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FULL PAPER

Polyphyly of intraspecific groups of *Umbelopsis ramanniana* and their genetic and morphological variation

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Abstract The taxonomic positions of three intraspecific groups of *Umbelopsis ramanniana* in the genus *Umbelopsis* were analyzed based on the nucleotide sequences of their nuclear large subunit ribosomal DNA (nLSU rDNA) D1/D2 region. The examined members of the genus *Umbelopsis* were resolved into two major clades, Clades I and II. The intraspecific groups of *U. ramanniana* were nested within Clade II together with *U. westeae*, *U. swartii*, *U. autotrophica, U. gibberisopra*, *U. angularis*, and *U. fusiformis*. In this major clade, the intraspecific groups of *U. ramanniana* were split into three polyphyletic subclades. This suggests that *U. ramanniana* is an assemblage of several genetically distinct species. Interestingly, in spite of the diverse sporangiospore shapes of the members of Clade II, the genetic variation among them was small. It is considered that their flexible sporangia membranes make it possible for them to develop various sporangiospore shapes.

Keywords Molecular phylogeny · Mucorales · nLSU rDNA D1/D2 region · Taxonomic position · Umbelopsidaceae

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Introduction

On the basis of sequence analyses of the nuclear ribosomal DNA (nrDNA) internal transcribed spacer (ITS) 1 region and restriction fragment length polymorphism, *Umbelopsis ramanniana* (Möller) W. Gams was recombined from *Mortierella ramanniana* var. *ramanniana* (Möller) Linnem. by Meyer and Gams (2003). The noticeable characteristics of this species include the marked variation in its sporangiospore shape and its ubiquitous distribution from boreal to tropical areas.

These morphological and ecological characteristics of *U. ramanniana* imply that some intraspecific groups that display genetic variation may occur in this species. Ogawa et al. (2005) showed that, based on the nucleotide sequences of the ITS regions of nrDNA, *U. ramanniana* could be resolved into three intraspecific groups and that these intraspecific groups differed from each other in their sporangiospore shapes.

However, it is unclear whether these three intraspecific groups are monophyletic in the genus *Umbelopsis* Amos & H.L. Barnett or whether they are polyphyletic, forming clades containing other species of the genus. Sugiyama et al. (2003) determined the nucleotide sequences of the nuclear large subunit ribosomal DNA (nLSU rDNA) including the D1/D2 region of the members of the genus *Umbelopsis* to investigate the taxonomic positions of two new species, *U. gibberispora* M. Sugiy., Tokum. & W. Gams, and *U. angularis* W. Gams & M. Sugiy. In the present study, referring to the nucleotide sequences of nLSU rDNA provided by Sugiyama et al. (2003), we studied the taxonomic relationship among the three intraspecific groups of *U. ramanniana* and other members of the genus *Umbelopsis*. Furthermore, we discuss the extent of genetic variation in the nLSU rDNA D1/D2 region in members of the genus *Umbelopsis* and its relationship to the variation in their sporangiospore shapes.

Materials and methods

Strains examined

The species names, strain numbers, and nucleotide sequence database (DDBJ/EMBL/GenBank) accession numbers used in the present study are summarized in Table 1. Sequences of the nLSU rDNA D1/D2 regions of 16 *U. ramanniana* strains with sigla YODK or CBS were analyzed in the present study, and another 12 sequences were retrieved from the databases. Four CBS strains of *U. ramanniana* were used for the morphological observation of sporangiospores.

Morphological observation of *U. ramanniana* strains

Sporangiospore sizes and shapes of the four CBS strains of *U. ramanniana* were observed and compared with those of YODK strains of the species reported by Ogawa et al. (2005). Cultures established from single sporangiospores of the CBS strains were grown at room temperature on Miura agar medium (0.1% glucose, 0.02% $MgSO_4 \cdot 7H_20$, 0.02% KCl, 0.2% $NaNO_3$, 0.02% Difco yeast extract, 1.3% agar; Miura and Kudo 1970). The diameters of the sporangiospores were measured by scanning electron microscopy (SEM). Sporangiospore specimens were prepared for SEM as described by Ogawa et al. (2005) and were observed with an S-2300 scanning electron microscope (Hitachi, Tokyo, Japan) at 20 kV.

