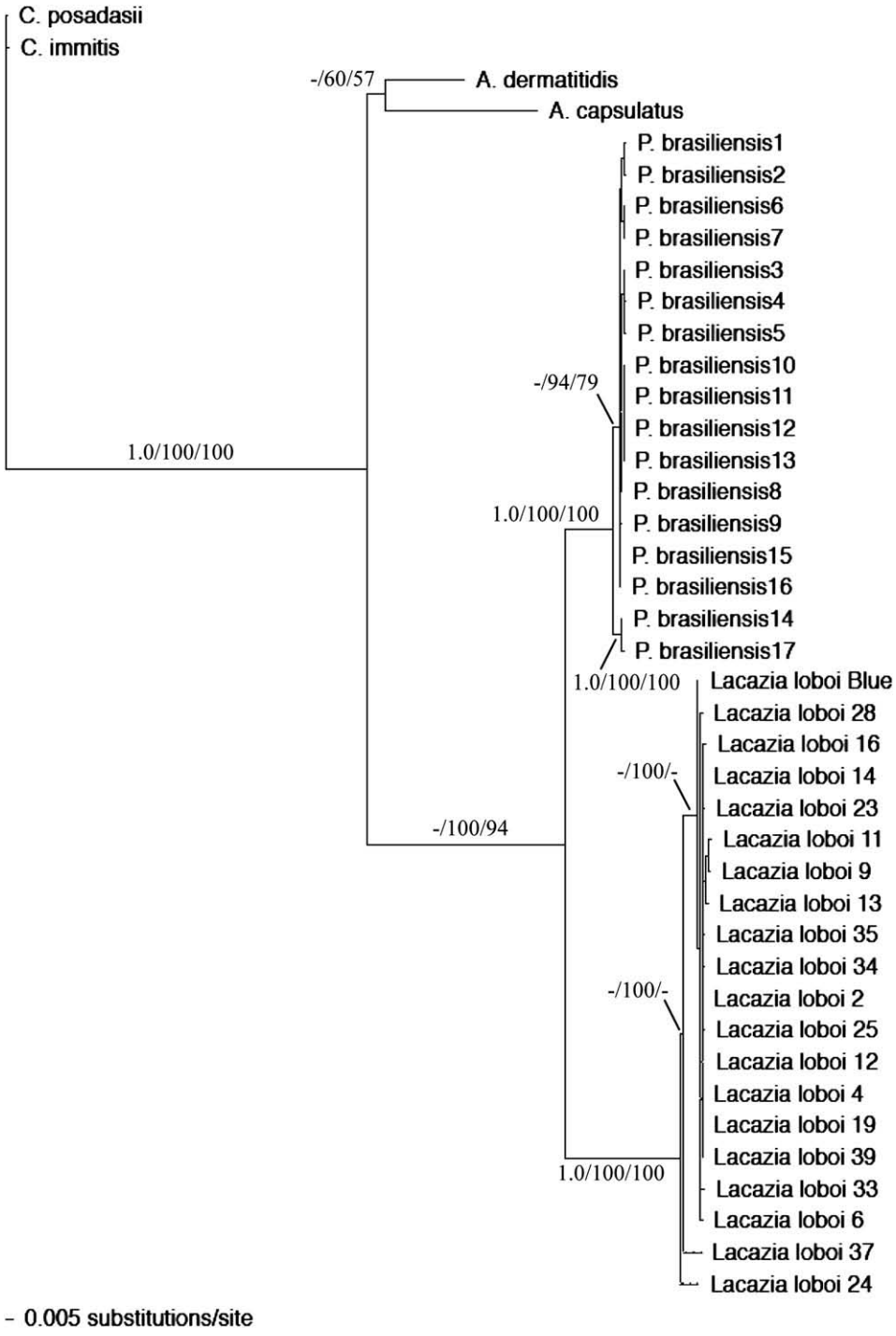

Fig 1 Phylogenetic analysis of individuals of *Ajellomyces*, *Coccidioides*, *Lacazia* and *Paracoccidioides* species based on analyses of four partial gene sequences. The topology presented here is based on neighbor-joining analysis of maximum-likelihood estimates of genetic distance. Support on key branches is the Bayesian probability for that branch followed by the percentage of 1000 bootstrap resampled data sets containing the branch in neighbor-joining analyses of maximum-likelihood distances followed by the percentage of 1000 bootstrap resampled data sets containing the branch in parsimony analyses using heuristic searches. *Coccidioides* species were designated as the outgroup based on published analyses of larger samples of Onygenalean fungi (Untereiner *et al.* 2004). The scale bar represents the number of substitutions per nucleotide position based on the neighbor-joining analysis. The tree contains DNA sequences used by Carrero *et al.* (2008) and Matute *et al.* (2006). The accession numbers other than *Lacazia loboi* DNA sequences (Table 1) are as follows (the *ADP-rf* and *CHS4* sequences related to strains 14 and 17 were not available): ADP-Ribosylation factor: *Ajellomyces capsulatum* AF072357; *Ajellomyces dermatitidis* AY013310; *Coccidioides immitis* XM001243101; digits before accession numbers identified each strain of *Paracoccidioides brasiliensis* in the tree. 1-DQ004112; 2-DQ004109; 3-DQ004104; 4-DQ004067; 5-DQ004064; 6-DQ004058; 7-DQ004057;

bootstrap, 94 % nj bootstrap) (Fig 1). Within the *L. loboi* and *P. brasiliensis* clades, there were many different topologies found by parsimony analysis as expected for individuals within a species. In *P. brasiliensis*, with the exception of the branch uniting isolates 14 and 17, none of the branches was consistently well supported. Within the *L. loboi* clade, branches placing isolate 