

## D1/D2 Domain of Large-Subunit Ribosomal DNA for Differentiation of *Orpinomyces* spp.<sup>∇</sup>

Sumit S. Dagar, Sanjay Kumar, Priti Mudgil, Rameshwar Singh, and Anil K. Puniya\*

Dairy Microbiology Division, National Dairy Research Institute, Karnal-132001, India

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**This study presents the suitability of D1/D2 domain of large-subunit (LSU) ribosomal DNA (rDNA) for differentiation of *Orpinomyces joyonii* and *Orpinomyces intercalaris* based on PCR-restriction fragment length polymorphism (RFLP). A variation of G/T in *O. intercalaris* created an additional restriction site for AluI, which was used as an RFLP marker. The results demonstrate adequate heterogeneity in the LSU rDNA for species-level differentiation.**

Anaerobic fungi constitute nearly 20% of the total microbial biomass in the rumen (17). These are regarded as the primary colonizers (1) and most active lignocellulose degraders in the biological world (23, 25). Hence, their role is very critical in the digestion, especially in tropical regions, where forage is generally fibrous and of low quality (10). Taxonomically, these fungi belong to the phylum Neocallimastigomycota, class Neocallimastigomycetes, and order Neocallimastigales (9). Based on morphological features like growth pattern (monocentric or polycentric), thallus morphology (filamentous or bulbous), and number of flagella per zoospore, 6 genera divided into 20 species have been described (7). Within the genus *Orpinomyces*, two species, *O. joyonii* and *O. intercalaris*, have been identified, based on the position of sporangia, but the paucity of morphological features presents a big problem in resolving their taxonomy (2). Often, the polycentric taxa (including *Orpinomyces*) fail to produce sporangia, making their identification and differentiation very difficult. Therefore, various molecular approaches employing small subunit (SSU; 18S) ribosomal DNA (rDNA) or internal transcribed spacer (ITS) regions have been tried (2, 5, 24), but the highly conserved nature of the SSU rDNA and abundant intraindividual variations of ITS makes these regions inappropriate for diversity studies (3). Hence, the present study was conducted to explore the D1/D2 domain of large subunit (LSU; 28S) rDNA region for its potential application in species-level differentiation in *Orpinomyces* spp.

For this purpose, rumen liquor was collected from fistulated Murrah buffaloes maintained at the cattle yard of the National Dairy Research Institute (NDRI), Karnal, India, into O<sub>2</sub>-free, CO<sub>2</sub>-flushed, and preautoclaved bottles after 30 to 40 min of feeding (12). The liquor was immediately brought to the laboratory, flushed with O<sub>2</sub>-free CO<sub>2</sub> (1 min), and diluted (10<sup>-1</sup> dilution) in anaerobic diluent (13) containing 2% (vol/vol) antibiotic solution from a stock of benzylpenicillin, streptomycin sulfate, and chloramphenicol (each at 5 mg/ml). Roll tubes were prepared by injecting 0.5 ml of a 10<sup>-1</sup> dilution into 50-ml

serum bottles (14) containing 5 ml cellobiose agar medium (pH 6.7 ± 1) with antibiotics (1, 15). The medium composition was slightly modified (i.e., tryptone and cellobiose at 5 g/liter, cysteine-HCl at 1 g/liter, and hemin at 2 ml from a stock of 0.05% hemin mixed in 1:1 ethanol and 0.05 M NaOH). Serum bottles were incubated at 39 ± 1°C for 2 to 3 days for the development of colonies. Morphologically distinct colonies were transferred to a fresh medium using a bent needle and purified through repeated roll tube culturing. Cultures were maintained by routine subculture in 50-ml serum bottles containing 10 ml medium with wheat straw (5 g/liter) as the sole carbon source every fifth day. Features such as thallus morphology, growth patterns, and positions of sporangia were examined by phase-contrast and fluorescence microscopy using bisbenzimidazole (4). Genomic DNA was isolated from the 3-day-old culture (grown in cellobiose broth) by the cetyltrimethylammonium bromide (CTAB) method (7). PCR amplification of the D1/D2 domains of the LSU was performed with NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') according to reference 5. The amplified PCR products were visualized on 1.5% agarose gel and purified using the MinElute PCR purification kit (Qiagen, India), before sequencing (Xcelris Genomic Centre, Ahmadabad, Gujarat). The sequences obtained were edited, compiled, and aligned using BioEdit software (8). Sequence similarity searches were performed using GenBank Blastn. *In silico* digestion was done using Cleaver (11). A phylogenetic tree was generated using the neighbor-joining algorithm in MEGA5 (22). For restriction fragment length polymorphism (RFLP) analysis, 12 μl (≈1 μg) of amplified PCR product was digested overnight at 37°C using 2.5 U of AluI (recognition sequence, AG'CT) and 1× buffer in a 20-μl reaction volume. The digested products were separated by electrophoresis (65 V for 4 h; Biometra Power Pack P25) on 3% agarose gel containing ethidium bromide (2.0 μg/ml) in 1× Tris-borate-EDTA (TBE) buffer.

