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Small Subunit Ribosomal DNA Sequence Shows *Paracoccidioides brasiliensis* Closely Related to *Blastomyces dermatitidis*

RALF BIALEK,[1]* AIDA IBRICEVIC,[1] ANNETTE FOTHERGILL,[2] AND DOMINIK BEGEROW[3]

Institut für Tropenmedizin, Universitätsklinikum Tübingen,[1] and Botanisches Institut, Universität Tübingen,[3] Tübingen, Germany, and Fungus Testing Laboratory, University of Texas Health Science Center at San Antonio, San Antonio, Texas[2]

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The similarities of paracoccidioidomycosis and blastomycosis are highly suggestive of a close relation of the two etiological agents. Whereas the agent of the first disease is exclusively endemic in Latin America, the agent of the latter one is endemic in North America and Africa. In symptomatic travelers visiting both areas of endemicity, differentiation of the diseases might be impossible, even though therapy and prognosis for these two diseases differ significantly. In order to identify differences in the 18S rRNA gene (rDNA) for use as molecular diagnostic tools, we sequenced this gene from five isolates of *Paracoccidioides brasiliensis* and compared them to known sequences of other fungi. Neighbor-joining, maximum parsimony, and maximum likelihood analyses and, finally, the Kishino-Hasegawa test revealed that *P. brasiliensis*, *Blastomyces dermatitidis*, and *Emmonsia parva* are more closely related than *Histoplasma capsulatum* and *B. dermatitidis*, whose teleomorphic forms belong to one genus, *Ajellomyces*. In accordance with the work of other investigators who have used internal transcribed spacer and large subunit rDNA sequences, our small subunit rDNA data show that the dimorphic fungus *P. brasiliensis* must be grouped within the order *Onygenales* and is closely related to members of the family *Onygenaceae*. There are hints in the molecular phylogenetic analysis that the family *Onygenaceae* might be further divided into two families. The subgroup that includes *P. brasiliensis* comprises all zoopathogenic species. The differences in the 18S rDNAs appear to be too small to allow species identification of the members of the family *Onygenaceae* pathogenic for humans by use of target sequences within this gene.

Paracoccidioidomycosis is endemic in Latin America from 20°N to 35°S. Its etiological agent, the dimorphic fungus *Paracoccidioides brasiliensis*, has rarely been isolated from nature. Its habitat and its teleomorph are still unknown. The former name of the disease, South American blastomycosis, indicates the clinical similarities of paracoccidioidomycosis and blastomycosis. The latter is endemic in North America and Africa. For patients with travel histories to both areas of endemicity, the differential diagnosis of these diseases by clinical aspects is impossible. Histopathology and culture might be misleading, and even commercial gene probes fail to distinguish isolates of these two species (4). Since therapy and prognosis for these two diseases differ, discrimination is essential. Recently, PCR assays have been introduced for the detection of systemic fungal infections, as have hybridization techniques for the identification of pathogens. The 18S rRNA gene (rDNA) is often used as a target because a high degree of sensitivity can be anticipated due to the presence of several gene copies per genome. When we used common primers prepared from sequences from within this region to identify parts of the yet unpublished 18S rDNA of *P. brasiliensis*, a cross-reactivity to *Blastomyces dermatitidis* was unavoidable, and sequencing showed a high degree of homology of our DNA targets of these two fungi. In order to identify a target sequence unique to *P. brasiliensis*, we sequenced the complete 18S rRNA gene and used it for a phylogenetic analysis.

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* Corresponding author. Mailing address: Institut für Tropenmedizin, Universitätsklinikum Tübingen, Keplerstrasse 15, D-72074 Tübingen, Germany. Phone: 49 7071-298 2367. Fax: 49 7071 29 5267. E-mail: ralf.bialek@med.uni-tuebingen.de.

MATERIALS AND METHODS

Five isolates of *P. brasiliensis* (isolates R-2978 to R-2982), originating from A. Restrepo in Colombia, were grown on potato flakes agar at 30°C for 2 weeks and were identified in the Fungus Testing Laboratory in San Antonio, Tex. Colonies