24 as sister to all other isolates, and within the remaining isolates, placing isolate 37 as sister to the remaining isolates well supported by neighbor-joining bootstrap (both at 100 %), but not by parsimony bootstrap or by Bayesian probabilities. The test for conflict among individual gene trees (ILD or PHT for *CHS4*, *ADP-rf*, *gp43-like* protein, *ITS*, and *kex*) found no conflict ($p = 1.0$) when no more than two individuals of each well-supported clade were included (*Coccidioides immitis*, *Coccidioides posadasii*, *A. dermatitidis*, *A. capsulatum*, *P. brasiliensis* 1, 2, 14, 17; *L. loboi* 16, 24, 28, 37) to avoid the obvious conflict expected among recombining or potentially recombining individuals.

Accession numbers

The accession numbers for every one of the four loci, including four *kex* sequences, used in this study are shown in Table 1.

Discussion

Beginning with the first reported human case of Jorge Lobo's disease, its etiologic agent, *Lacazia loboi*, has been at the center of a taxonomic dispute. The fungus was described as *Loboa loboi* Ciferri *et al.* (1956) but subsequent morphological and serological studies argued that *L. loboi* was a *Paracoccidioides* species (Silva *et al.* 1968; Fonseca & Lacaz 1971; Baruzzi *et al.* 1979; Lacaz *et al.* 1986). Recent phylogenetic analyses with three strains established that *L. loboi* was indeed closely related to *Paracoccidioides brasiliensis*, and perhaps was an independent taxon (Herr *et al.* 2001; Vilela *et al.* 2005). Our phylogenetic data strongly support the placement of *L. loboi* in its own species separate from all known *P. brasiliensis* phylogenetic species (Matute *et al.* 2006; Carrero *et al.* 2008). Given the relatively short lengths of branches among *P. brasiliensis* species and the long branch found between the *Paracoccidioides* and *Lacazia* clades, it seems reasonable to retain the genus *Lacazia*.