The *Orpinomyces* spp. were identified by polycentric growth pattern (many sporangia on a single rhizoid) and nucleated rhizoids (Fig. 1A and B). Sporangia were mostly spherical, developing on the terminal end of a simple or branched sporangiophore in *O. joyonii* (Fig. 2A), while the sporangial position was intercalary in the case of *O. intercalaris* (Fig. 2B). The

\* Corresponding author. Mailing address: Dairy Microbiology Division, National Dairy Research Institute, Karnal-132001, India. Phone: 91-184-2259176. Fax: 91-184-2250042. E-mail: puniya1234@rediffmail.com.

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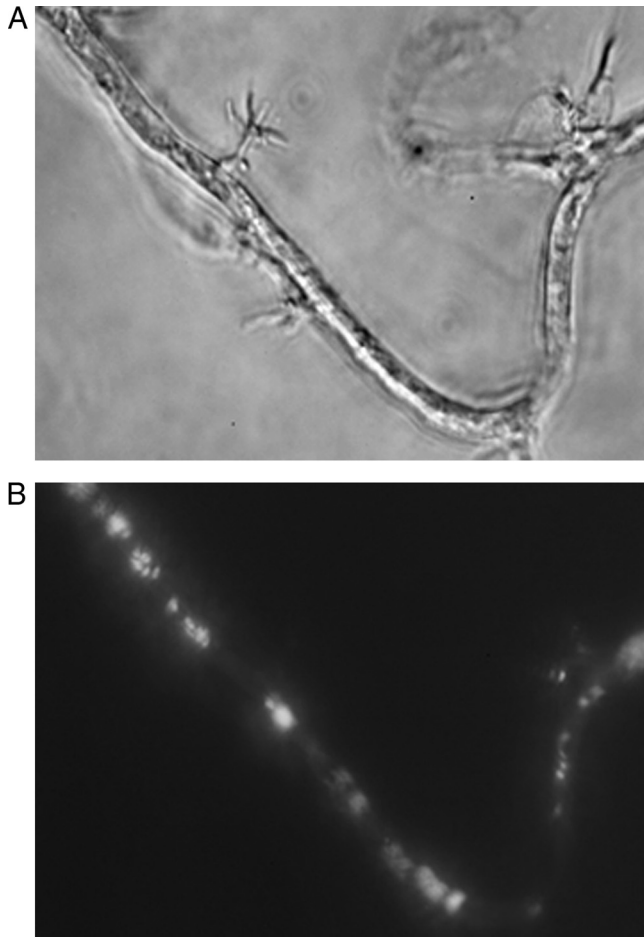


FIG. 1. Nucleated rhizoids of *Orpinomyces intercalaris* by phase-contrast microscopy (A) and fluorescence microscopy (B). Magnification,  $\times 400$ .