Polymerase chain reaction (PCR) and DNA sequencing

According to a direct PCR method (Suyama et al. 1996), sequences of the nLSU rDNA D1/D2 region were amplified with primers NL1 and NL4 (O'Donnell 1993). The conditions for PCR were as follows: 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.

The PCR products were purified using a QIAquick PCR purification kit (QIAgen, Hilden, Germany) and sequenced directly using a CEQ 8000 genetic analyzer (Beckmann

Table 1 *Umbelopsis* strains examined in the present study

Species	Strain	Accession no.
Umbelopsis angularis W. Gams & M. Sugiy.	CBS 603.68	AB090294
Umbelopsis autotrophica (E. H. Evans) W. Gams	CBS 212.72	AB090295
Umbelopsis fusiformis H. Y. Yip	CBS 385.85	AB090296
Umbelopsis gibberispora M. Sugiy., Tokum. & W. Gams	CBS101745	AB090297
	CBS109328	AB090298
Umbelopsis isabellina (Oudem.) W. Gams	NRRL 1757	AF157220
Umbelopsis nana (Linnem.) Arx	NRRL 22420	AF157221
Umbelopsis ramanniana (Möller) W. Gams	CBS 112.08[a]	AB435498[b]
	CBS 219.47[a]	AB435499[b]
	CBS 243.58[a]	AB435500[b]
	CBS478.63[a]	AB440225[b]
	YODK 004	AB435486[b]
	YODK 028	AB435487[b]
	YODK 036	AB435488[b]
	YODK 101	AB435489[b]
	YODK 106	AB435490[b]
	YODK 119	AB435491[b]
	YODK 120	AB435492[b]
	YODK 122	AB435493[b]
	YODK 126	AB435494[b]
	YODK 129	AB435495[b]
	YODK 130	AB435496[b]
	YODK 170	AB435997[b]
Umbelopsis roseonana (W. Gams & Gleeson) Arx	CBS 473.74	AB090302
Umbelopsis swartii H. Y. Yip	CBS 868.85	AB090303
Umbelopsis vinacea (Dixon-Stewart) Arx	CBS 222.29	AB090304
Mucor hiemalis f. *hiemalis* Wehmer	NRRL 3624	AF113468
Mucor circinelloides f. *lusitanicus* (Bruderl.) Schipper	ATCC1216b	AJ271061

[a] Sporangiospore morphology studied in this study

[b] DNA sequence determined in this study

Coulter, Fullerton, CA, USA) with the dye terminator method according to the manufacturer's instructions (Quick Start Kit; Beckmann Coulter).

Alignment

DNA sequences were aligned through a profile/structure alignment process in CLUSTAL W ver. 1.71 (Thompson et al. 1997). The obtained sequences were added individually to the alignment data set deposited by Sugiyama et al. (2003) in TreeBASE (http://www.treebase.org./treebase/) as S870. Their alignment set was constructed based on the alignment of representative nLSU rDNA sequences downloaded from the rRNA www server at the University of Antwerp (http//:rrna.uia.ac.be/), where the secondary structure of nLSU rDNA was taken into account for the alignment. Finally, the aligned data were checked and optimized manually. The alignment was deposited in TreeBASE as SN4428 (P. I. N Number 23241).

Phylogenetic analyses

Phylogenetic relationships of the examined strains were analyzed by the neighbor-joining (NJ) (Saitou and Nei 1987), maximum parsimony (MP), and maximum likelihood (ML) methods using PAUP* version 4.0 beta 10 (4.0b10) (Swofford 2001). The examined operational taxonomic units provided 664 aligned sites including sequence gaps. All indels were excluded from the phylogenetic analyses. *Mucor circinelloides* f. *lusitanicus* (Bruder.) Schipper and *M. hiemalis* f. *hiemalis* Wehmer were selected as outgroups.

In the NJ analysis, distances were estimated by the Hasegawa, Kishino, and Yano (HKY85) model (Hasegawa et al. 1985) without the assumption of rate heterogeneity. The topology of the tree was assessed by 1000 bootstrap replications.

In the MP analysis, the heuristic search option was employed, using tree bisection and reconnection (TBR) for branch swapping. The topology of the starting tree used for the heuristic search was obtained from the random stepwise addition option with 100 replicates. Bootstrap analysis was performed for 1000 random resample data using a heuristic search of the TBR branch swapping and the random stepwise addition option (10 replicates) for the starting topologies.

