Phylogenetic analysis of the ballistosporous anamorphic genera *Udeniomyces* and *Bullera*, and related basidiomycetous yeasts, based on 18S rDNA sequence

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The small subunit nuclear ribosomal DNA (18S rDNA) sequence was determined for twelve species of basidiomycetous anamorphic yeasts, i.e. three species of *Udeniomyces*, seven species of *Bullera*, *Cryptococcus albidus* and *Phaffia rhodozyma*. For phylogenetic analysis, these sequences were aligned with published sequences for 36 other fungal species. Molecular phylogenetic analysis of maximum likelihood and parsimony showed that the 44 species of basidiomycetes analysed were divided into three major lineages. The ballistosporous yeast genera *Udeniomyces* and *Bullera* were clearly separated. On the phylogenetic tree, *Udeniomyces megalosporus*, *U. puniceus* and *U. piricola* showed a very close relationship with one another, and composed a lineage with *Mrakia frigida*, *P. rhodozyma* and *Cystofilobasidium capitatum* at high bootstrap confidence level. On the other hand, eight species of *Bullera* made lineages with selected species of *Tremella* (Tremellaceae), *Filobasidium* and *Filobasidiella* (Filobasidiaceae), *Cryptococcus albidus* and *Trichosporon cutaneum*. The molecular phylogeny deduced from the 18S rDNA sequence showed a possibility of heterogeneity among the species of *Bullera* at the generic level.

Keywords: basidiomycetes, ballistosporous yeasts, *Bullera*, *Udeniomyces*, 18S rDNA

INTRODUCTION

Since the establishment of the genus *Bullera* (Derx, 1930), the symmetrical (rotationally symmetrical) ballistospores and whitish- to pale-coloured colonies have been considered to be important criteria for distinguishing this genus from the genus *Sporobolomyces*, whose ballistospores are asymmetrical (bilaterally symmetrical) and whose colonies are deeply pigmented (Lodder & Kreger-van Rij, 1952). Chemotaxonomically, the genus *Bullera* has been characterized as containing Q-10 as the major ubiquinone and xylose in the cells (Nakase *et al.*, 1993a). Nakase (1987, 1989) divided the species of *Bullera* into two

groups, a group of typical *Bullera* and the *piricola* group, based on the morphology of ballistospores and the colour of colonies, and suggested that the *piricola* group represented a different genus from *Bullera*. Typical *Bullera* species produce symmetrical ballistospores and pale-coloured or orange colonies. The *piricola* group comprises species which produce large asymmetrical ballistospores and pinkish-white to pale pink colonies. Later, a new genus *Udeniomyces* was proposed in the Cryptococcaceae for the *piricola* group of *Bullera* (Nakase & Takematsu, 1992).

Nakase *et al.* (1993b) determined the partial sequence (about 170 bp) of 18S ribosomal RNA of 46 species of ballistosporous yeasts, and found that the *piricola* group of *Bullera* (= *Udeniomyces*) differed from the typical *Bullera* species based on the comparison of these sequence data.

In studying the phylogeny of fungi, the 18S rRNA gene (rDNA) sequence has been considered to be a useful criterion, and recently complete or almost complete sequences of 18S rDNA of several fungi have been

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The nucleotide sequence data reported in this paper will appear in the GSD, DDBJ, EMBL and NCBI nucleotide sequence databases with the following accession numbers: D31648 (*Bullera crocea*), D31649 (*B. den-drophila*), D31650 (*B. globospora*), D31651 (*B. miyagiana*), D31652 (*B. oryzae*), D31654 (*B. variabilis*), D31655 (*Cryptococcus albidus*), D31656 (*Phaffia rhodozyma*), D31657 (*Udeniomyces megalosporus*), D31658 (*U. puniceus*), D31659 (*U. piricola*), and D31660 (*B. pseudoalba*).

reported (e.g. Berbee & Taylor, 1992a, b, 1993; Bruns *et al.*, 1992; Hendriks *et al.*, 1989, 1991a, b, 1992; Nishida & Sugiyama, 1993; Suh & Sugiyama, 1993; Swann & Taylor, 1993; Van de Peer *et al.*, 1992; Wilmotte *et al.*, 1993). In basidiomycetous yeasts, however, the complete or almost complete 18S rDNA sequence from only a few selected species has been reported (Berbee & Taylor, 1993; Suh & Sugiyama, 1993; Swann & Taylor, 1993; Van de Peer *et al.*, 1992; Wilmotte *et al.*, 1993).