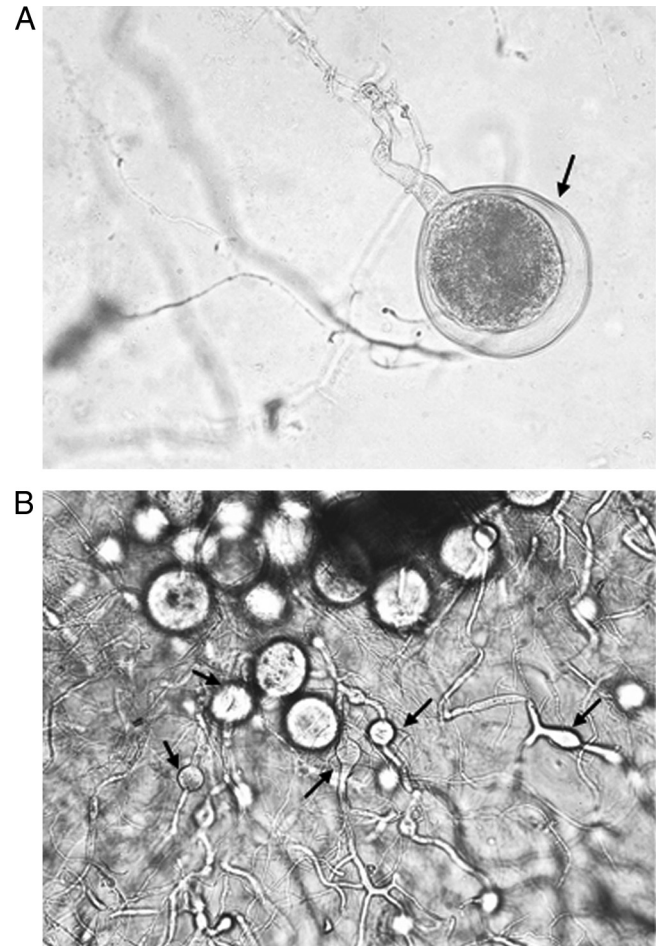


FIG. 2. Different patterns (as indicated by arrows) of sporangial development. (A) Terminal end in *O. joyonii*. Magnification,  $\times 400$ . (B) Intercalary position in *O. intercalaris*. Magnification,  $\times 200$ .

zoospores of both species were polyflagellated, and no distinct morphological differences were observed. Five isolates each of *O. joyonii* and *O. intercalaris* were chosen for further study. The amplified region showed a product of  $\approx 780$  bp (Fig. 3) in both species. The sequence similarity searches showed various similarities of 98 to 99% with the *Orpinomyces* sp. strain OUS1 partial 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, and partial 28S rRNA, clone V5-1 (AJ864475.1), the only sequence available with related matching sequence and which, therefore, was taken as a reference. The DNA sequence alignment showed a total of 10 nucleotide variations between both species (Fig. 4). Four variations each were observed for both A/G and T/C, while two variations were found for G/T. *In silico* digestion showed restriction sites for AfaI, BseRI, AluI, DdeI, HpyF3I, MaeII, MnlI+, RsaI, and TscI. Based on the availability, AluI was selected. The AluI digestion showed common bands of approximately 379, 79, 57, 40, and 13 bp, while discriminatory bands of 212 bp and 138 bp were found in *O. joyonii* and *O. intercalaris*, respectively. It was observed that the variation of G/T created an extra restriction site for AluI in *O. intercalaris*, which resulted in cleavage of 212-bp regions in *O. joyonii* into two regions of 138 bp and 74 bp in *O. intercalaris*. A phylogenetic tree of all of the isolates grouped these into two

major clusters (Fig. 5), thereby confirming there was sufficient heterogeneity of this region for their differentiation. The reference sequence was found to be clustered with *O. joyonii*, suggesting their similar evolutionary relationship. Restriction

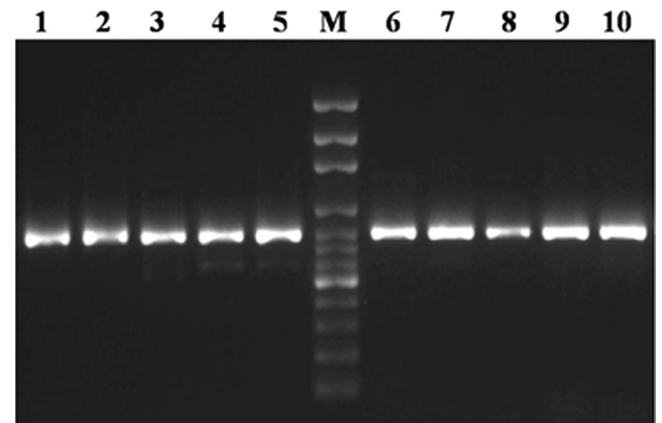


FIG. 3. PCR-amplified product ( $\approx 780$  bp) of the LSU region of *O. joyonii* (lanes 1 to 5) and *O. intercalaris* (lanes 6 to 10). M, 100-bp DNA marker.

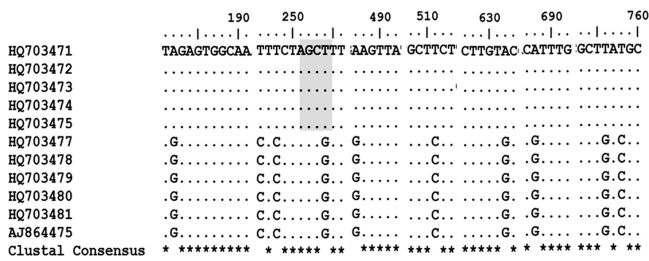


FIG. 4. Patterns of variation obtained after multiple sequence alignment of isolates with an extra restriction site (highlighted area) for AluI in *O. intercalaris*.