Our study suggests that the ancestor of *L. loboi* had the ability to grow in culture, as do members of extant onygenalean genera, and that *L. loboi* must have lost its ability to grow on laboratory media as it evolved and adapted to parasitic life (Fig 2). In this aspect, *L. loboi* is similar to the *Ajellomyces capsulatum* isolate from Peruvian monkeys that, unlike other *A. capsulatum* individuals, cannot be cultivated on laboratory media (Miller *et al.* 1998). Loss of the ability to grow in culture evolved independently: once on the branch leading to *L. loboi* (black bar Fig 2), and again on the branch leading to *Histoplasma capsulatum* collected from Peruvian monkeys (Miller *et al.* 1998) (grey bar Fig 2).

In *P. brasiliensis*, our analyses show that there are two, well-supported clades (Fig 1), one of which contains two strains (Pb14, Pb17), that are among those recently described by Carrero *et al.* (2008) as a putative new species. The DNA sequences used in this study did not, however, contain sufficient variation to distinguish the three phylogenetic *P. brasiliensis* species found by Matute *et al.* (2006).

Although we have not been able to recover *L. loboi* in culture (Lacaz *et al.* 1986; Vilela *et al.* 2007), it is possible that this pathogen has a saprobic form with mycelia and conidia similar to those displayed by other members of the Ajellomycetaceae. Support for the environmental acquisition of lacaziosis comes from epidemiological accounts. For example, a Caiabi Brazilian Indian tribe acquired numerous cases of the disease when they lived in Xingu National Park, but not after they were relocated to the margins of the Paranatinga and Peixes rivers in the late 1960's (Baruzzi *et al.* 1973; Baruzzi *et al.* 1979; Lacaz *et al.* 1986). Acquisition of lacaziosis from the environment could certainly involve conidia, as seen in other Ajellomycetaceae (Bagagli *et al.* 2006; Lacaz *et al.* 1986; Terçarioli *et al.* 2007).

L. loboi and *P. brasiliensis* have different geographic distributions in South America, based on differences in the occurrence of paracoccidioidomycosis and lacaziosis (Wanke & Londero 1994; Lacaz *et al.* 2001; Terçarioli *et al.* 2007). Infections caused by *P. brasiliensis* are more frequently diagnosed south and north of the Amazon basin, whereas *L. loboi* infections are reported only within the areas of the Amazon basin and some nearby tributaries. Following the evolutionary divergence of these species, they must have adapted to mutually exclusive environments (Bagagli *et al.* 2006; Terçarioli *et al.* 2007). The divergence of *L. loboi* and *P. brasiliensis* probably took place after Africa split from South America during the break up of Pangea, because autochthonous human cases of either disease have never been documented outside the endemic areas of Latin America (Wanke & Londero 1994; Lacaz *et al.* 2001). Neither *L. loboi* nor *P. brasiliensis* are known to infect domesticated animals and, perhaps due to this fact, they appear not to have been spread by human activity, as has been

8-DQ004053; 9-DQ004052; 10-DQ004111; 11-DQ004110; 12-DQ004097; 13-DQ004096; 15-DQ004091; 16-DQ004090; *CHS4*: *A. capsulatum* HCAG_06002; *C. immitis* XM001245579; *Coccidioides posadasii* AF533442; *P. brasiliensis* 1-EF638846; 2-EF638857; 3-EF638847; 4-EF638858; 5-EF638848; 6-EF638859; 7-EF638860; 8-EF638850; 9-EF638861; 10-EF638851; 11-EF638862; 12-EF638852; 13-EF638863; 15-EF638864; 16-EF638854; *gp43*: *A. capsulatum* XM001540694; *P. brasiliensis* 1-DQ003728; 2-DQ003727; 3-DQ003778; 4-DQ003777; 5-DQ003745; 6-DQ003739; 7-DQ003733; 8-AY005420; 9-AY005433; 10-AY626378; 11-Y619000; 12-DQ003767; 13-DQ003750; 14-AB304693 (Pb01-like); 15-DQ003771; 16-DQ003772; 17-EU870196 (Pb01); ITS: *A. capsulatum* AB071828; *A. dermatitidis* AF322388; *C. immitis* AB232894; *C. posadasii* AB232900. *P. brasiliensis* 1-AY374336; 2-AY374337; 3-AY374339; 4-AB038164; 5-AY631235; 6-AY631234; 7-AY631236; 8-AY631237; 9-AF322389; 10-AY618999; 11-AF038360; 12-AF416745; 13-AB035710; 14-AB304443 (Pb01-like); 15-AB304445; 16-AB304447; 17-AF092903 (Pb01).