In this paper, we report the nuclear 18S rDNA of twelve basidiomycetous anamorphic yeast species, i.e. three species of *Udeniomyces*, seven species of *Bullera*, *Cryptococcus albidus* and *Phaffia rhodozyma* in an attempt to deduce the phylogenetic relationships of these species and to clarify their classification in higher fungi.

METHODS

Yeast strains. The following yeast strains were used for sequencing: *Bullera crocea* JCM 2961^T (= CBS 6714); *B. dendrophila* JCM 5357^T (= CBS 6704); *B. globospora* JCM 2957^T (= CBS 6981); *B. miyagiana* JCM 7536^T; *B. oryzae* JCM 5281^T (= CBS 7194); *B. pseudoalba* JCM 5290^T (= IFO 10792); *B. variabilis* JCM 5275^T (= CBS 7347); *Cryptococcus albidus* JCM 2334^T (= CBS 142); *Phaffia rhodozyma* JCM 9042^T (= IFO 10129); *Udeniomyces megalosporus* JCM 5269^T (= CBS 7236); *U. piricola* JCM 2958^T (= CBS 6754); *U. puniceus* JCM 1535^T (= AJ 4764). Strains with a superscript T are derived from the type.

PCR and sequencing. The yeasts were grown in YM broth at 17 °C or 25 °C. DNA was obtained from cells broken by sonication. The gene of the 18S rRNA coding region was amplified by polymerase chain reaction (PCR) (Saiki *et al.*, 1988) with the primers P1 5'-ATCTGGTTGATCCTGCCAGT-3' and P2 5'-GATCCTTCCGCAGGTTCCACC-3' as described by Nishida & Sugiyama (1993). PCR products were purified by agarose gel electrophoresis, and sequenced with Sequenase v. 2.0 (US Biochemical).

Phylogenetic analysis. We sequenced the 18S rDNA of the 12 yeast species mentioned above and added known sequence data for 36 additional fungal species for analysis. The published sequence data used in this study were obtained from the nucleotide sequence libraries (EMBL, GenBank, and DDBJ). All sequences were aligned using the multialignment program CLUSTAL V (Higgins *et al.*, 1992; UNIX version). The DNAML program in the PHYLIP 3.5c package (Felsenstein, 1993) was used for maximum likelihood analysis (Felsenstein, 1981). From the parsimony analysis, a bootstrap 50% majority consensus tree was constructed by PAUP 3.0s (Swofford, 1991) with bootstrap analysis of 100 random resamplings.

RESULTS AND DISCUSSION

Phylogenetic relationships among the basidiomycetes based on 18S rDNA sequence data

As the result of sequencing, nucleotides of about 1750 bases were determined for each strain from the PCR amplification product of 18S rDNA. The sequence data were well aligned for all 48 fungi, including 36 sets of published data, and phylogenetic trees (Fig. 1) were constructed using the ascomycetes *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, *Candida albicans* and *Neurospora crassa* as an outgroup.

The 44 species of basidiomycetes used in this analysis were divided into three major lineages described as groups 1, 2, and 3 in the maximum likelihood tree (Fig. 1a). The results showed good agreement with those of several reports on fungal evolution (e.g. Bruns *et al.*, 1992; Suh & Sugiyama, 1993; Swann & Taylor, 1993; Wilmotte *et al.*, 1993).

