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FULL PAPER

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Intraspecific groups of *Umbelopsis ramanniana* inferred from nucleotide sequences of nuclear rDNA internal transcribed spacer regions and sporangiospore morphology

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Abstract *Umbelopsis ramanniana* is a well-known species in this genus. A characteristic morphological feature of this fungus is the remarkable variation in the sporangiospore shape, which implies the genetic variations occur in the nucleotide sequences of the internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA (nrDNA) in the *U. ramanniana* isolates. The relationship between the variations of the sequences of the nrDNA ITS regions and those of the sporangiospore morphology was investigated for 12 isolates of *U. ramanniana* collected in Europe. Neighbor-joining and parsimony analyses on the sequences suggested that these isolates split into three groups. Precise examination of the morphology showed that the isolates of those respective groups were different from each other in their sporangiospore shape. The present study implies at least three intraspecific groups exist in *U. ramanniana* and that the variations in the nucleotide sequences of the nrDNA ITS regions correlate well with those in the sporangiospore shape in these intraspecific groups.

Key words Intraspecific groups · Nuclear ribosomal DNA sequences · Sporangiospore morphology · *Umbelopsis ramanniana*

Introduction

Umbelopsis ramanniana (A. Möller) W. Gams is a well-known species in this genus. Since the fungus was first described as *Mucor ramannianus* A. Möller (Möller 1903), its genus name has changed several times with the transfer of its taxonomic position. The history of the transfers was detailed by Sugiyama et al. (2003). After decades of controversy (Linnemann 1941; Turner 1963; Gams 1977; von Arx 1984; Yip 1986), Meyer and Gams (2003) subsumed all taxa of *Umbelopsis* Amos & Barnett (1966) and *Micromucor* (W. Gams) v. Arx (von Arx 1984) under *Umbelopsis*, the oldest suitable name, on the basis of the analyses of the restriction fragment length polymorphism (RFLP) including whole nrDNA and the sequences of nrDNA internal transcribed spacer 1 (ITS-1). At that time, they proposed a new family, Umbelopsidaceae W. Gams & W. Meyer, and raised *Mortierella ramanniana* var. *ramanniana* to species rank as *U. ramanniana*.

A characteristic morphological feature of *U. ramanniana* is the remarkable variation in the sporangiospore shape. Sugiyama et al. (2003) referred to the variations of the spore shape of *U. ramanniana*. The spore shape of 75 isolates varied continuously from nearly subglobose to ellipsoidal so that they could not delimit the isolates on the basis of the spore shape.

We think that such remarkable variations in the sporangiospore shape imply that genetic variations occur in the sequences of the nrDNA ITS regions in *U. ramanniana*. The sequences of the nrDNA ITS regions have provided reliable information concerning intra- and interspecific variations (Nazar et al. 1991; Carbone and Kohn 1993; Morales et al. 1993; Zambino and Szabo 1993; Henrion et al. 1994; Sreenivasaprasad et al. 1994; Takamatsu et al. 1998). In the present study, we elucidate what variations occur in the sequences of nrDNA ITS regions, including 5.8S rDNA, and how these genetic variations correlate with the morphological variations of *U. ramanniana*.

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Materials and methods

Strains examined

Strains of *U. ramanniana* used for the present study are summarized in Table 1. All strains were isolated

Table 1. *Umbelopsis* strains examined in the present study

Species	Strain	Database accession no.	Origin	Substratum
U. ramanniana	YODK004	AB193533	Copenhagen, Denmark	*Picea* sp.
U. ramanniana	YODK028	AB193534	Copenhagen, Denmark	*Pinus* sp.
U. ramanniana	YODK036	AB193535	Pedersted, Denmark	*Picea abies*
U. ramanniana	YODK101	AB193536	Altai mountains, Russia	*Pinus sibirica*
U. ramanniana	YODK106	AB193537	Altai mountains, Russia	*Pinus sibirica*
U. ramanniana	YODK119	AB193538	Fontainebleau, France	*Pinus* sp.
U. ramanniana	YODK120	AB193539	Fontainebleau, France	*Pinus* sp.
U. ramanniana	YODK122	AB193540	Fontainebleau, France	*Pinus* sp.
U. ramanniana	YODK126	AB193541	Mön Is., Denmark	*Pinus* sp.
U. ramanniana	YODK129	AB193542	Mön Is., Denmark	*Pinus* sp.
U. ramanniana	YODK130	AB193543	Mön Is., Denmark	*Pinus* sp.
U. ramanniana	YODK170	AB193544	Kivik, Sweden	*Pinus* sp.
U. isabellina	YODK039	AB193545	Pedersted, Denmark	*Picea abies*
U. isabellina	YODK041	AB193546	Pedersted, Denmark	*Picea abies*

from leaf litter of conifers (*Pinus* and *Picea*) collected in Europe.

Morphological observations

Morphological observation was performed using single-sporangiospore or single-sporangium isolates grown at room temperature on Miura agar medium (0.1% glucose, 0.02% $MgSO_4 \cdot 7H_20$, 0.02% KCl, 0.2% $NaNO_4$, 0.02% Difco yeast extract, 1.3% agar; Miura and Kudo 1970). For line drawings and measurements of morphological characteristics such as lengths of sporangiophores and diameters of micro- and macrochlamydospores, small pieces of the fungal strains growing on Miura agar plates were mounted in water and observed under a phase contrast microscope (Nikon, Tokyo, Japan).

For precise examination of sporangiospores, specimens were prepared for scanning electron microscopy (SEM) as follows: the fungal materials were prefixed with 0.5% OsO_4 and 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7) for 2 h and postfixed with 1% OsO_4 in the buffer for 2 h, then dehydrated through a graded ethanol series. After critical point drying with a Hitachi HCP-2 (Hitachi, Tokyo, Japan), the materials were coated with platinum-palladium of 15-nm thickness in an ion sputter (Hitachi E-102), and then observed with a scanning electron microscope (Hitachi S-2300) at 20 kV.

PCR and DNA sequencing

The isolate were grown on Miura agar plates. A small amount of the mycelium was suspended in a polymerase chain reaction (PCR) tube with 24 μl sterilized distilled water. Using 25 μl HotStarTaq Master Mix (Qiagen, Hilden, Germany) and 0.5 μl of each primers (25 μM), direct PCR was performed by the modified method described by Suyama et al. (1996).