In the ML analysis, the starting tree was obtained by NJ with the HKY85 model. A heuristic search was performed

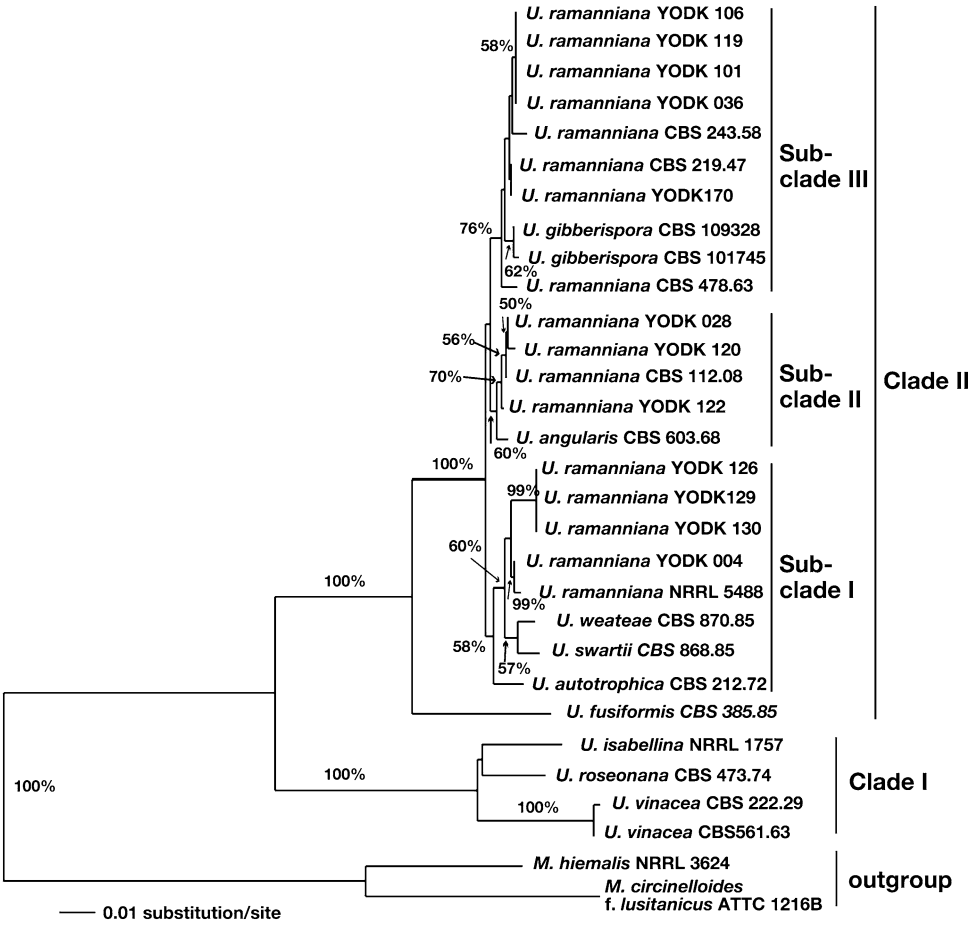

Fig. 1 Taxonomic position of three intraspecific groups of *Umbelopsis ramanniana* inferred from neighbor-joining (NJ) analysis of the nuclear large subunit ribosomal DNA (nLSU rDNA) D1/D2 region. For the NJ analysis, distances were determined by the Hasegawa, Kishino, and Yano (HKY85) model. *Percentages beside the branches* are bootstrap values of 1000 replicates

using the empirical nucleotide frequency, and the transition/transversion ratio was estimated via maximum likelihood from the NJ tree, and TBR branch swapping. The topology of the trees was assessed by 100 bootstrap replications.

Results

The NJ and MP analyses yielded almost identical topologies (Figs. 1, 2). Two major clades, Clades I and II, were recognized with high bootstrap values. Clade I consisted of *U. isabellina* (Oudem.) W. Gams, *U. roseonana* (W. Gams & Gleeson) Arx, and *U. vinacea* (Dixon-Stew.) Arx; whereas Clade II consisted of *U. westeae* H. Y. Yip, *U. swartii* H. Y. Yip, *U. ramanniana*, *U. autotrophica* (E. H. Evans) W. Gams, *U. gibberisopra*, *U. angularis*, and *U. fusiformis* H. Y. Yip.