The first major lineage (group 1) in the basidiomycetes included the smut-like, teliospore-forming yeasts (*Leucosporidium scottii*, *Sporidiobolus johnsonii*, *Rhodosporeidium toruloides*) and the related yeasts *Sporobolomyces roseus* and *Erythrobasidium basegawianum*. The rust fungi *Cronartium ribicola* and *Peridermium barknessii* were also included in this lineage. The second major lineage (group 2) was composed of the smut fungi (Ustilaginales), represented by *Ustilago maydis*, *U. hordei* and *Tilletia caries*. The third major lineage (group 3) could be divided into four sub-groups (3a, b, c, and d; Fig. 1a) which were well supported statistically at high bootstrap levels of 96–100% (Fig. 1b). The Dacrymycetales, represented by *Dacrymyces chrysospermus*, *D. stillatus*, *Calocera cornea* and *Heterotextus alpinus*, made up group 3a. Group 3b was composed of the 'teliospore-forming' filobasidiaceous yeasts *Mrakia frigida* and *Cystofilobasidium capitatum*, together with species of the anamorphic yeast genera *Udeniomyces* and *Phaffia*. Selected species of homobasidiomycetes and Auriculariales were included in group 3c. The Filobasidiaceae, represented by *Filobasidium floriforme* and *Filobasidiella neoformans*, and the Tremellaceae, represented by *Tremella foliacea*, *T. globospora* and *T. moriformis* composed group 3d together with *Bullera* spp., *Trichosporum cutaneum* and *Cryptococcus albidus*. The three major lineages in basidiomycetes were also found in the tree by the neighbour-joining method (tree not shown). Groups 2 and 3 were not well separated in the most parsimonious consensus tree (Fig. 1b) because the bootstrap confidence level was low (< 50%).

Most of the basidiomycetous yeasts were included in groups 1 and 3. The teleomorphic yeast genera of group 1, *Leucosporidium*, *Rhodosporeidium*, *Sporidiobolus* and *Erythrobasidium*, are characterized by having simple pores and no xylose in the cells (Table 1). In contrast, genera of group 3, *Mrakia*, *Cystofilobasidium*, *Filobasidiella*, *Filobasidium* and *Tremella* (with a yeast phase), have dolipores and xylose in the cells (Table 1). The basidiomycetous anamorphic yeast species, *Sporobolomyces roseus*, *Bullera* spp., *Cryptococcus albidus* and *Trichosporon cutaneum*, were also located in groups 1 and 3 of Fig. 1(a), and they also correlated well with the presence or absence of xylose in the cells. The value of septal pore ultrastructure and cellular xylose as taxonomic characters in basidiomycetous yeasts has been discussed based on the comparison of 18S rDNA sequence (Suh & Sugiyama, 1993) or 18S and 26S rRNA partial sequence (Guého *et al.*, 1989; Nakase *et al.*, 1993b). Walker & Doolittle (1982, 1983) and Blanz & Unseld (1987) concluded, from 5S rRNA sequence comparisons, that septal pore structures were of great value as a taxonomic tool in basidiomycetes. The molecular phylogeny of this study strongly supported the results of the studies mentioned above.

Fig. 1. Phylogenetic relationships of basidiomycetes determined from 18S rDNA sequence. Tree (a) is the result of searching for the best tree by the maximum likelihood method (Felsenstein, 1981) using the DNAML program of the PHYLIP 3.5c package (Felsenstein, 1993). Tree (b) is the result of parsimony analysis (bootstrap 50% majority consensus tree) by PAUP 3.0s (Swofford, 1991). Each number in tree (b) indicates the percentage of bootstrap samplings supporting the internal branches.

Phylogeny among the ballistosporous yeasts *Bullera* and *Udeniomyces*, and related basidiomycetous yeast genera

Nakase & Takematsu (1992) proposed a new genus, *Udeniomyces*, in the Cryptococcaceae for the *piricola* group of *Bullera*, which produce large asymmetrical ballistospores and pinkish-white to pale pink colonies. The typical *Bullera* species produce symmetrical ballistospores and pale-coloured or orange colonies. The genus *Udeni-*

myces comprised three species, *Udeniomyces piricola* (= *Bullera piricola*; the type species), *U. puniceus* (= *B. punicea*) and *U. megalosporus* (= *B. megalospora*), which were transferred from *Bullera*.

Nakase *et al.* (1993b) determined the partial sequence of 18S ribosomal RNA (positions 1451–1618 in *Saccharomyces cerevisiae*) of 46 species of ballistosporous yeasts and found that the *piricola* group of *Bullera* (= *Udeniomyces*) differed from the typical *Bullera* species based on the comparison

Table 1. Taxonomic characteristics of the basidiomycetous yeast genera used in this study

All the data are from the literature (see text for references).