Nuclear ribosomal DNA ITS regions were amplified using the primers ITS1 and ITS2, and ITS3 and ITS4 (White et al. 1990), under the following thermal conditions: an initial incubation of 95°C for 15 min, 25 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, and a final extension period of 72°C for 5 min. After electrophoresis, the amplified DNA was extracted from an agarose gel by using a GENECLEAN kit (BIO 101, Carlsbad, CA, USA). The extracted DNA was cloned on pGEM-T Easy Vector (Promega, Madison, WI, USA) and transformed into JM109 competent cells. After an insert check, the JM109 cells that were transformed from the plasmid vectors were cultured on LA agar plates (1% Difco tryptone, 0.5% Difco yeast extract, 1% NaCl, 10% ampicillin sodium, 1.5% agar). For each strain, three colonies of the transformed cells were grown in Terrific broth and the plasmid vectors were collected using a QIAprep Spin Miniprep Kit (Qiagen).

The collected plasmid DNA was labeled using a Thermo Sequenase Cycle Sequencing Kit (Amersham Bioscience, Piscataway, OH, USA) with M13 forward and M13 reverse RID 800 Infrared Labeled Dye primers (ALOKA, Tokyo, Japan). DNA sequences of both directions were determined by the Sanger method (Sambrook and Russell 1989) with a DNA Analyzer GENE READIR 4200 (LI-COR, Lincoln, NE, USA). Polymorphous sequences, which mainly consist of several successive As or Ts, were sometimes detected. In such cases, additionally, the DNA sequences of both directions were determined twice. Then, the sequences that got a majority were selected as the sequences at polymorphous positions.

Alignment and phylogeny analysis

DNA sequences were aligned through a multiple sequence alignment program in Clustal W ver. 1.71 (an updated version of Clustal W ver. 1.4; Thompson et al. 1994), and visually corrected. The alignment was deposited in TreeBASE (http://www.treebase.org/treebase/) as S1362. Phylogenetic analyses were performed with neighbor-joining (NJ) using the Hasegawa, Kishino, and Yano (HKY85) model (Hasegawa et al. 1985) and maximum-parsimony analysis (MP) with the heuristic search option in PAUP*4.0b10 (Swofford 2001). All indels were excluded from the phylogenetic analyses. Using NJ option and heuristic search

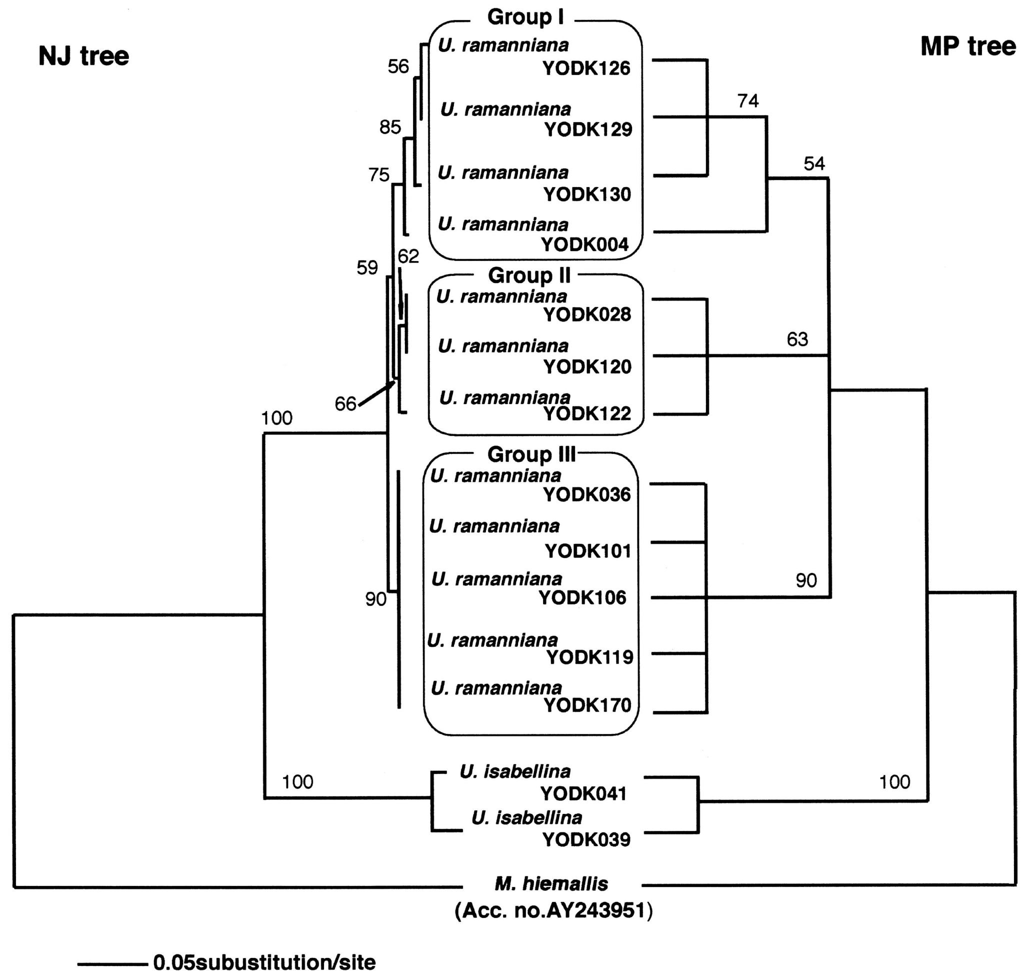

Fig. 1. Intraspecific relations of *Umbelopsis ramanniana* inferred from neighbor-joining (*NJ*) and maximum-parsimony (*MP*) analyses of internal transcribed spacer (ITS)-1, ITS-2, and 5.8S rDNA. For the NJ tree, distances were determined by the Hasegawa, Kishino, and Yano (HKY) model. In MP analysis, the strict consensus tree was reconstructed from two equally parsimonious trees (length = 293 steps, CI = 0.969, RI = 0.940, RC = 0.912). *Numbers beside the branches* are the bootstrap values (>50%) of 1000 replicates in the NJ tree and 100 replicates in the MP tree

option implemented with PAUP*4.0b10, stability of clades was assessed by 1000 and 100 bootstrap replications for the NJ tree and MP tree, respectively.