These analyses indicated that the intraspecific groups of *U. ramanniana* were polyphyletic. In Clade II, the examined *U. ramanniana* strains were resolved into three subclades. In the NJ and MP analyses, five strains of *U. ramanniana*, YODK 004, YODK 126, YODK 129, YODK 130, and NRRL 5488 formed Subclade I together with *U. westeae* CBS 870.85, *U. swartii* CBS 868.85, and *U. autotrophica* CBS 212.72. Also, eight strains of *U. ramanniana*, YODK 036, YODK 101, YODK 106, YODK 119, YODK 170, CBS 219.47, CBS 243.58, and CBS 478.63 formed Subclade III with two strains of *U. gibberispora*, CBS 109328 and CBS 101745. Four strains of *U. ramanniana*, YODK 028, YODK 120, YODK 122, and CBS 112.08 formed Subclade II with *U. angularis* CBS 603.68 according to the NJ analysis, whereas the former five strains of *U. ramanniana* formed a separate subclade according to the MP analysis. *Umbelopsis fusiformis* branched at the root of Clade II, distinct from the other three subclades.

Figure 3 shows the phylogenetic tree inferred from the ML analysis. The topology of the ML tree was slightly different from the NJ and MP trees. In the ML tree, the YODK 028, YODK 120, YODK122, and CBS 112.08 strains did not form a subclade, although the other

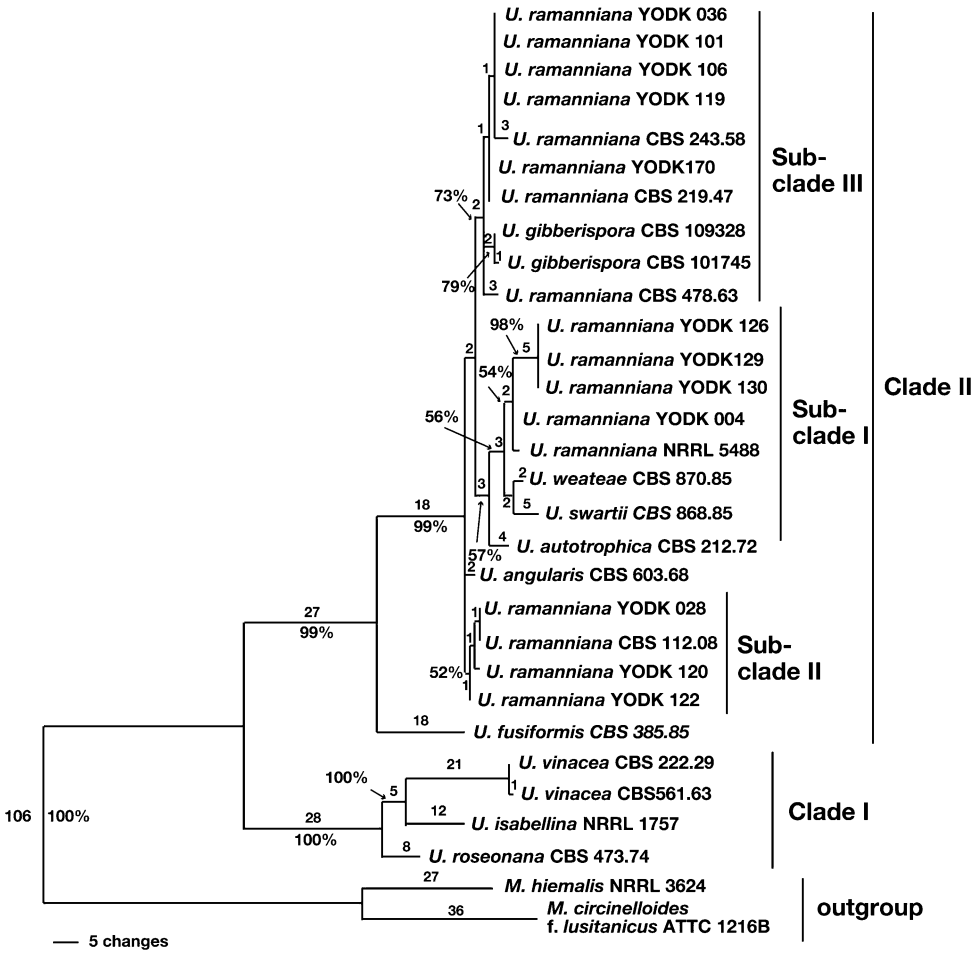

Fig. 2 Taxonomic position of three intraspecific groups of *Umbelopsis ramanniana* inferred from maximum parsimony (MP) analysis of the nLSU rDNA D1/D2 region. The tree is one of 68 equally parsimonious trees (length = 355 steps, CI = 0.780, RI = 0.850, RC = 0.663, HI = 0.2209). *Percentages and numbers beside the branches* are bootstrap values of 100 replicates and numbers of substitutions per branch