Genus	Xylose in cell wall	Septal pore	Ballistospore	Major ubiquinone
Teleomorph				
<i>Leucosporidium</i>	—	Simple pore	—	Q-9/Q-10
<i>Rhodospiridium</i>	—	Simple pore	—	Q-9/Q-10
<i>Sporidiobolus</i>	—	Simple pore	+	Q-10
<i>Erythrobasidium</i>	—	Simple pore	—	Q-10 (H ₂)
<i>Cystofilobasidium</i>	+	Dolipore	—	Q-8
<i>Mrakia</i>	+	Dolipore	—	Q-8
<i>Filobasidium</i>	+	Dolipore	—	Q-9/Q-10
<i>Filobasidiella</i>	+	Dolipore	—	Q-10
<i>Tremella</i>	+	Dolipore	—	Q-10
Anamorph				
<i>Bullera</i>	+	?	+	Q-10
<i>Cryptococcus</i>	+	?	—	Q-8/Q-9/Q-10
<i>Phaffia</i>	+	?	—	Q-10
<i>Trichosporon</i>	+	?	—	Q-9/Q-10
<i>Udeniomyces</i>	+	?	+	Q-10
<i>Sporobolomyces</i>	—	?	+	Q-10†

* *Bulleromyces albidus* (teleomorph of *Bullera alba*) has dolipores.

† One species has Q-10(H₂).

of these sequence data. However, in the study of Nakase *et al.* (1993b), it was not enough to clearly explain their phylogenetic positions, because the sequence data were too short by one molecule (about 10% of complete 18S rRNA sequence) and most of the main lineages of the tree were not supported well statistically.

In the trees based on 18S rDNA sequence (Fig. 1), the three species of *Udeniomyces*, i.e. *U. piricola*, *U. puniceus* and *U. megalosporus*, made a single branch and were included in group 3b with *M. frigida*, *C. capitatum* and *P. rhodozyma*. On the other hand, the species of *Bullera* were located in group 3d. Eight species of *Bullera* made up a lineage with *F. neoformans*, *Tremella globospora*, *T. moriformis* and *Trichosporon cutaneum* at the 95% bootstrap confidence level (Fig. 1b), but they showed a tendency to be heterogeneous at the generic level. *Bullera dendrophila* was placed on the same branch as *Filobasidiella neoformans*, and *Bullera pseudoalba* and *B. alba* showed a relationship with *Tremella moriformis*. The possibility of heterogeneity in *Bullera* species was also supported by the results of parsimony (Fig. 1b). The three species of *Udeniomyces* were closely related to one another based on the evolutionary distance as shown in Table 2. On the other hand, *Bullera* species were located at positions with varying evolutionary distances. *Bullera miyagiana* and *B. variabilis* were the species most distant to each other among eight species of *Bullera* examined. However, as shown in Fig. 1(b), the relationships between each species of *Bullera* and other related basidiomycetes species were difficult to clarify as most branches were not supported well statistically.

The molecular phylogeny suggests that the presence or absence of ballistospores is not a rational criterion for the higher taxa in basidiomycetous yeasts, because the ballistosporeous yeasts *Sporidiobolus*, *Sporobolomyces*, *Udeniomyces* and *Bullera* belong to different phylogenetic groups (Fig. 1). However, the morphology of ballistospores may be important in the taxonomy of ballistosporeous yeasts at generic or specific level as in the case of *Udeniomyces* and *Bullera*.

Boekhout *et al.* (1991) described the occurrence of sexual reproduction in *Bullera alba* and proposed a new basidiomycetous teleomorphic genus *Bulleromyces*. This genus was placed in the Tremellales based on basidial morphology and septal pore ultrastructure. Several authors have postulated a close relationship between the Filobasidiaceae and Tremellaceae (Jülich, 1981; Moore, 1978; Slodki *et al.*, 1966). Oberwinkler (1990) included the Filobasidiaceae as a family of the Tremellales in a study of septal pore ultrastructure. From molecular phylogeny analysis Swann & Taylor (1993) found that *Filobasidiella neoformans* and *Tremella foliacea* made a branch in the most parsimonious tree of 18S rDNA sequence. Van de Peer *et al.* (1992) reported the 18S rDNA sequence of *B. alba* (= *Bulleromyces albus*) and found that it showed a close relationship with *F. neoformans* of the Filobasidiaceae. In Fig. 1, eight species of *Bullera* grouped with the species of *Tremella* (Tremellaceae), *Filobasidium* and *Filobasidiella* (Filobasidiaceae), *Trichosporon* and *Cryptococcus*. Our results strongly support the close relationship between the families Filobasidiaceae and Tremellaceae.