Results

Intraspecific groups of *U. ramanniana*

In the 12 examined strains of *U. ramanniana*, the nucleotide lengths of the nrDNA ITS regions (ITS-1, 5.8S rDNA, and ITS-2) ranged from 545 to 556 bp. The shortest sequence was observed in YODK120 and the longest in YODK119.

Following phylogenetic analysis, we investigated intraspecific relation of the 12 isolates of *U. ramanniana*. The operational taxonomic units consisted of 12 isolates of *U. ramanniana*, 2 isolates of *U. isabellina* (Oudemans) W. Gams, and an isolate of *Mucor hiemalis* f. *luteus* (Linnemann) Schipper as a outgroup. These OTUs provided 602 aligned sites including sequence gaps. Of these, 144 sites from ITS-1, 163 sites from ITS-2, and 166 sites from 5.8S rDNA were used for the analyses.

Neighbor-joining analysis showed that *U. ramanniana* isolates were separated into three groups, although bootstrap values were low (Fig. 1). Group I consisted of YODK004, YODK126, YODK129, and YODK130, group II of YODK028, YODK120, and YODK122, and group III of YODK036, YODK101, YODK106, YODK119, and YODK170. Maximum-parsimony analysis using same data set as the NJ analysis (95 parsimony-informative sites) generated two equally parsimonious trees with 293 steps, and the strict consensus tree showed *U. ramanniana* strains were also separated into three groups, which were also supported by not so high bootstrap values (Fig. 1).

Intraspecific variations in the nrDNA ITS regions

Tables 2 and 3 show the inter- and intragroup variations in the nrDNA ITS-1 and ITS-2 sequences, respectively.

Table 2. The inter- and intragroup variations in the nrDNA ITS-1 sequences of the *Umbelopsis ramanniana* strains[a]

	Group I				Group II			Group III				
	YODK 004	YODK 126	YODK 129	YODK 130	YODK 028	YODK 120	YODK 122	YODK 036	YODK 101	YODK 106	YODK 119	YODK 170
Group I												
YODK004		2.3	1.7	2.3	7.9	3.4	5.6	10.2	10.2	10.2	10.7	10.2
YODK126	4		1.7	1.1	6.8	4.5	5.6	11.3	11.3	11.3	11.9	11.3
YODK129	3	3		0.6	6.2	4.0	5.1	10.7	10.7	10.7	11.3	10.7
YODK130	4	2	1		6.8	4.5	5.6	11.3	11.3	11.3	11.9	11.3
Group II												
YODK028	14	12	11	12		4.5	7.9	9.0	9.0	9.0	9.6	9.6
YODK120	6	8	7	8	8		4.5	9.0	9.0	9.0	9.6	9.0
YODK122	10	10	9	10	14	8		9.6	9.6	9.6	10.2	9.6
Group III												
YODK036	18	20	19	20	16	16	17		0.0	0.0	0.6	0.6
YODK101	18	20	19	20	16	16	17	0		0.0	0.6	0.6
YODK106	18	20	19	20	16	16	17	0	0		0.6	0.6
YODK119	19	21	20	21	17	17	18	1	1	1		1.1
YODK170	18	20	19	20	17	16	17	1	1	1	2	

[a] 177 positions including gaps were compared: lower left, the number of different positions; upper right, the percentage of the different position to total

Table 3. The inter- and intragroup variations in the nrDNA ITS-2 sequences of the *Umbelopsis ramanniana* strains[a]

	Group I				Group II			Group III				
	YODK 004	YODK 126	YODK 129	YODK 130	YODK 028	YODK 120	YODK 122	YODK 036	YODK 101	YODK 106	YODK 119	YODK 170
Group I												
YODK004		2.6	2.6	2.2	9.2	7.0	7.0	10.0	9.2	10.0	10.0	10.0
YODK126	6		0.0	1.3	8.7	9.2	9.2	11.4	10.5	11.4	11.4	11.4
YODK129	6	0		1.3	8.7	10.0	9.2	11.4	10.5	11.4	11.4	11.4
YODK130	5	3	3		8.3	8.7	8.7	10.9	10.9	10.5	10.9	10.9
Group II												
YODK028	21	20	20	19		2.6	2.6	8.7	7.9	8.7	8.7	9.2
YODK120	16	21	23	20	6		0.9	7.4	6.6	7.4	7.4	7.9
YODK122	16	21	21	20	6	2		11.8	11.4	11.8	11.8	10.9
Group III												
YODK036	23	26	26	25	20	17	27		0.9	0.0	0.0	0.9
YODK101	21	24	24	25	18	15	26	2		0.9	0.9	1.7
YODK106	23	26	26	24	20	17	27	0	2		0.0	0.9
YODK119	23	26	26	25	20	17	27	0	2	0		0.9
YODK170	23	26	26	25	21	18	25	2	4	2	2	

[a] 229 positions including gaps were compared: lower left, the number of different positions; upper right, the percentage of the different position to total

The variations were 3.4%–7.9% (6–14 bp) in ITS-1 and 7.0%–10.0% (16–23 bp) in ITS-2 between group I and group II, 10.2%–11.9% (18–21 bp) in ITS-1 and 9.2%–11.4% (21–26 bp) in ITS-2 between group I and group III, and 9.0%–10.2% (16–18 bp) in ITS-1 and 6.6%–11.8% (15–27 bp) in ITS-2 between group II and group III.

Compared with the intergroup variations of the sequences, the intragroup variations of those were remarkably small in groups I and III. The sequences of group I varied 0.6%–2.3% (1–4 bp) in ITS-1 and 0.0%–2.6% (0–6 bp) in ITS-2, and the sequences of group III 0.0%–1.1% (0–2 bp) in ITS-1 and 0.0%–1.7% (0–4 bp) in ITS-2. Also, the intragroup variations of ITS-2 sequences were low in group II. They varied 0.9%–2.6% (2–6 bp). However, the sequences of ITS-1 in group II were more divergent. The sequences of ITS-1 varied 7.9% (14 bp) between YODK028 and YODK122, 4.5% (8 bp) between YODK028 and YODK120, and 4.5% (8 bp) between YODK120 and YODK122. Nevertheless, because the nrDNA ITS sequences of members of groups I and III were highly resemble each other within respective groups, YODK028, YODK120, and YODK122 were inferred not to belong to group I or III but to form another group, group II.