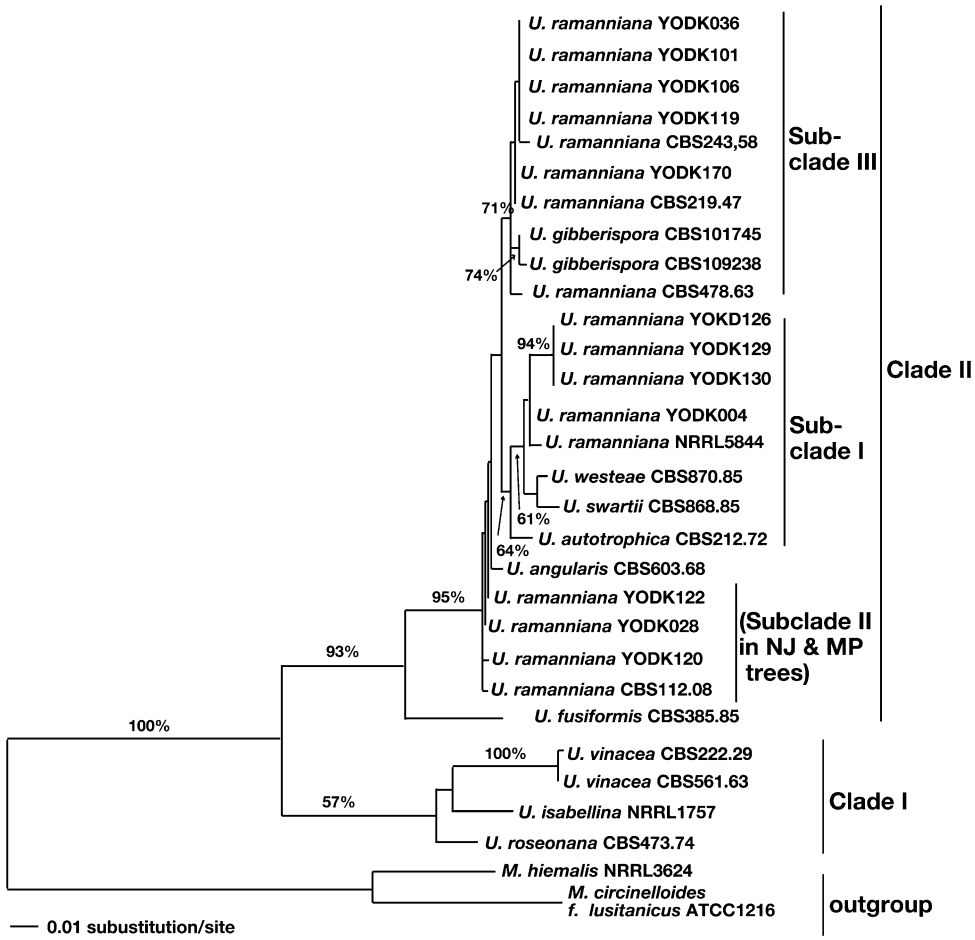

Fig. 3 Taxonomic position of three intraspecific groups of *Umbelopsis ramanniana* inferred from maximum-likelihood (ML) analysis of the nLSU rDNA 28S D1/D2 region. *Percentages beside the branches* are bootstrap values of 100 replicates