TABLE 1. Names and GenBank accession numbers of species used for analyses

Species	Accession no.
Paracoccidioides brasiliensis (Splendore) Almeida	AF227151
Blastomyces dermatitidis Gilchrist et Stokes	M55624
Emmonsia parva (Emmons et Ashburn) Cifferri et Montemartini	U29390
Histoplasma capsulatum var. *duboisii* (Vanbreuseghem) Kwon-Chung	Z75306
Histoplasma capsulatum var. *farciminosum* Rivolta et Micelloni	Z75307
Malbranchea dendritica Sigler et Carmichael	L28064
Auxarthron zuffianum (Morini) Orr et Kuehn	L28062
Coccidioides immitis Rixford et Gilchrist	X58571
Onygena equina Persoon ex Fries	U45442
Renispora flavissima Sigler, Gaur, Lichtwardt & Carmichael	U29393
Uncinocarpus reesii Sigler et Orr	U29394
Malbranchea albolutea Sigler et Carmichael	L28063
Arthroderma benhamiae Ajello et Cheng	Z34927
Trichophyton rubrum (Castellani) Sabouraud	Z34928
Microsporum audouinii Gruby	Z34924
Epidermophyton floccosum (Harz) Langeron et Milochevitch	Z34923
Ctenomyces serratus Eidam	U29391
Gymnoascus petalosporus (Orr, Roy et Ghosh) von Arx	U29392
Aspergillus fumigatus Fresenius	M55626
Eurotium rubrum König, Spieckermann et Bremer	U00970

were scraped off the agar, dissolved in sterile water, frozen, and stored at −20°C. After thawing, two 200-μl aliquots of each suspension were used for DNA extraction. After three cycles of freezing in liquid nitrogen for 30 s and then boiling for 5 min, proteinase K (Qiagen, Hilden, Germany) was added to a final concentration of 2 mg/ml. After incubation at 50°C for 30 min, the DNA was extracted with the QIAamp Tissue Kit (Qiagen) by following the manufacturer's instructions.

The universal fungal primers (primers NS1 to NS8) and reaction conditions described by White et al. (7) were used. The reaction mixture consisted of 10 μl of DNA extract in a total volume of 50 μl with final concentrations of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$ (Perkin-Elmer [10× buffer II and $MgCl_2$ solution; Roche Molecular Systems, Inc., Branchburg, N.J.]), one primer set with each primer (Roth, Karlsruhe, Germany) at a concentration of 1 μM, 1.5 U of AmpliTaq DNA polymerase (Perkin-Elmer), and each deoxynucleotide triphosphate (Promega, Madison, Wis.) at a concentration of 100 μM. The reaction mixture was thermal cycled once for 5 min at 94°C, 30 times (94°C for 30 s, 55°C for 30 s, 72°C for 60 s), and once at 72°C for 5 min before cooling to 4°C. In the reaction mixtures with primers NS7 and NS8, the annealing temperature was raised from 55 to 60°C, with all other conditions kept identical to those described above. The PCR products were analyzed by electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and visualized on a UV transilluminator. The PCR products were purified by using the QIAquick PCR Purification Kit (Qiagen). Automated sequencing was done with a BigDye Terminator Cycle Sequencing Kit and PCR primers in accordance with the recommendations of the manufacturer, and the sequences were analyzed on an ABI 373 automated DNA sequencer (Applied Biosystems Division, Perkin-Elmer Biosystems, Foster City, Calif.). Sequences were generated from both strands, edited, and initially aligned with Sequence Navigator software (Applied Biosystems). The accession numbers of the sequences used are given in Table 1.

The alignment of 1,699 bp was done in Megalign (Lasergene; DNASTAR Inc.)