The variations of nucleotides in 5.8S rDNA were very small. All isolates other than YODK122 possessed the same sequence in this region, whereas YODK 122 differed by two positions from other isolates.

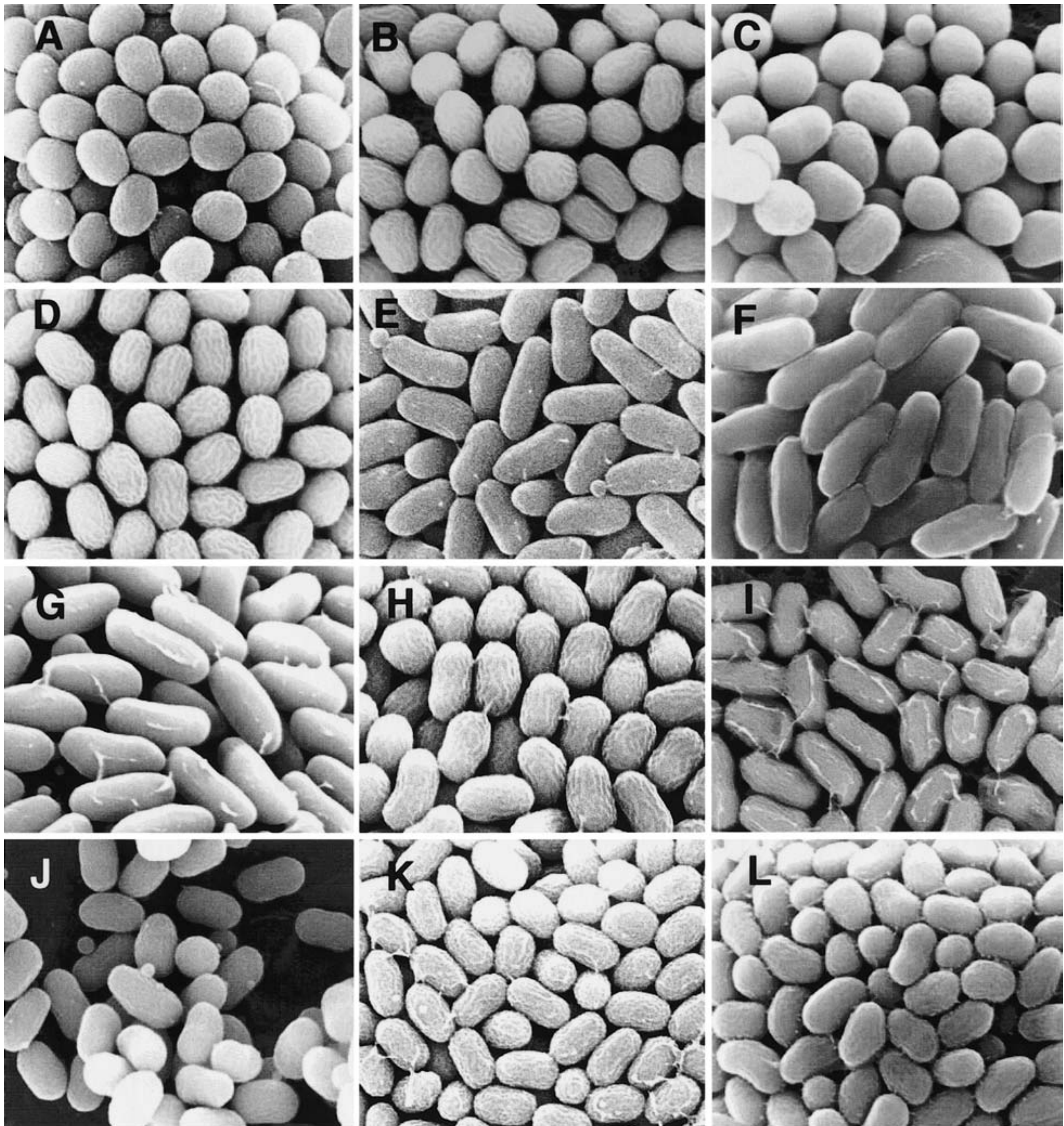

Fig. 2. Scanning electron microscopy (SEM) images of sporangiospores of three intraspecific groups among *U. ramanniana*. **A–D** Sporangiospores of group I: YODK004 (**A**), YODK126 (**B**), YODK129 (**C**), and YODK130 (**D**). **E–G** Sporangiospores of group II: YODK028 (**E**), YODK120 (**F**), and YODK122 (**G**). **H–L** Sporangiospores of group III: YODK036 (**H**), YODK101 (**I**), YODK106 (**J**), YODK119 (**K**), and YODK170 (**L**). *Bar* 5 μm

Morphological characteristics of three groups of *U. ramanniana*

We examined whether the grouping implied from nrDNA sequences reflected some morphological characteristics. Figure 2 shows the scanning electron microscopy (SEM) images of sporangiospores of the 12 isolates examined in the present study. Their sizes are summarized in Table 4. Variations in the spore shape corresponded well to those in the nrDNA sequences. The spores of group I were subglobose, 2.2–2.6 × 1.6–2.0 μm, those of group II were rounded oblong, 3.5–3.9 × 1.5–1.7 μm, and those of group III were ellipsoidal, 2.3–2.9 × 1.3–1.4 μm. The ratios of the length to the width (L/W ratio) revealed differences among the groups; the ratios were 1.2–1.4, 2.2–2.6, and 1.7–2.1 in group I, group II, and group III, respectively. The L/W ratio

Table 4. Comparison of spore size of *U. ramanniana* strains (μm)[a]