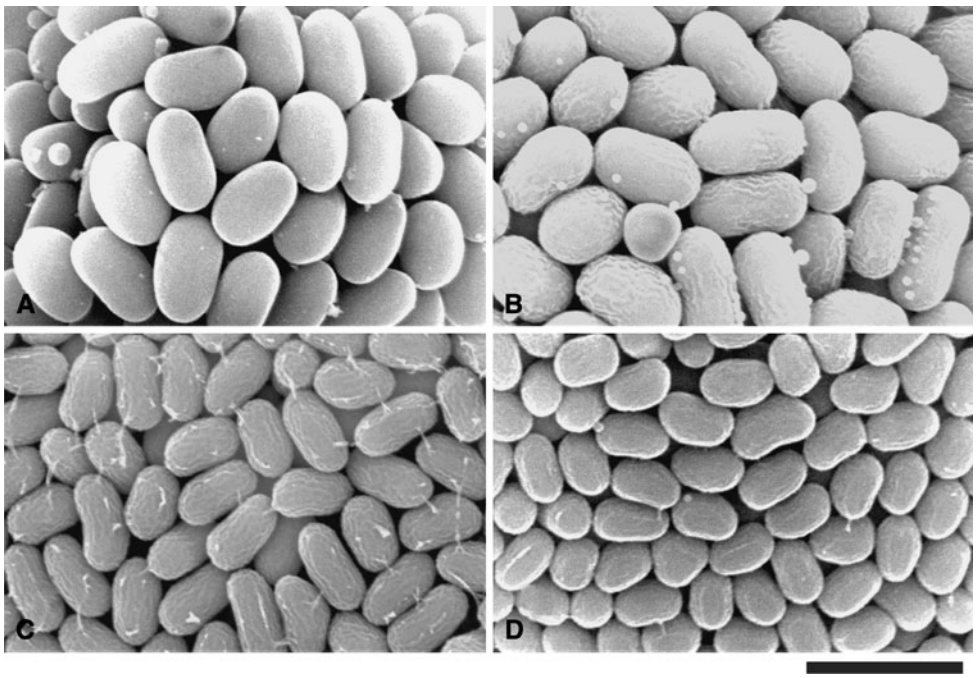

Fig. 4 Scanning electron microscopy (SEM) images of sporangiospores of four CBS strains of *U. ramanniana*. **a** CBS112.08, **b** CBS 219.47, **c** CBS 246.58, **d** CBS 478.63. *Bar* 5 μm

Table 2 Sporangiospore size of CBS strains of *Umbelopsis ramanniana*

	Sporangiospore length (μm)	Width (μm)	Length/width
CBS 112.08	3.2 ± 0.08	1.8 ± 0.05	1.8 ± 0.05
CBS 219.47	3.4 ± 0.06	1.7 ± 0.04	2.0 ± 0.06
CBS 243.58	2.5 ± 0.04	1.4 ± 0.02	1.8 ± 0.04
CBS 478.63	2.3 ± 0.03	1.4 ± 0.02	1.6 ± 0.03

± shows 95% confidence interval estimated from Student's *t*-test

U. ramanniana strains formed nearly the same subclades as those resolved in the NJ and MP analyses.

The spore shapes of CBS strains of *U. ramanniana* were compared with those of the YODK strains of *U. ramanniana*, which were reported in a previous paper (Ogawa et al. 2005). Figure 4 shows SEM images of the sporangiospores of four CBS strains of *U. ramanniana*, and Table 2 summarizes the morphological characteristics of these spores. The sporangiospores of these CBS strains were ellipsoidal, 2.3–3.4 μm in length, 1.4–1.8 μm in width, and had a 1.6–2.0 length/width ratio (Table 2).

Discussion

Based on the sequences of the nLSU rDNA D1/D2 region, Sugiyama et al. (2003) studied the taxonomic position of their new species, *U. gibberispora*, within *Umbelopsis*. In their study, they showed that 4 strains of *U. ramanniana*, CBS 219.47, CBS 243.58, CBS 112.08, and NRRL 5844, belonged to three different subclades together with other species of the genus, and these authors suggested that *U. ramanniana* was polyphyletic. In the present study, the 16 strains of *U. ramanniana* formed three polyphyletic subclades, supporting the conclusions of Sugiyama et al. (2003).

The nucleotide sequences of the coding regions of the conserved ribosomal RNA have provided good resolution for phylogenetic analysis among distantly related organisms from genera to kingdom (Berbee et al. 1995; Okada et al. 1997; Cantrell and Hanlin, 1997; Ko et al. 1997). The fact that the *U. ramanniana* strains belonged to three subclades based on analyses of the nLSU rDNA D1/D2 region suggested that these intraspecific groups may represent phylogenetically distinct species.

