J. Microbiol. Biotechnol. (2000), **10**(3), 363–366

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Phylogenetic Analysis of Genus *Sporobolomyces* Based on Partial Sequences of 26S rDNA

HONG, SOON GYU, JONGSIK CHUN, JIN SIK NAM, YOON-DONG PARK, AND KYUNG SOOK BAE*

Korean Collection for Type Cultures, Genetic Resources Center, Korea Research Institute of Bioscience and Biotechnology, P.O. Box 115 Yusong, Taejon 305-600, Republic of Korea

Received: January 26, 2000

Abstract The sequences of the D1/D2 region of 26S rDNA from seven *Sporobolomyces* species, *Bensingtonia subrosea*, and *Rhodosporidium toruloides* were determined and compared with those from representatives of the genera *Leucosporidium*, *Rhodosporidium*, *Rhodotorula*, and *Sporidiobolus*. The five species of *Sporobolomyces* analyzed were distantly related to a monophyletic clade consisting of species of Sporidiobolaceae and Sporobolomycetaceae. *Sporobolomyces falcatus* was found to be closely related to *Tremella exigua*. The members of Sporidiobolaceae and Sporobolomycetaceae were divided into four groups. Group 1 was composed of *Leucosporidium scottii* and two *Rhodotorula* species, and group 2 contained three *Rhodotorula* species. Group 3 was designated as the *Sporobolomyces/Sporidiobolus* core group, as it contained *Sporidiobolus johnsonii*, the type species of *Sporidiobolus* and the teleomorphic state of *Sporobolomyces salmonicolor* (the type species of *Sporobolomyces*). Group 4, named the *Rhodotorula/Rhodosporidium* core group, included *Rhodosporidium toruloides* and *Rhodotorula glutinis*, the type species of the genera *Rhodosporidium* and *Rhodotorula*, respectively. The four groups were differentiated on the basis of their physiological characteristics including the assimilation of D-glucosamine, glucuronate, 2-keto-gluconate, L-arabinitol, raffinose, methyl-α-glucoside, and starch. The taxonomy of the genera *Leucosporidium*, *Rhodosporidium*, *Rhodotorula*, *Sporidiobolus*, and *Sporobolomyces* will require a major revision when more data becomes available.

Key words: 26S rDNA, phylogeny, *Sporobolomyces*

Sporobolomyces is an anamorphic genus that consists of ballistoconidia-forming yeasts [1]. The genus is currently defined by ellipsoidal, subglobose, fusiform or cylindroidal cells, which are mostly polar, rarely lateral or multilateral budding, bilaterally symmetrical ballistoconidia, pink, orange, red, yellowish or pale colonies, and a presence of hyphae or pseudohyphae. Other physiological and chemical characteristics are as follows: no fermentation of sugars, Diazonium Blue B and urease positive, absence of xylose from the whole-cell hydrolyzates, and Q-10 or Q-10(H_2) as the major ubiquinone [2]. It has been proposed that *Rhodotorula* is closely related to *Sporobolomyces* on the basis of cell wall and carbohydrate analyses [4, 17]. The major difference between the genera *Sporobolomyces* and *Rhodotorula* is the formation of ballistoconidia in the former. However, it is well known that this characteristic is unreliable since it might be lost during subculturing on agar media [12]. Apart from ballistoconidia formation, the two genera show similar morphological and physiological profiles.

Fell *et al.* [8] conducted a phylogenetic study based on about 200 bp long sequences of 26S rDNA and concluded that the members of two genera may not be monophyletic. It is therefore essential to carry out further comprehensive phylogenetic study. In the present investigation, partial sequences covering the D1/D2 regions of 26S rDNA from seven *Sporobolomyces* species, *Bensingtonia subrosea*, and *Rhodosporidium toruloides* were determined and compared with physiological data to elucidate the taxonomic structure of the genus *Sporobolomyces* and related taxa.

Materials and Methods

Test Strains

B. subrosea JCM 5735^T, *R. toruloides* KCTC 7134^T, *Sporobolomyces alborubescens* KCTC 7792^T, *Sporobolomyces elongatus* KCTC 7793^T, *Sporobolomyces gracilis* JCM 2963^T, *Sporobolomyces griseoflavus* KCTC 7794^T, *Sporobolomyces linderae* KCTC 7796^T, *Sporobolomyces sasicola* KCTC 7795^T, and *Sporobolomyces singularis* KCTC 7534^T were all grown on a YM agar at 24°C for days.