	Sporangiospore			Sporangiophore length	Macrochlamydospore diameter	Microchlamydospore diameter
	Length	Width	Length/width			
Group I						
YODK126	2.4 ± 0.08	1.7 ± 0.05	1.4 ± 0.07	251 ± 27	–[b]	6.7 ± 0.6
YODK129	2.6 ± 0.10	2.0 ± 0.08	1.2 ± 0.07	313 ± 31	–	6.5 ± 0.6
YODK130	2.4 ± 0.07	1.6 ± 0.04	1.4 ± 0.07	274 ± 28	–	6.4 ± 0.5
YODK004	2.2 ± 0.05	1.6 ± 0.05	1.3 ± 0.04	390 ± 62	–	5.4 ± 0.4
Group II						
YODK028	3.5 ± 0.08	1.5 ± 0.03	2.2 ± 0.08	479 ± 29	29 ± 1.4	5.9 ± 0.4
YODK120	3.9 ± 0.11	1.5 ± 0.05	2.6 ± 0.10	560 ± 39	33 ± 1.5	6.8 ± 0.5
YODK122	3.9 ± 0.09	1.7 ± 0.04	2.2 ± 0.08	702 ± 35	30 ± 2.2	7.0 ± 0.5
Group III						
YODK036	2.4 ± 0.06	1.4 ± 0.03	1.8 ± 0.07	375 ± 30	33 ± 2.2	7.5 ± 0.8
YODK101	2.9 ± 0.09	1.4 ± 0.07	2.1 ± 0.10	318 ± 20	29 ± 2.2	7.4 ± 0.6
YODK106	2.5 ± 0.09	1.3 ± 0.05	1.9 ± 0.09	483 ± 33	32 ± 2.4	4.9 ± 0.4
YODK119	2.4 ± 0.07	1.4 ± 0.03	1.7 ± 0.07	340 ± 30	28 ± 3.5	9.1 ± 0.8
YODK170	2.3 ± 0.05	1.4 ± 0.05	1.7 ± 0.07	523 ± 49	29 ± 2.0	6.2 ± 0.6

[a] ± shows 95% confidence interval estimated from Student's *t* test
[b] Macrochlamydospores were not observed under the examined conditions

of YODK101 in group III was comparable to those in group II. However, the spore length of YODK101 is distinctly shorter than those of group II.

The 95% confidence intervals estimated from Student's *t* values implied that the L/W ratios significantly differed among the groups. Furthermore, we performed one-way analysis of variance (ANOVA) and Bonferroni's multiple comparison test (Prism ver. 4; Motulsky 2003) to confirm whether the differences of the L/W ratios among the groups were significant. Both statistics analyses showed that differences among the averages of the L/W ratios of each group were extremely significant ($P < 0.001$) and that the spores of each group were derived from different populations.

We also investigated the correlations among the variations of the nrDNA ITS sequences and other morphological characteristics than the sporangiospore shape (see Table 4; Figs. 3–5). The variations in the length of sporangiophore loosely reflected the variations in the nrDNA ITS sequences. Shorter sporangiophores were observed in group I, intermediate ones in group III, and longer ones in group II. However, ranges of variations of the sporangiophores overlapped among groups. The length of sporangiophores varied from 251 ± 27 μm of YODK126 to 390 ± 62 μm of YODK004 in group I, 318 ± 20 μm of YODK101 to 523 ± 49 μm of YODK170 in group III, and 479 ± 29 μm of YODK028 to 702 ± 35 μm of YODK122 in group II.

It is noteworthy that the isolates of group I produced no macrochlamydospores under the examined conditions, whereas the isolates in groups II and III abundantly produced macrochlamydospores that were similar to each other (see Table 3; Figs. 3–5). The microchlamydospores were produced in all strains, which were similar in size and shape (Table 3; Figs. 3–5).

Columellae that were subglobose to flattened were observed in all three groups. The branching manner of sporangiophores was essentially the same. Most of them produce the sympodially proliferated sporangiophores. Rarely, we observed umbellate sporangiophores (Figs. 3–5).

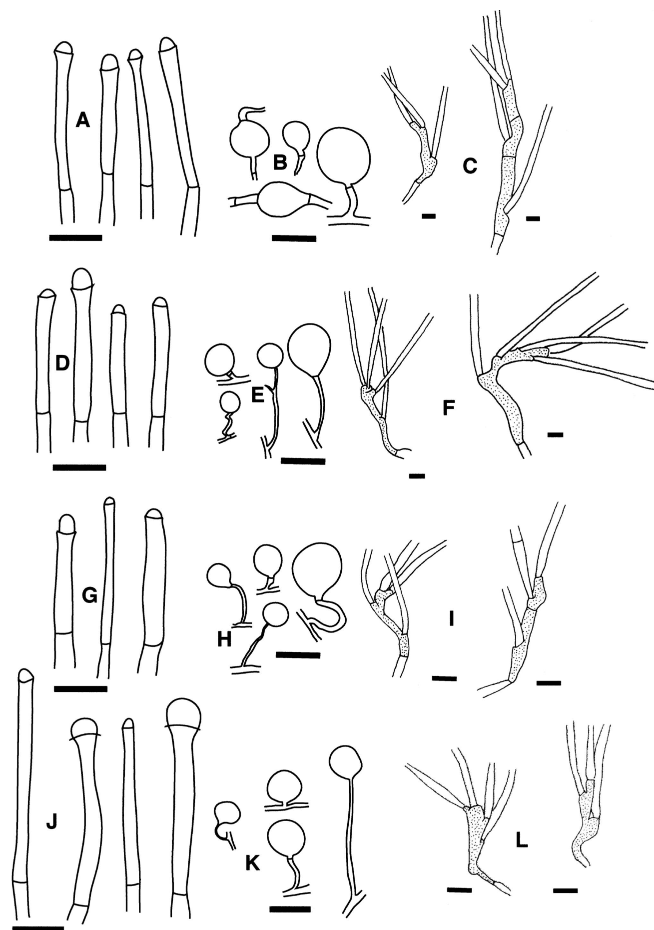

Fig. 3. Some morphological characteristics of group I. **A–C** YODK004; **D–F** YODK126; **G–I** YODK129; **J–L** YODK130; **A, D, G, J** columellae; **B, E, H, K** microchlamydospores; **C, F, I, L** lower portion of sporangiophores. *Bars* 10 μm