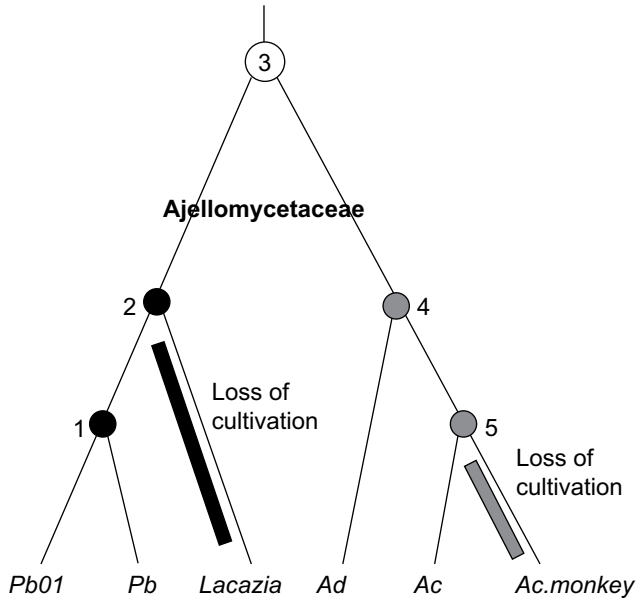

Fig 2 Diagram of relationships among Onygenales based on phylogenetic trees in this and other studies (Carrero *et al.* 2008; Herr *et al.* 2001; Kasuga *et al.* 2003; Matute *et al.* 2006; Untereiner *et al.* 2004) depicting the ancestors (white, grey, and black circles) and some of the current members in the family Ajellomycetaceae (bottom of the tree). The last common ancestor of all the taxa, ancestor 3 (white circle), was also the ancestor of postulated species that are ancestors (4, grey circle) of *A. dermatitidis* (*Ad*) and *A. capsulatum* (Ac). Within *A. capsulatum*, we postulate an ancestor (5, grey circle) for both cultivated (Ac) and uncultivated strains collected from monkeys (*Ac monkeys*, Miller *et al.* 1998). In the other principal clade, there is an ancestor (2, black circle) for all *Lacazia* and *Paracoccidioides* strains, and an ancestor (1, black circle) for the three *P. brasiliensis* phylogenetic species (*Pb*) of Matute *et al.* (2006) and the Pb01-like strains recently reported as possible new species by Carrero *et al.* (2008). The independent losses of cultivation, perhaps as a result of more complete adaptation to parasitic life, are shown for *Lacazia* (black bar) and *A. capsulatum* recovered from monkeys (grey bar).

hypothesized for *A. capsulatum* (Kasuga *et al.* 2003) and *Coccidioides posadasii* (Fisher *et al.* 2001).

No sexual stage has been found for *L. loboi*, and neither has one been found for *P. brasiliensis*, although the teleomorphic stages of closely related *Ajellomyces* species are readily induced in cultural media (Kwon-Chung KJ. 1972; McDonough & Lewis 1967). Analyses of nucleic acid variation in *P. brasiliensis* (Matute *et al.* 2006) and *A. capsulatum* (Kasuga *et al.* 2003) are consistent with recombining population structures, so it seems likely that a sexual state exists for *P. brasiliensis* and possible that one exists for *L. loboi*. If a sexual state is found for *L. loboi* (or *P. brasiliensis*), and because generic concepts in fungi rely on sexual morphology, it is possible that these fungi would be transferred to the genus *Ajellomyces*. However, until that time they should be maintained in separate genera.

Acknowledgements

This research was supported in part by the Biomedical Laboratory Diagnostics Program, Michigan State University to LM and in part by NIH, NIAID RO1 AI070891and NSF DEB 05-16511 to JWT.

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