*Corresponding author
Phone: 82-042-860-4610; Fax: 82-042-860-4677;
E-mail: ksbae@mail.kribb.re.kr

DNA Amplification and Sequence Analysis

The nuclear 26S rDNA was amplified and sequenced using the primer pair No. 4 (ACCCGCTGAAYTTAAGCATAT) and No. 11 (CTCCTTGGTCCGTGTTTCAAGACGG) [16]. The amplified 26S rDNAs were purified using a Wizard PCR prep (Promega, Madison, U.S.A.). The nucleotide seuquences were determined using BigDye terminator cycle sequencing kits (PE Applied Biosystems, Foster City, U.S.A.) following the manufacturer's instructions. The gel electrophoresis and data collection were performed on an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems, Foster City, U.S.A.). The sequences were proofread, edited, and merged into composite sequences using the PHYDIT program version 3.0 [3]. The sequences were then submitted to the GenBank database under accession number AF207882-AF207890.

Phylogenetic Analysis

The nine newly determined D1/D2 domain sequences of 26S rDNA were aligned with other LSU rDNA sequences from Sporidiales, Tremellales and related anamorphic taxa on the basis of similarity in the primary and secondary structures using the PHYDIT program. Ambiguously aligned sites were excluded from the phylogenetic analysis, however, similarity values were calculated for all the aligned sites. A tree topology was reconstructed with the Kimura's 2-parameter distance model [11] and neighbor-joining method [14] using the PHYLIP 3.05 package [10]. *Saccharomyces cerevisiae* was used as the outgroup. Confidence values for the individual branches of the resulting tree were assessed by a bootstrap analysis [9] in which 1,000 bootstraped trees were generated from the resampled data. The resultant phylogenetic trees were visualized using the TreeView program [13]. The parsimony tree reconstructed by the heuristic search option of the PAUP program version 4.0 beta [15] was then compared with the distance tree.

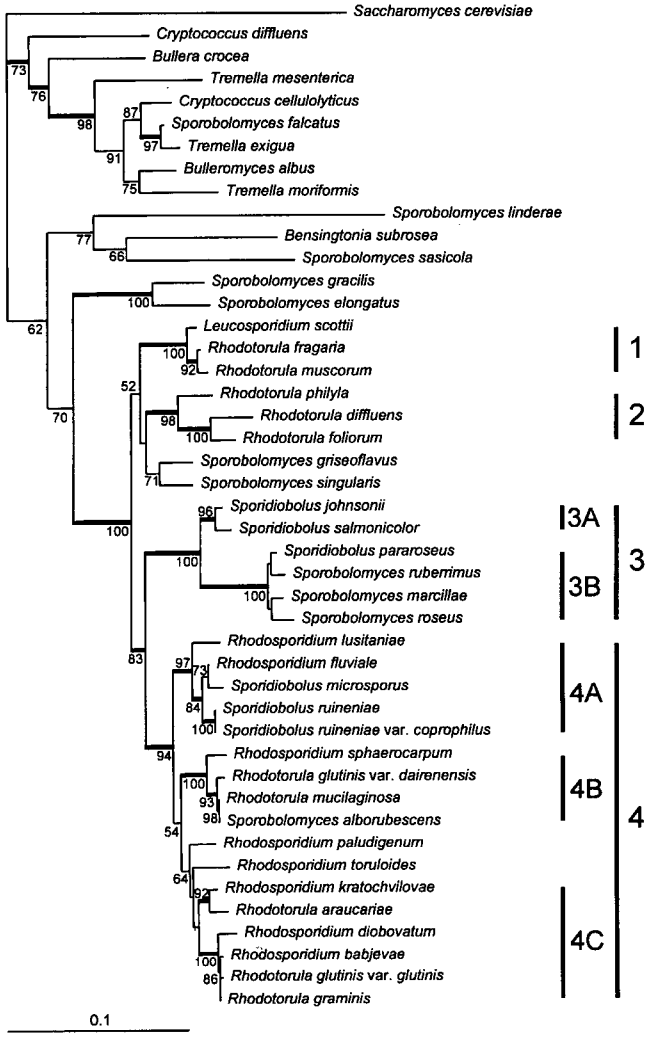

Fig. 1. Distance tree based on 44 partial 26S rDNA sequences from basidiomycetous yeasts, constructed by means of the neighbour-joining method. Bootstrap percentages higher than 50% are placed alongside the node considered. Branches supported by bootstrap values higher than 70% in both distance and parsimony analyses are indicated by bold lines. The scale bar indicates 0.1 substitutions per nucleotide.