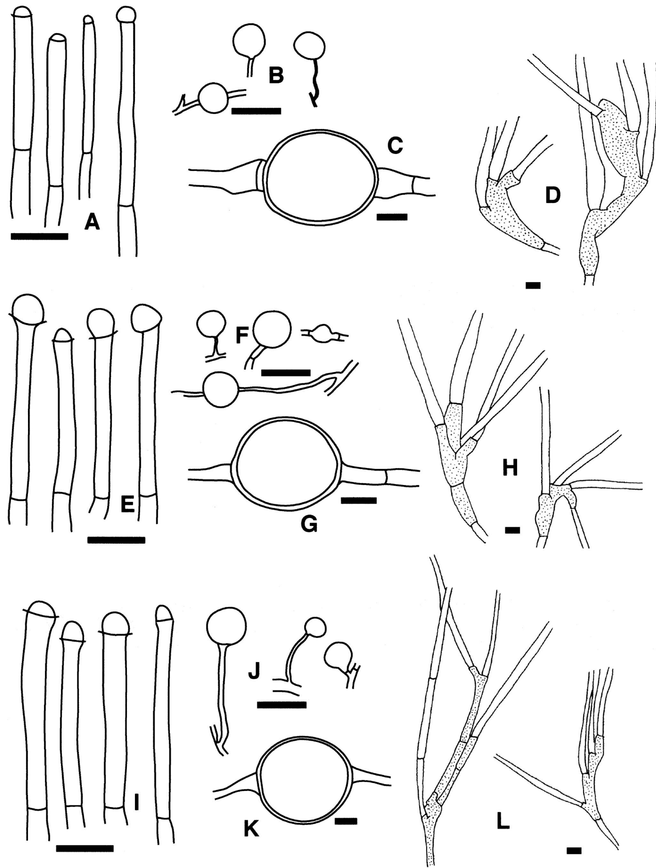

Fig. 4. Some morphological characteristics of group II. **A–D** YODK028. **E–H** YODK120. **I–L** YODK122. **A, E, I** Columellae. **B, F, J** Microchlamydospores. **C, G, K** Macrochlamydospores. **D, H, L** Lower portion of sporangiophores. *Bars* 10 μm

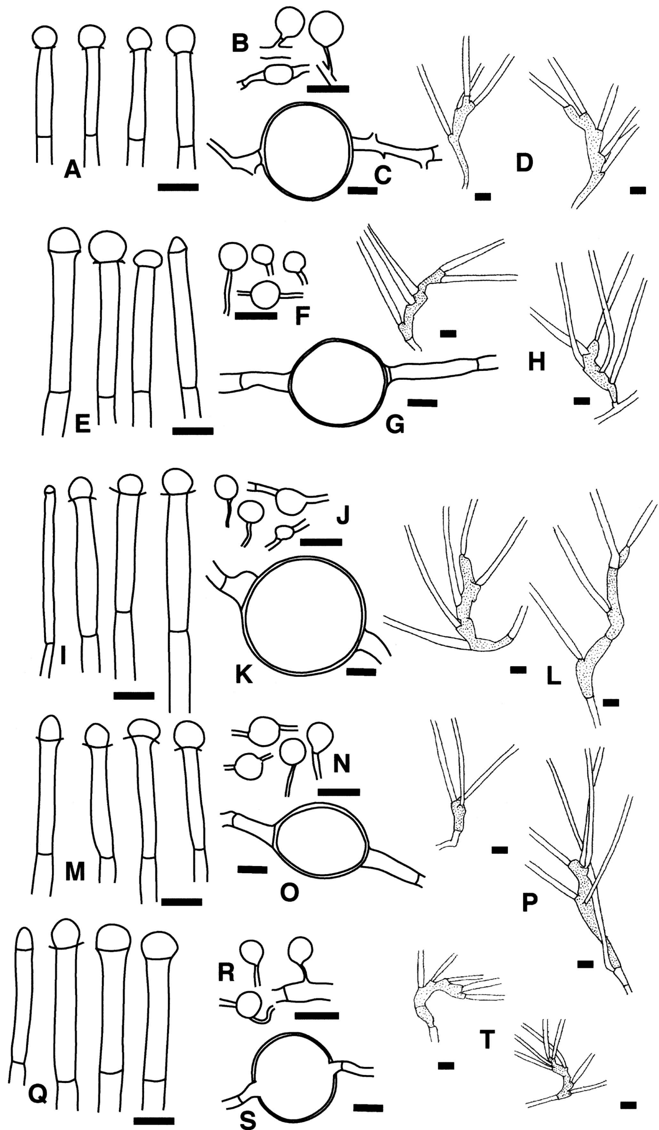

Fig. 5. Some morphological characteristics of group III. **A–D** YODK036. **E–H** YODK101. **I–L** YODK106. **M–P** YODK119. **Q–T** YODK170. **A, E, I, M, Q** Columellae. **B, F, J, N, R** Microchlamydospores. **C, G, K, O, S** Macrochlamydospores. **D, H, L, P, T** Lower portion of sporangiophores. *Bars* 10 μm

Discussion

Many researchers have investigated intraspecific variability of the nucleotides sequences in the nrDNA ITS regions for various fungal species. *Fusarium sambucinam* Fuckel showed 4.6%–15.0% variability in ITS-1 and ITS-2 sequences (O'Donnell 1992). In addition, such large variations in the regions were obtained for *Trichoderma harzianum* Rifai. The variations were in the range of 22.9% in ITS-1 (Muthumeenakshi et al. 1994) and of 0.0%–33.0% in ITS-1 and 0.0%–14.4% in ITS-2 (Kuhls et al. 1997). On the other hand, lower variability was observed in *Aspergillus tamarii* Kita (Peterson 2000), many *Penicillium* species (Skouboe et al. 2000), *Trichoderma* species that belong to sect. *Longibrachiatum* (Kuhls et al. 1997), several *Metarhizium* species (Driver et al. 2000), and *Phytophthora* (Lee and Taylor 1992). The variations were slight in these fungal groups (up to 5%). Additional data on the variability of the nrDNA ITS sequences were given by Seifert et al. (1995). The present study showed that variability in the nrDNA ITS regions among the *U. ramanniana* isolates (see Tables 2 and 3) was comparable to the higher class of the intraspecies variability in other fungal species.