Indeed, in some cases, the intraspecific base differences of *U. ramanniana* strains that belonged to different subclades were comparable to or larger than the interspecific base differences between *Umbelopsis* species that belonged to the same subclade (Table 3). The intraspecific base differences of *U. ramanniana* were 7–16, 9–18, and 8–14 bp between Subclade I and Subclade II, between Subclade I and Subclade III, and between Subclade II and Subclade III, respectively. On the other hand, in Subclade I, the *U. ramanniana* strains differed by 9–14 bp from *U. autotrophica*, CBS 212.72, and 11–14 bp from *U. westeae*, CBS 870.9. In Subclade II, the *U. ramanniana* strains differed only by 3–7 bp from *U. angularis*, CBS 603.38. In Subclade III, the *U. ramanniana* strains differed by only 6–11 bp from two strains of *U. gibberispora*, CBS 101745 and CBS 109328 (Table 3).

Considering the sequence variations of the nLSU rDNA D1/D2 region of the three intraspecific groups of *U. ramanniana* and the conserved nature of this region, we should raise these intraspecific groups to species rank. However, as Ogawa et al. (2005) pointed out, we could not find the necessary morphologically distinct criteria to raise them to species rank within these groups. Therefore, at present, we lump these intraspecific groups together as a single species. Genealogical approaches based on multiple genes have revealed the existence of cryptic species in some fungal species (Taylor et al. 2000). Such approaches will clarify the taxonomic relationship among the intraspecific groups of *U. ramanniana.*

It is noticeable that the *Umbelopsis* species in Clade II have developed a divergent sporangiospore morphology. In Clade II, the spore shapes of *U. ramanniana* range from subglobose to oblong; *U. swartii*, *U. westeae*, and *U. gibberispora* are basically ellipsoidal with peculiar polar appendages or lateral cell wall thickening; *U. autotrophica* is subglobose; and *U. angularis* is angular.

Interestingly, such morphological variation in sporangiospore shape occurred in the presence of relatively low genetic variation in the nLSU rDNA D1/D2 region. In Clade II, except for *U. fusiformis*, the maximum base difference was 24 bp between *U. swartii*, CBS 868.85 and two strains of *U. ramanniana*, YODK 120 and CBS 243.58; whereas in Clade I, the maximum base difference was 38 bp between *U. isabellina*, NRRL 1757 and both strains of *U. vinacea*, CBS 222.29 and CBS 561.63 (Table 3).

The divergent sporangiospore morphology may be related to the nature of their sporangial walls. Tokumasu et al. (1990) theorized that the polygonal sporangiospores of some *Umbelopsis* species resulted from the packing of spores in a rigid sporangial wall that did not allow the development of round-shaped spores at the mature stage. According to their hypothesis, the species in Clade II might have a flexible sporangial wall that provides the necessary free space for the development of various shapes of spores. The present study suggested that divergent spore morphology occurred in the presence of relatively low genetic variation among the species of Clade II.

We previously reported on the correlation between genetic variation of the sequence of the nrDNA ITS region

Table 3 Intra- and interspecific base differences in nLSU rDNA D1–D2 region of the genus *Umbelopsis*[a]