RESULTS AND DISCUSSION

Based on a distance analysis of 568 unambiguously aligned sites out of the 738 sites of the 26S rDNA D1/D2 domain, it is clear that the members of the genus *Sporobolomyces* are phylogenetically heterogeneous and polyphyletic (Fig. 1). Two closely related species (sequence similarity 93.0%), *S. gracilis* and *Sporobolomyces elongatus*, did not exhibit a high sequence similarity when compared with any other taxa (the highest similarity value was 85.7% between *S. elongatus* and *Rhodotorula sphaerocarpum*). Similarly, *S. linderae* and *S. sasicola* did not show close relationships with other species of *Sporobolomyces* and *B. subrosea*. It was also evident that they are not related to Tremellales, based on phylogenetic and sequence similarity (70.8–76.8%) analyses. More molecular data from Sporidiobolaceae and Sporobolomycetaceae are needed to clarify the phylogenetic positions of these four species.

Sporobolomyces falcatus was found to be related to a species of Tremellales. The most closely related taxon was *Tremella exigua* (97.4%). The relationship was supported by the high bootstrap values in both distance and parsimony analyses. However, the addition of *S. falcatus* to the genus *Tremella* should be waived until the taxonomic structure of Tremellales is unravelled, as members of this order have been shown to be polyphyletic [6].

Except for the five species of *Sporobolomyces* discussed above, the species of *Leucosporidium*, *Rhodosporidium*, *Rhodotorula*, *Sporidiobolus*, and *Sporobolomyces* formed a monophyletic group that was supported by high bootstrap

values in both distance and parsimony analyses. This group was divided into four major monophyletic groups based on monophyly, bootstrap support, and similarity values. Groups 3 and 4 were subdivided into two and three subgroups, respectively (Fig. 1).

Group 1 consisted of *Leucosporidium scottii*, *Rhodotorula fragaria*, and *Rhodotorula muscorum* with an average similarity of 98.6%. Group 2 contained three *Rhodotorula* species, namely *R. philyla, R. diffluens*, and *R. foliorum*. The average similarity, i.e. 93.8%, was significantly lower than that of group 1. Two taxa, *Sporobolomyces griseoflavus* and *Sporobolomyces singularis* whose phylogenetic positions were not clear, have not been assigned to any group. Members of groups 1 and 2 could all assimilate D-glucosamine, glucuronate, and 2-keto-gluconate as a carbon source and ethylamine as a nitrogen source. In particular, the assimilation of D-glucosamine and glucuronate was only found in the members of groups 1 and 2 except for *Rhodosporidium lusitaniae* of group 4A. Groups 1 and 2 could be distinguished by their assimilation of raffinose and methyl-α-glucoside, as only the former could use these substances. Although these two characteristics were very homoplasious in groups 3 and 4, they were significantly conserved in groups 1 and 2. *Sporobolomyces griseoflavus* and *S. singularis* shared assimilation ability of glucuronate, 2-keto-gluconate, and ethylamine with groups 1 and 2. These two species could not utilize raffinose and methyl-α-glucoside as a carbon source, which indicates that they are closely related to group 2. However, they differed from groups 1 and 2 in their assimilation of D-glucosamine. At present, the phylogenetic relationship of groups 1 and 2, *S. griseoflavus*, and *S. singularis* is unclear based on the currently available molecular and physiological data.

Group 3A consisted of *Sporidiobolus johnsonii* and *Sporidiobolus salmonicolor*, and group 3B contained *Sporidiobolus pararoseus*, *Sporobolomyces marcillae, Sporobolomyces roseus*, and *Sporobolomyces ruberrimus*. Groups 3A and 3B were relatively homogeneous (mean similarity 98.8% and 97.9%, respectively), however, group 3 was in general rather diverse in sequence similarity (95.5%). It was designated as the core group of *Sporidiobolus* and *Sporobolomyces* as it contained *S. johnsonii*, the species of the genus *Sporidiobolus* and the teleomorphic state of *Sporobolomyces salmonicolor* (the type species of the genus *Sporobolomyces*). Group 3 was characterized by its lack of ability to assimilate D-glucosamine, glucuronate, 2-keto-gluconate, and L-arabinitol as a carbon source. However, the ability to assimilate starch was unique to group 3, although this characteristic was not conserved in all the members of group 3. Groups 3A and 3B could be distinguished from each other based on their assimilation of ethylamine as a nitrogen source and tolerance to cycloheximide. Members of group 3A showed a tolerance of 0.1% as well as 0.01% cycloheximide.