Fungal species with high divergence of the nrDNA ITS regions seems to be an assemblage of some species or populations. To explain the high divergence in the nrDNA ITS regions of *F. sambucinam*, O'Donnell (1992) suggested the presence of some morphologically cryptic populations in *F. sambucinam*. Muthumeenakshi et al. (1994) split the examined *T. harzianum* strains into three groups on the basis of ITS-1 sequences and RFLP and random amplified polymorphic DNA (RAPD) analyses. More recently, Kuhls et al. (1997) compared their ITS-1 sequences with those obtained by Muthumeenakshi et al. (1994), and confirmed that group 1 and group 2 by Muthumeenakshi et al. (1994) corresponded to *T. harzianum* strains in section *Pachybasium*. Additionally, group 2 was distinguished from

group 1 by aggressive colonization of mushroom compost (Muthumeenakshi et al. 1994). Group 3 was closely related to strains of *T. harzianum/atroviride* reidentified by Gams (Kuhls et al. 1997).

The present study shows that some intraspecific groups exist in *U. ramanniana* as in *F. sambucinam* and *T. harzianum*. The variations of the nrDNA ITS sequences among the three groups of *U. ramanniana* sometimes exceed the interspecific variations reported for species of *Penicillium* (Skouboe et al. 2000), *Trichoderma* sect. *Longibrachiatum* (Kuhls et al. 1997), and *Metarhizium* (Driver et al. 2000). In addition, the variations in the sequences of the nrDNA ITS regions correlated well with those in sporangiospore shape. Although the bootstrap values that supported the intraspecific groups were low, the grouping by NJ and MP analyses seems not to be caused accidentally. The probability of the coincidence of both groupings by NJ and MP analyses and by sporangiospore morphology should be very low. Furthermore, the production of macrochlamydospores was not observed in group I. Turner (1963) pointed out that chlamydospore production of the member of *Umbelopsis* is variable even in the same species. The isolates of group I revealed such intraspecific variations of chlamydospore production. These facts emphasize that there are at least three intraspecific groups in *U. ramanniana*.

Nevertheless, we hold these three groups within *U. ramanniana*, taking account of the probability distributions of the L/W ratios of their sporangiospores. Assuming that the data follow Gaussian distribution, we estimated the probability distribution curves of the L/W ratios (Fig. 6). Although one-way analysis of variance (ANOVA) and Bonferroni's test showed that spores of each group belonged to different populations with extreme significance, the L/W ratios of each population overlapped each other at the ends of the Gaussian distributions. Therefore, there is no distinct morphological boundary to delimit the intraspecific groups of *U. ramanniana* based on the L/W ratios, and thus it would be difficult to classify these three groups as species of the genus *Umbelopsis* or as varieties of *U. ramanniana*. Furthermore, there is no rational guideline on the variations of the nrDNA ITS sequences to define species or variety because the variability of these regions is considerably different depending on the fungal groups (Seifert et al. 1995).

There is a possibility that other intraspecific groups exist in *U. ramanniana*. Meyer and Gams (2003) presented data that might suggest the existence of another intraspecific groups of *U. ramanniana*. They studied ITS-1 sequences of two strains of *U. ramanniana* (CBS219.47 and CBS478.63) and *Umbelopsis* sp. (CBS101744 and CBS101226) together with those of other members of the genus *Umbelopsis*. The variability in the nrDNA ITS-1 sequences were 0.6% and 0.0% between CBS219.47 and CBS478.63 and between CBS101744 and CBS101226, respectively. According to the line drawings by Meyer and Gams (2003) and Sugiyama et al. (2003), sporangiospores of CBS219.47 and CBS101744 were rounded oblong and those of CBS478.63 and CBS101226 were subglobose. These facts may suggest other intraspecific groups of which the members resemble one another genetically but differ morphologically.

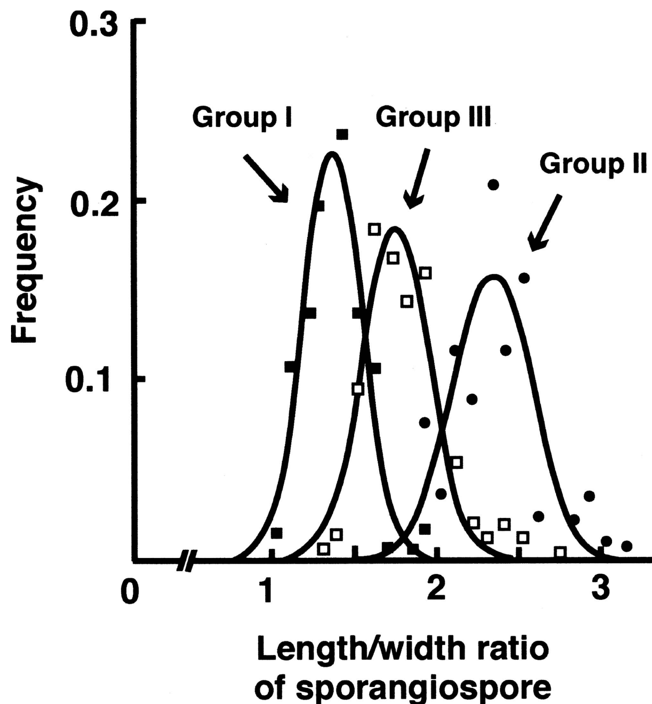

Fig. 6. Probability distribution curves and observed frequencies of length/width ratios (L/W ratios) of sporangiospores of groups I, II, and III. The curves were estimated from the means and the standard deviations of the L/W ratios on the assumption that the probability distribution curves follow the Gaussian distribution. The equations of the curves are $f(x)=\frac{0.1}{0.17\sqrt{2\pi}}e^{-\frac{(x-1.36)^2}{2\cdot(0.17)^2}}$ for group I; $f(x)=\frac{0.1}{0.27\sqrt{2\pi}}e^{-\frac{(x-2.34)^2}{2\cdot(0.27)^2}}$ for group II; and $f(x)=\frac{0.1}{0.26\sqrt{2\pi}}e^{-\frac{(x-1.81)^2}{2\cdot(0.26)^2}}$ for group III. *Closed squares* are the observed frequencies of the L/W ratios of group I; *closed circles*, group II; *open squares*, group III

Meyer and Gams (2003) referred to some strains of *Umbelopsis*, which morphologically closely resembled *U. ramanniana*, as *Umbelopsis* sp. on the basis of 2.8% nucleotide difference of the nrDNA ITS-1 sequences. Considering the high variability in the nrDNA ITS sequences in *U. ramanniana*, we think that these *Umbelopsis* sp. should be retained within *U. ramanniana*.