Table 2. Evolutionary distance among the species of *Bullera* and *Udeniomyces*, and *Sporobolomyces roseus*

Evolutionary distance is percentage divergence (/100) calculated by the CLUSTAL V (Higgins *et al.*, 1992).

Species	1	2	3	4	5	6	7	8	9	10	11
1. <i>Bullera alba</i>	—										
2. <i>Bullera crocea</i>	0.0323	—									
3. <i>Bullera dendrophila</i>	0.0353	0.0429	—								
4. <i>Bullera globospora</i>	0.0247	0.0376	0.0329	—							
5. <i>Bullera miyagiana</i>	0.0470	0.0476	0.0464	0.0447	—						
6. <i>Bullera oryzae</i>	0.0335	0.0253	0.0429	0.0364	0.0511	—					
7. <i>Bullera pseudoalba</i>	0.0123	0.0347	0.0329	0.0282	0.0488	0.0276	—				
8. <i>Bullera variabilis</i>	0.0611	0.0523	0.0623	0.0558	0.0705	0.0564	0.0605	—			
9. <i>Udeniomyces megalosporus</i>	0.0552	0.0588	0.0699	0.0652	0.0799	0.0635	0.0576	0.0828	—		
10. <i>Udeniomyces puniceus</i>	0.0552	0.0599	0.0711	0.0664	0.0811	0.0646	0.0588	0.0852	0.0047	—	
11. <i>Udeniomyces piricola</i>	0.0541	0.0599	0.0711	0.0640	0.0811	0.0646	0.0576	0.0840	0.0024	0.0047	—
12. <i>Sporobolomyces roseus</i>	0.1146	0.1175	0.1187	0.1199	0.1287	0.1181	0.1193	0.1316	0.1105	0.1110	0.1099

The genera *Cystofilobasidium* and *Mrakia* have been distinguished from *Rhodospiridium* and *Leucosporidium* mainly by ultrastructural and chemotaxonomic data and have been assigned to the Filobasidiaceae (Oberwinkler *et al.*, 1983; Yamada & Komagata, 1987). Suh & Sugiyama (1993) found that *Cystofilobasidium capitatum* and *M. frigida* were well separated from *R. toruloides* and *L. scottii*, and showed a relationship with species of the Filobasidiaceae based on 18S rDNA sequence comparison. In Fig. 1, *C. capitatum* and *M. frigida* are located in group 3 with other filobasidiaceous yeasts. However, the trees showed that it may be possible to separate these two genera from other filobasidiaceous yeasts because the branch including *Cystofilobasidium* and *Mrakia* (group 3b) makes a lineage with selected species of homobasidiomycetes and Auriculariales (group 3c) and not with other filobasidiaceous yeasts (group 3d). These two genera could be distinguished from other Filobasidiaceae by the formation of teliospores and possession of the Q-8 ubiquinone system.

Weijman & Rodrigues de Miranda (1988) transferred *Phaffia rhodozyma*, the single species of the genus, to *Cryptococcus* based on the monosaccharide composition of whole cell hydrolysates. However, Yamada *et al.* (1990) advocated retention of the genus *Phaffia* based on the comparison of 18S and 26S rRNA partial sequence. As shown in the present study (Fig. 1), *P. rhodozyma* made a branch with *Cystofilobasidium capitatum* in group 3b, while *Cryptococcus albidus* was located in group 3d together with species of *Tremella*, *Filobasidiella*, *Filobasidium*, *Bullera* and *Trichosporon*. The same result was obtained from the most parsimonious tree (Fig. 1b). This fact strongly supports the separation of the two genera, *Phaffia* and *Cryptococcus*.

In this study, we have discussed the phylogeny of basidiomycetes from 18S rDNA sequence data of 44 selected species. Most of our results showed good agreement with those reported by other investigators as mentioned above. However, more sequence data from several classes of basidiomycetes may be needed to discuss

clearly the phylogenetic relationships of these taxa. Also, the sequence data of other species of the genus and related ballistosporous yeast taxa, particularly species of *Itersonilia* and *Kockovaella*, will be needed to clarify the phylogenetic position of *Bullera* species, although phylogenetic heterogeneity among species of *Bullera* has been suggested in the present study.

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