Group 4 was a large and diverse group (mean similarity 94.7). It consisted of seven species of *Rhodosporidium* including *R. toruloides*, the type species of the genus, five species of *Rhodotorula* including *R. glutinis*, the type species of the genus, three species of *Sporidiobolus*, and *Sporobolomyces alborubescens*. Group 4 was equated with the *Rhodosporidium/Rhodotorula* core group, as it contained type species of both genera. Among the three subgroups, group 4C was not supported by bootstrap values. However, the bootstrap value became high in both distance and parsimony analyses, when *Rhodosporidium paludigenum* and *Rhodosporidium toruloides* were excluded. Therefore, it can be concluded that the cause for the low bootstrap support for group 4C originated from the uncertain phylogenetic positions of *R. paludigenum* and *R. toruloides*. Group 4 contained many taxa compared to groups 1, 2, and 3, and was heterogeneous in its physiological characteristics. The common characteristics of all the members of group 4 were the lack of assimilation of D-glucosamine and starch as a carbon source. The three subgroups of group 4 could be differentiated by the growth rate using the combination of the assimilation of 2-keto-gluconate without thiamin.

Boekhout [1] considered that *Sporidiobolus microsporus* (nom. nud.) and *Sporidiobolus ruineniae* var. *coprophilus* are synonyms of *Sporidiobolus ruineniae* on the basis of their physiological and morphological properties. The synonomy of *S. ruineniae* and *S. ruineniae* var. *coprophilus* was supported by identical D2 domain sequences [8]. However, the synonomy of *S. microsporus* and *S. ruineniae* was questioned as they differed in their clamp connection, colony color, and assimilation of maltose [7]. Fell *et al.* [5] validated and described *S. microsporus* sp. nov. based on phenotypic and molecular data. In this study, *Sporidiobolus microsporus* and *S. ruineniae* were shown to be closely related to the *Rhodosporidium* species based on physiological as well as molecular data, and were contained in the *Rhodosporidium/ Rhodotorula* core group (group 4). Therefore, it is clear that the two species, *S. microsporus* and *S. ruineniae*, should be renamed as *Rhodosporidium microsporus* and *Rhodosporidium ruineniae*, respectively.

Sporobolomyces alborubescens had an identical sequence with *Rhodotorula mucilaginosa*. However, the two species had considerable physiological differences, including the assimilation of L-sorbose, L-rhamnose, methyl-α-glucoside, cellobiose, gluconate, galacturonic acid, and propanediol as a carbon source, D-glucosamine and D-tryptophan as a nitrogen source, tolerance to 0.01% cycloheximide, and growth temperature. Although these physiological characteristics were generally homoplasious, the assimilation of propanediol was only lacking in three taxa, *Rhodotorula fragaria* (group 1), *Sporobolomyces roseus* (group 3B), and *R. mucilaginosa* (group 4B), and the assimilation of D-glucosamine as a nitrogen source was unique to *S. alborubescens*. The ability to grow at 37°C was only found in *S. alborubescens* among

those species assigned to group 4B. Because of an inconsistency between molecular and physiological data, more data on DNA-DNA hybridization, mating, and fatty acid composition are required to determine whether the two species should be merged.

A phylogenetic analysis of 26S rDNA sequences revealed that the genus *Sporobolomyces* is polyphyletic. Some species of *Sporobolomyces* were not closely related to the main lineage of Sporidiobolaceae and Sporobolomycetaceae. Accordingly, their phylogenetic relationship and classification should be revised when additional molecular data from related taxa such as *Bensingtonia*, *Kurtzmanomyces*, and *Sterigmatomyces* becomes available. It was also evident that two anamorphic genera, namely *Sporobolomyces* and *Rhodotorula*, and three teleomorphic genera, namely *Sporidiobolus*, *Rhodosporidium*, and *Leucosporidium*, were intermixed, based on 26S rDNA phylogeny. Therefore, it would appear that ballistosporic characteristics have been overestimated in the taxonomy of basidiomycetous yeasts.

Acknowledgments

The present research was supported by grant BSKB1130 from the Ministry of Science and Technology of Korea. We would also like to thank Dr. T. Nakase from the Japan Collection of Microorganisms (JCM) for the gift of the strains.

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