Further studies on the variations of the nrDNA and morphological characteristics may bring about other intraspecific groups of *U. ramanniana* and may change the grouping. However, it would be certain that *U. ramanniana* is an assemblage of morphologically and genetically divergent intraspecific groups.

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References

Amos RE, Barnett HL (1966) *Umbelopsis versiformis*, a new genus and species of the Imperfects. Mycologia 58:805–808

Carbone I, Kohn LM (1993) Ribosomal DNA sequence divergence within internal transcribed spacer 1 of the Sclerotiniaceae. Mycologia 85:415–427

Driver F, Milner RJ, Trueman WH (2000) A taxonomic revision of *Metarhizium* based on a phylogenetic analysis of rDNA sequence data. Mycol Res 104:134–150

Gams W (1977) A key to the species of *Mortierella*. Persoonia 9:381–391

Hasegawa M, Kishino H, Yano T (1985) Dating of the human-ape splitting by a molecular clock of mitochondria DNA. J Mol Evol 22:160–174

Henrion B, Chevalier G, Martin F (1994) Typing truffle species by PCR amplification of the ribosomal DNA spacers. Mycol Res 98:37–43

Kuhls K, Lieckfeldt E, Samuels GJ, Meyer W, Kubicek CP, Börner T (1997) Revision of *Trichoderma* sect. *Longibrachiatum* including related teleomorphs based on analysis of ribosomal DNA internal transcribed spacer sequences. Mycologia 89:442–460

Lee SB, Taylor JW (1992) Phylogeny of five fungus-like protoctistan *Phytophthora* species, inferred from the internal transcribed spacers of ribosomal DNA. Mol Biol Evol 9:636–653

Linnemann G (1941) Die Mucorineen-Gattung *Mortierella* Coemans. Pflanzenforschung, Heft 23. Fischer, Jena

Meyer W, Gams W (2003) Delimitation of *Umbelopsis* (Mucorales, Umbelopsidaceae fam. nov.) based on ITS sequence and RFLP data. Mycol Res 107:339–350

Miura K, Kudo MY (1970) An agar medium for aquatic hyphomycetes. Trans Mycol Soc Jpn 11:116–118

Möller A (1903) Untersuhungen über ein- und zweijährige Kiefern im märkishen Sandboden. Z Forst- Jagdwiss 35:321–338

Motulsky HJ (2003) Prism 4 statistics guide: statistical analyses for laboratory and clinical researchers. GraphPad Software, San Diego

Morales VM, Pelcher LE, Taylor JL (1993) Comparison of the 5.8S rDNA and internal transcribed spacer sequences of isolates of *Leptosphaeria maculans* from different pathogenicity groups. Curr Genet 23:490–495

Muthumeenakshi S, Mills PR, Brown AE, Seaby DA (1994) Intraspecific molecular variation among *Trichoderma harzianum* isolates colonizing mushroom compost in the British Isles. Microbiology 140:769–777

Nazar RN, Hu X, Schmidt J, Culham D, Robb J (1991) Potential use of PCR-amplified ribosomal intergenic sequences in the detection and differentiation of *Verticillium* with pathogens. Physiol Mol Plant Pathol 39:1–11

O'Donnell K (1992) Ribosomal DNA internal transcribed spacers are highly divergent in the phytopathogenic ascomycete *Fusarium sambucinam* (*Gibberella pulicaris*). Curr Genet 22:213–220

Peterson SW (2000) Phylogenetic relationships in *Aspergillus* based on rDNA sequence analysis. In: Samson RA, Pitt JI (eds) Integration of modern taxonomic methods for *Penicillium* and *Aspergillus* classification. Harwood, Amsterdam, pp 323–355

Sambrook J, Russell DW (1989) Molecular cloning. A laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, New York

Seifert KA, Wingfield BD, Wingfield MJ (1995) A critique of DNA sequence analysis in the taxonomy of filamentous ascomycetes and ascomycetous anamorphs. Can J Bot 73(suppl 1):S760–S767

Skouboe P, Taylor JW, Frisvad JC, Lauritsen D, Larsen L, Alboek C, Boysen M, Rossen L (2000) Molecular methods for differentiation of closely related *Penicillium* species. In: Samson RA, Pitt JI (eds) Integration of modern taxonomic methods for *Penicillium* and *Aspergillus* classification. Harwood, Amsterdam, pp 179–188

Sreenivasaprasad S, Mills PR, Brown AE (1994) Nucleotide sequences of the rDNA spacer 1 enables identification of isolate of *Colletotrichum* as *C. acutatum*. Mycol Res 98:186–188

Sugiyama M, Tokumasu S, Gams W (2003) *Umbelopsis gibberispora* sp. nov. from Japanese leaf litter and a clarification of *Micromucor ramannianus* var. *angulisporus*. Mycoscience 44:217–226

Suyama Y, Kawamuro K, Kinoshita I, Yoshimura K, Tsumura Y, Takahara H (1996) DNA sequence from a fossil pollen of *Abies* spp. from Pleistocene peat. Genes Genet Syst 71:145–149

Swofford DL (2001) PAUP*: phylogenetic analysis using parsimony and other methods (PAUP* version 4.0 beta 10). Sinauer, Sunderland

Takamatsu S, Hirata T, Sato Y (1998) Phylogenetic analysis and predicted secondary structures of the rDNA internal transcribed spacers of the powdery mildew fungi (Erysiphaceae). Mycoscience 39: 441–453

Thompson JD, Higgins DG, Gibson TJ (1994) The Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Turner M (1963) Studies in the genus *Mortierella* 1. *Mortierella isabellina* and related species. Trans Br Mycol Soc 46:262–272

von Arx (1984[1982]) On Mucoraceae s. str. and other families of the Mucorales. Sydowia 35:10–26

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, New York, pp 315–322

Yip H-Y (1986) Two new species of *Umbelopsis* from soil in Australia. Trans Br Mycol Soc 87:243–248

Zambino PJ, Szabo LJ (1993) Phylogenetic relationship of selected cereal and grass rusts based on rDNA sequence analysis. Mycologia 85:401–414