patterns (Fig. 6) generated after digestion with AluI clearly differentiated both species. Two types of banding patterns were observed among the isolates examined. The bands were identical for the isolates pertaining to the same species. In both species, two bands of 379 bp and  $\approx$ 79 bp were common, while the discriminatory bands were 212 bp and 138 bp in *O. joyonii* and *O. intercalaris*, respectively. The bands below the size of  $\approx$ 79 bp were not visible in the gel. The highly pleomorphic morphological features (10) and unsuitability of SSU and ITS regions (3, 16, 24) for the genus- and species-level differentiations have necessitated the need for better alternative genetic markers like the LSU, RNA polymerase II subunits, actin-coding genes, translation elongation factor 1- $\alpha$ ,  $\beta$ -tubulin, or mitochondrial cytochrome *c* oxidase. Since the anaerobic fungi lack mitochondria (26), design of mitochondrially based genetic markers is not feasible. In addition, many of the above markers are single copy; therefore, their amplification from low-quantity samples is problematic, while for others, universal primer designing is very difficult (16). On the other hand, the

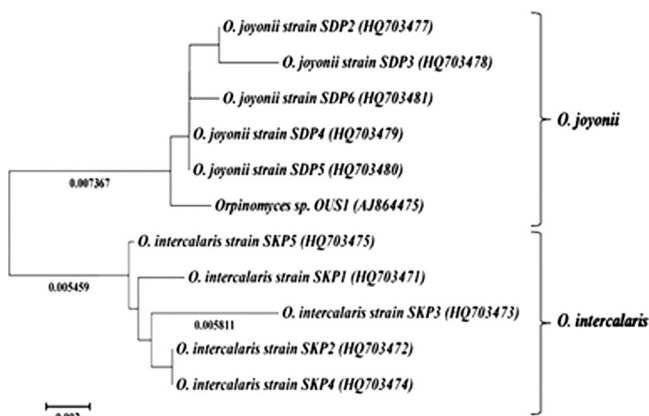


FIG. 5. Neighbor-joining phylogenetic tree of *Orpinomyces* spp. obtained from the sequences of the LSU. The evolutionary history was inferred using the neighbor-joining method (19). The optimal tree with the sum of branch length of 0.03107997 is shown. The tree is drawn to scale, with branch lengths (below the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method (21) and are in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. The codon positions included were the 1st, 2nd, 3rd, and noncoding positions. All positions containing gaps and missing data were eliminated. There were a total of 743 positions in the final data set. Evolutionary analyses were conducted in MEGA5 (22).

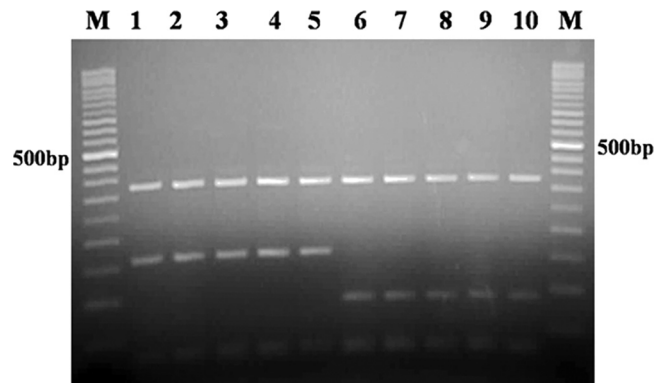


FIG. 6. AluI-digested PCR product of the LSU regions of *O. joyonii* (lanes 1 to 5) and *O. intercalaris* (lanes 6 to 10). M, 50-bp DNA marker.

multicopy LSU gives a possibility to design universal primers that can be easily amplified and has been shown to possess less intraindividual polymorphism (20). Moreover, this region has also been used for genus-level differentiation in anaerobic fungi (5) and species-level differentiation in yeasts (6, 18). In this study, the sequenced products of morphologically distinct *Orpinomyces* spp. showed proper alignment, rare intraindividual polymorphism, and a high degree of variation, sufficient enough to differentiate at the species level.

It can be concluded that the LSU has the potential to be used as a taxonomic marker for species-level differentiation in anaerobic fungi. We also report that AluI can be used for differentiating species of *Orpinomyces*, suggesting that PCR-RFLP can be used for carrying out genetic heterogeneity studies without the need for sequencing.

**Nucleotide sequence accession numbers.** The sequences reported in this paper have been deposited in the GenBank database under accession no. HQ703471, HQ703472, HQ703473, HQ703474, and HQ703475 for *O. intercalaris* and HQ703477, HQ703478, HQ703479, HQ703480, and HQ703481 for *O. joyonii*.

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