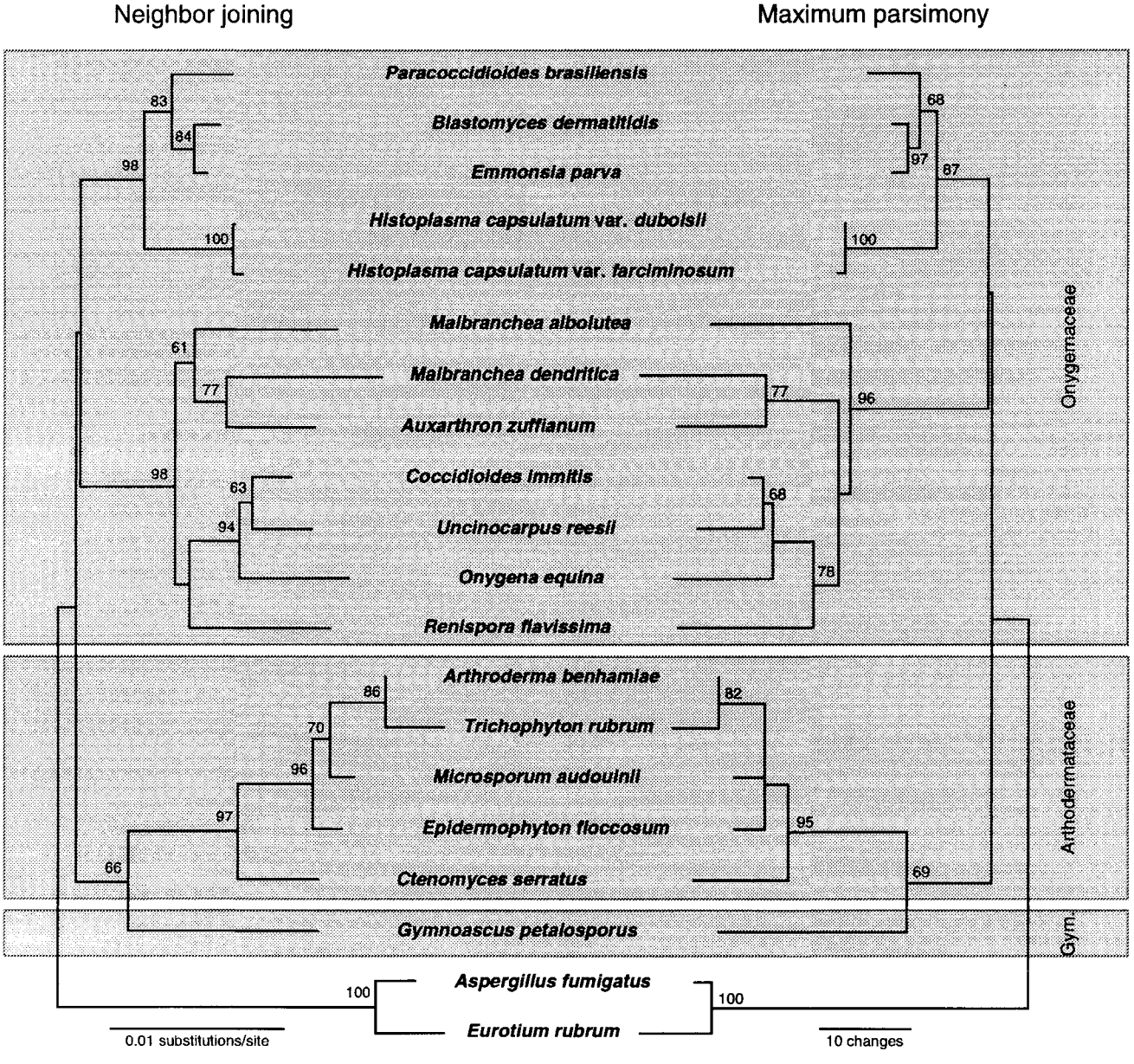

FIG. 1. Topologies obtained by neighbor-joining analysis are shown on the left. Tree number 3 of the eight most parsimonious trees is presented on the right. The phylogenetic hypotheses are based on the alignment of 1,699 bp of the small subunit of the rDNA from 18 species of the order *Onygenales*. They were rooted with *Aspergillus fumigatus* and *Eurotium rubrum*. Bootstrap values lower than 50% are not shown.