		Clade II																								Clade I		
		Subclade I								Subclade II					Subclade III													
		U.wes CBS 870.85	U.swa CBS 868.85	U.ram YODK 004	U.ram YODK 126	U.ram YODK 129	U.ram YODK 130	U.ram NRRL 5844	U.aut CBS 212.72	U.ram YODK 028	U.ram YODK 120	U.ram YODK 122	U.ram CBS 112.08	U.ang CBS 603.38	U.ram YODK 036	U.ram YODK 101	U.ram YODK 106	U.ram YODK 119	U.ram YODK 170	U.ram CBS 219.47	U.ram CBS 243.58	U.ram CBS 478.63	U.gib CBS 101745	U.gib CBS 109328	U.fus CBS 385.85	U.isa NRRL 1757	U.ros CBS 473.74	U.vin CBS 222.29
Clade II																												
Subclade I																												
U.wes	CBS 870.85																											
U.swa	CBS 868.85	14																										
U.ram	YODK 004	11	16																									
U.ram	YODK 126	12	21	5																								
U.ram	YODK 129	12	21	5	0																							
U.ram	YODK 130	12	21	5	0	0																						
U.ram	NRRL 5844	14	19	3	8	8	8																					
U.aut	CBS 212.72	16	19	9	14	14	14	12																				
Subclade II																												
U.ram	YODK 028	19	22	9	14	14	14	12	11																			
U.ram	YODK 120	21	24	10	15	15	15	13	13	3																		
U.ram	YODK 122	18	21	7	12	12	12	10	10	2	3																	
U.ram	CBS 112.08	20	23	11	16	16	16	14	12	3	5	4																
U.ang	CBS 603.38	18	22	8	12	12	12	11	11	5	6	3	7															
Subclade III																												
U.ram	YODK 036	19	20	10	13	13	13	13	11	8	10	7	9	8														
U.ram	YODK 101	19	20	10	13	13	13	13	11	8	10	7	9	8	0													
U.ram	YODK 106	19	20	10	13	13	13	13	11	8	10	7	9	8	0	0												
U.ram	YODK 119	19	20	10	13	13	13	13	11	8	10	7	9	8	0	0	0											
U.ram	YODK 170	18	19	9	12	12	12	12	10	7	9	6	8	7	1	1	1	1										
U.ram	CBS 219.47	18	19	9	12	12	12	12	10	7	9	6	8	7	1	1	1	1	0									
U.ram	CBS 243.58	23	24	14	17	17	17	17	15	12	14	11	13	10	4	4	4	4	5	5								
U.ram	CBS 478.63	21	22	14	18	18	18	17	15	12	14	11	12	10	8	8	8	8	7	7	0							
U.gib	CBS 101745	17	21	12	17	17	17	15	13	10	12	9	11	8	6	6	6	6	5	5	8	9						
U.gib	CBS 109328	20	22	14	19	19	19	17	15	12	14	11	13	10	8	8	8	8	7	7	10	11	4					
U.fus	CBS 385.85	46	52	46	45	45	45	49	45	41	42	41	43	41	43	43	43	43	43	43	45	45	45	49				
Clade I																												
U.isa	NRRL 1757	90	96	89	86	86	86	92	88	85	87	85	85	87	89	89	89	89	88	88	91	88	91	93	89			
U.ros	CBS 473.74	86	94	89	84	84	84	92	90	83	85	84	83	86	87	87	87	87	86	86	89	87	89	89	85	29		
U.vin	CBS 222.29	94	98	95	92	92	92	98	94	89	91	90	89	92	95	95	95	95	94	94	97	93	95	97	95	38	35	
U.vin	CBS 561.63	94	96	94	91	91	91	97	93	88	90	89	88	91	94	94	94	94	93	93	96	91	95	97	95	38	37	3

[a] 664 positions including gaps were compared

nLSU rDNA, nuclear large subunit ribosomal DNA. The abbreviations of species names are as follows: U.wes, *U. westae*; U.swa, *U. swartii*; U.ram, *U. ramanniana*; U.aut, *U. autotrophica*; U.ang. *U.angularis*; U.gib, *U.gibberispora*; U.fus, U. *fusiformis*; U.isa, U. *isabellina*: U.ros, U. *roseonana*; U.vin, U. *vinacea*

of *U. ramanniana* strains and morphological variation of their sporangiospores (Ogawa et al. 2005). However, the genetic variation in the nLSU D1/D2 region did not correlate clearly with the morphological variation of sporangiospores. The sporangiospores of CBS 112.08, nested within Subclade II in both the NJ and MP trees, were large and ellipsoidal (Fig. 4), whereas those of other *U. ramanniana* strains of this subclade were oblong (Ogawa et al. 2005). The spores of CBS 219.47 (3.4 × 1.7 μm; Table 2) in Subclade III were ellipsoidal but larger than those of other *U. ramanniana* strains (2.3–2.9 × 1.3–1.4 μm; Ogawa et al. 2005) in this subclade. Taking the divergent spore morphology in each subclade in Clade II into account, it is reasonable that *U. ramanniana* strains with large ellipsoidal spores were found to form clades with the strains with oblong or smaller ellipsoidal spores. The small sample size used in our previous study (Ogawa et al. 2005) elucidated a clear correlation between the genetic variation of the ITS regions and the morphological variation in sporangiospore shape. *Umbelopsis ramanniana* strains with oblong spores and those with ellipsoidal spores may dominate within Subclades II and III, respectively. When we isolate a small number of samples, however, the probability of obtaining only the dominant strains is high, and so the correlation detected in the present study would have been due to the exclusion of non-dominant strains such as CBS 112.08 and CBS 219.47.

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