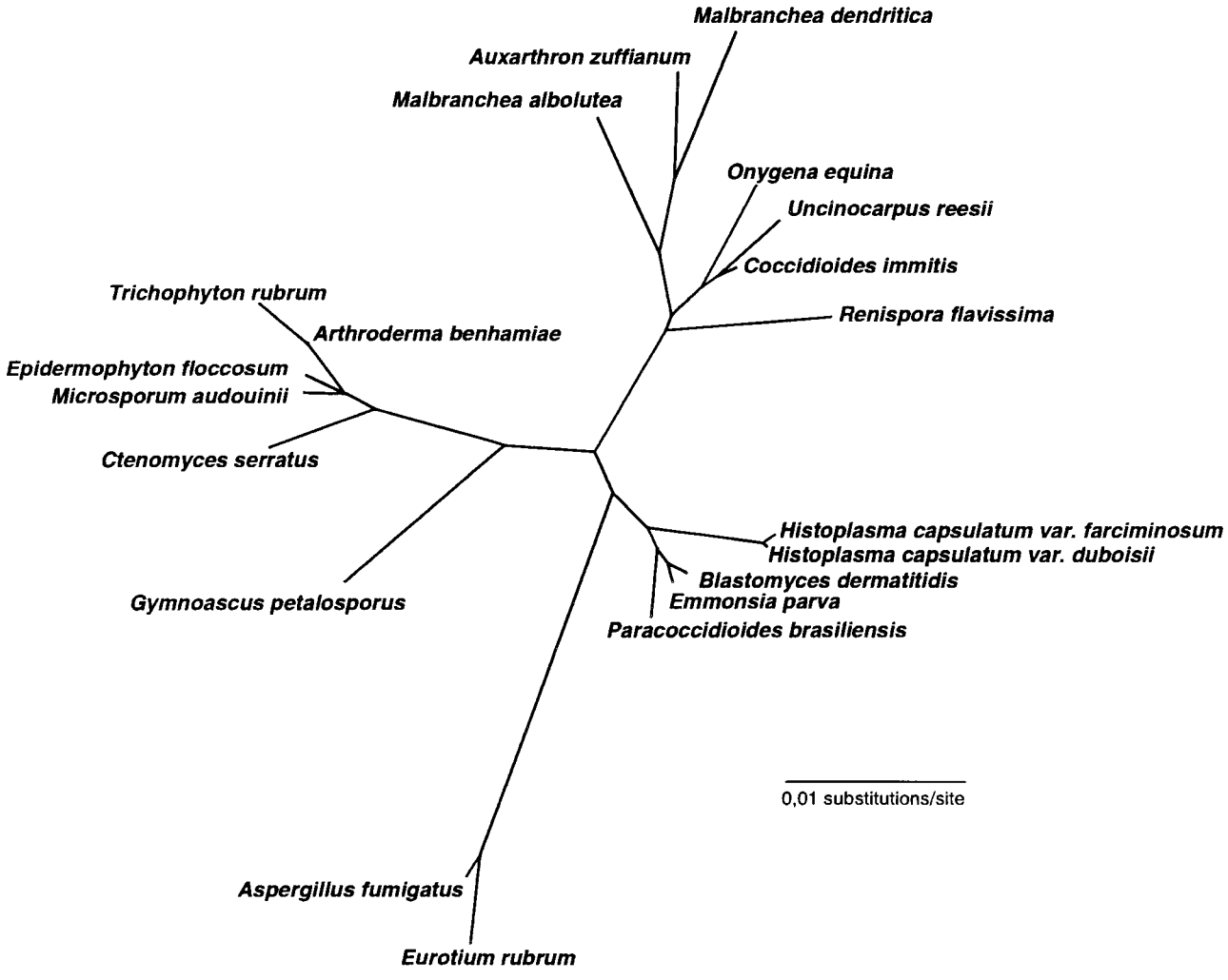

FIG. 2. Topology from maximum likelihood analysis. The phylogenetic hypothesis is based on the alignment of 1,699 bp of the small subunit of the rDNA from 18 species of the order *Onygenales* together with *Aspergillus fumigatus* and *Eurotium rubrum*.

and was optimized visually. The sequences of *Arthroderma*, *Trichophyton*, *Microsporum*, and *Epidermophyton* were available only from positions 518 to 1365.

Phylogenetic analyses were done with PAUP, version 4.0b3a, software (D. Swofford, Sinauer Associates). Neighbor-joining was calculated with all available distance models. Maximum parsimony was carried out in several analyses. In the first step we used 10,000 replicates of random addition without branch swapping to obtain all islands. In a second step we optimized the eight best trees under the maximum parsimony criterion using tree-bisection-reconnection for branch swapping. In a third step we did the optimization under the maximum likelihood criterion. Finally, we used the Kishino-Hasegawa test for comparison of the different trees. Bootstrap values were calculated for 10,000 replicates.

Nucleotide sequence accession number. The ribosomal small subunit sequence was submitted to GenBank at the National Center for Biotechnology Information, Washington, D.C., and given accession number AF227151.

RESULTS

The ribosomal small subunit sequences of the five strains studied in the present analysis show complete identity. The different tree-calculating methods showed a clear relationship between *P. brasiliensis* and the members of the family *Onygenaceae*.

The topology presented in the neighbor-joining tree of Fig. 1 was obtained with different distance models. Only if the maximum likelihood parameters were used (nucleotide frequencies of A, 0.25317; C, 0.21747; G, 0.27070; and T, 0.25866; four substitution types; invariable sites, 0.767166; gamma distribution shape parameter, 0.638474) for the neighbor-joining analysis was the same topology as that obtained by maximum likelihood analysis achieved. The maximum parsimony analysis resulted in eight islands with the same tree length of 255 steps. After 21,284 rearrangements swapped on these eight most parsimonious trees, we got no shorter tree. Tree number 3 is shown in Fig. 1. Optimization under the maximum likelihood criterion resulted in a topology that differed at the position of the *Paracoccidioides* group (Fig. 2), but the Kishino-Hasegawa test classified none of the 10 trees as significantly worse (Table 2). The bootstrap values for the monophyly of the family *Onygenaceae* are very low (Fig. 1), but there is also no evidence for a paraphyletic origin.

DISCUSSION

All analyses resulted in a monophyly of the members of the order *Onygenales* studied; i.e., it can be assumed that the species that belong to this order have a common ancestor. The species studied could be classified in the known families *Gymnoascaceae*, *Arthrodermaceae*, and *Onygenaceae*. Species of the family *Myxotrichiaceae* were not included. The topologies in

TABLE 2. Results of Kishino-Hasegawa test[a]

Test	Tree	−ln L	Diff −ln L	SD (diff)	*t*	*P*[b]
MP	1	3916.53730	7.23457	5.62549	1.2860	0.1986
MP	2	3915.63369	6.33096	5.98081	1.0585	0.2900
MP(Fig. 1, right)	3	3914.72430	5.42156	5.07218	1.0689	0.2853
MP	4	3914.72430	5.42156	5.07217	1.0689	0.2853
MP	5	3916.53730	7.23457	5.62549	1.2860	0.1986
MP	6	3915.63369	6.33096	5.98080	1.0585	0.2900
MP	7	3914.72430	5.42156	5.07218	1.0689	0.2853
MP	8	3915.63369	6.33096	5.98079	1.0585	0.2900
ML (Fig. 2)	9	3909.30274	Best			
NJ (K2P) (Fig. 1, left)	10	3912.88252	3.57979	3.82639	0.9356	0.3496
NJ (ML)	11	3910.80561	1.50287	2.12592	0.7069	0.4797

[a] Abbreviations: MP, maximum parsimony; ML, maximum likelihood analysis; NJ, neighbor-joining; Diff, difference; L, likelihood.
[b] Probability of getting a more extreme *t* value under the null hypothesis of no difference between the two trees (two-tailed test).

Fig. 1 demonstrate a close relationship of *P. brasiliensis* to *B. dermatitidis*, *Emmonsia parva*, and *Histoplasma capsulatum*. A similar tree was suggested by Bowman et al. (1), but *Paracoccidioides* was excluded due to missing data. Guého et al. (2) and Leclerc et al. (3) placed *P. brasiliensis* together with *Histoplasma* spp., separating it from *Emmonsia* and *Blastomyces*, but could not give a high bootstrap value to further prove their decision. This is in contrast to our findings and the description of Peterson and Sigler (5). Using ITS1, ITS2, 5.8S rDNA, and partial large subunit sequences of two isolates of *P. brasiliensis*, they placed *P. brasiliensis* between *Emmonsia crescens* on the one hand and *E. parva* and *B. dermatitidis* on the other hand and clearly separated *P. brasiliensis* from *Histoplasma* spp. Our data for the small subunit rDNA are in accordance with their findings.

There is some evidence from the trees in the neighbor-joining and maximum parsimony analyses (Fig. 1) that the family *Onygenaceae* is monophyletic, but the bootstrap values were too low (<50%) to support the hypothesis of monophyly. The maximum likelihood analysis (Fig. 2) is in favor of a paraphyletic origin, i.e., two different ancestors for its family members. At least the group that includes the pathogens *Paracoccidioides*, *Emmonsia*, *Blastomyces*, and *Histoplasma* spp. seems to be distinct with a separate evolutionary background and well separated from *Onygena* spp. and close relatives. However, the results of the comparison of 11 different trees by the Kishino-Hasegawa test (Table 2) are inconclusive because none of the trees including the three shown in Fig. 1 and 2 was rejected as being significantly worse than the optimal tree (Fig. 2). Further studies may demonstrate a fifth family within the order *Onygenales*, as proposed by Sugiyama et al. (6) as well. The human pathogen *Coccidioides immitis* appears in a totally different group, which is in favor of a convergent evolution of human pathogenicity.

The evolutionary analysis discloses relationships which have been assumed by the pattern of diseases and by classical mycology. The species of the first family, *Onygenaceae*, are all known zoopathogenic fungi, which might have evolved from a common ancestor. The grouping can help to identify common virulence factors and target sequences for diagnostic purposes, but it shows as well the limits of differentiation by gene probes within common sequences such as 18S rDNA. In order to determine the specificity of any diagnostic PCR assay, the isolates of fungal species grouped together must be examined, as should clinical specimens of patients infected with one of these pathogens. Clinicians must be aware of possible cross-reactions disclosed by genetic analysis.

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