Acta Biologica Hungarica 54 (3–4), pp. 393–402 (2003)

PHYLOGENETIC RELATIONSHIP OF THE GENUS *GILBERTELLA* AND RELATED GENERA WITHIN THE ORDER MUCORALES BASED ON 5.8 S RIBOSOMAL DNA SEQUENCES

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(Received: June 26, 2003; accepted: September 26, 2003)

The complete ITS (internal transcribed spacer) region coding the ITS1, the ITS2 and the 5.8S rDNA was amplified by polymerase chain reaction from two strains of *Gilbertella persicaria*, six strains in the Mucoraceae (*Mucor piriformis, M. rouxii, M. circinelloides, Rhizomucor miehei, R. pusillus* and *R. tauricus*) and four strains representing three species of the Choanephoraceae (*Blakeslea trispora*, *Choanephora infundibulifera* and *Poitrasia circinans*). Sequences of the amplified DNA fragments were determined and analysed. *G. persicaria* belongs to the monogeneric family (Gilbertellaceae), however, originally it was described as *Choanephora persicaria*. The goal of this study was to reveal the phylogenetic relationship among fungi belonging to Gilbertellaceae, Choanephoraceae and Mucoraceae. Our results support that the "intermediate" position of this family is between Choanephoraceae and Mucoraceae.

Keywords: Choanephoraceae – *Gilbertella* – Gilbertellaceae – Mucoraceae – phylogeny – rDNA

INTRODUCTION

Gilbertella persicaria belonging to the Mucorales is known to be a storage-rot microorganism primarily of peaches [6]. This species was originally described as *Choanephora persicaria* [4] and was included in the Choanephoraceae on the basis of the morphology of the sporangia and sporangiospores. A persistent sporangial wall with a longitudinal suture and sporangiospores with apical, hyaline appendages are characteristic of the Choanephoraceae species. Later, Hesseltine [8] introduced the genus *Gilbertella* for this species and placed this fungus in the Mucoraceae, because *Mucor*-type zygospores were observed. The mucoraceous zygosporangium is rough, thick walled and pigmented with opposed, equal suspensors [16] in contrast to the choanephoraceous hyaline and smooth-walled zygosporangium where the suspensors are apposed and basally entwined [12]. However, to increase the homogeneity of the Mucoraceae, *G. persicaria* was subsequently removed to the newly-creat-

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ed monogeneric family Gilbertellaceae [2]. The aim of the present study was to investigate the phylogenetic relations of *G. persicaria* and some related species belonging to the 3 families mentioned above (e.g. Gilbertellaceae, Choanephoraceae and Mucoraceae).

MATERIALS AND METHODS

Strains and cultivation

Sources of the strains used in this study are given in Table 1. Fungal strains were maintained on malt extract agar (0.5% malt extract, 0.5% yeast extract, 0.5% glucose, 1% KH_2PO_4, 1.5% agar) slants at 4 °C.

DNA isolation and PCR

For the PCR, total DNA was isolated using a variation of the rapid lithium chloride procedure [13, 18]. The ITS regions including the 5.8S rDNA were amplified by

Table 1
Fungal strains of Zygomycetes investigated in the present study and the sizes of their ribosomal regions (ITS1-5.8S-ITS2) with the EMBL accession numbers

Species	Strain number[a]	Source and geographic origin	Sequence length (bp)	EMBL accession No.
B. trispora	CBS 130.59	Soil, Panama	562	AJ278366
B. trispora	CBS 131.59	Unknown	561	AJ278367
C. infundibulifera	NRRL 2560	Unknown	589	AJ278358
P. circinans	NRRL 2545	Soil, Japan	589	AJ278369
G. persicaria	SzMC 1460	Peach, California	577	AJ278364
G. persicaria	IMI 101698	Trickling filter plant, Ohio	576	AJ278362
M. piriformis	NRRL 26211	Pear, Oregon	640	AJ278359
M. circinelloides	ATCC 1216b	Unknown	565	AJ271061
M. rouxii	NRRL 24905	Rice fermentation	570	AJ278363
R. miehei	NRRL 5901	Cow placent, USA	633	AJ278360
R. pusillus	IBP M.p./1	Unknown, Poland	559	AJ278361
R. tauricus	NRRL 3695	Forest soil, Ukraine	554	AJ278365

[a] Culture collection abbreviations: ATCC, American Type Culture Collection, Rockville, Maryland, USA; CBS, Centralbureau voor Schimmelcultures, Utrecht, The Netherlands; IBP, Institute of Fermentation Industry C. C., Warsaw, Poland; IMI, CABI Bioscience (formerly the International Mycological Institute), Egham, United Kingdom; NRRL, National Center of Agricultural Utilization Research (formerly the Northern Regional Research Laboratory), Peoria, Illinois, USA; SzMC, Department of Microbiology, University of Szeged, Szeged, Hungary. [b] syn. *conjuncta.* [c] syn. *racemosus.* [d] syn. *indicus.*

PCR using the standard primers ITS4 and ITS5 [29]. Amplification mixtures contained 1 × Taq polymerase buffer, 200 μM dNTPs, 1 μM of each primer, 5 U Taq polymerase and 50 ng of genomic DNA. Amplifications were carried out in a PTC-100-60 DNA thermocycler (MJ Research, Inc., Watertown, Massachusetts, USA) programmed for a denaturation step at 95 °C for 5 min, followed by 5 cycles at 95 °C for 1 min, 50 °C for 2 min and 72 °C for 2 min, and then 25 cycles at 95 °C for 1 min, 55 °C for 2 min and 72 °C for 2 min. The final cycle was followed by an extension step at 72 °C for 10 min.

Sequencing of the PCR fragments and phylogenetic analysis

Sequences of the amplified DNA fragments were determined in both directions by using an Applied Biosystem 373 DNA sequencer. Nucleotide sequences were initially aligned using the Clustal W program [24] and these alignments were manually adjusted (for alignments see www.sci.u-szeged.hu/microbiology/vcs.html). Phylogenetic analysis was performed with the PHYLIP 3.5 software package [5]. Phylogenetic reconstruction was carried out with the parsimony [23] and the neighbor-joining [19] methods. Evolutionary distances were calculated by Kimura's formula [11]. ITS sequence of *Gigaspora rosea* (Zygomycetes, Glomales; EMBL accession no. AF004696) was involved in the analysis as an outgroup. The ITS sequence of *Mucor circinelloides* ATCC1216b was obtained from the EMBL nucleotide sequence database (accession no. AJ271061).

RESULTS

Amplification and sequence alignments of the ITS fragments

A single amplicon was detected in each PCR reaction by agarose gel electrophoresis. The size range of the PCR products amplified with primers ITS1 and ITS4 from the 12 zygomycetous strains was 554 to 640 bp (Table 1). Comparison of the entire ITS region revealed a relatively good alignment in the 5.8S rDNA sequence, but considerable sequence variation in the ITS sequences. The total length of the multiple alignment obtained with the CLUSTAL W programme was 750 bp which from 150 were identical (20%) among the sequences of all strains used.

The percentage of homology of the aligned sequences are presented in Table 3. Sequence homology among the choanephoraceous strains varied from 94 to 99% and between the two *Gilbertella* strains was 99%. Sequences of the strains representing the Mucoraceae did not show such values of similarity. Although sequence homology between *M. circinans* and *M. rouxii* was 96% and between *Rhizomucor pusillus* and *R. tauricus* 98%, these values varied among all isolates belonging to the Mucoraceae in a very wide range (from 19 to 98%). The sequence similarity varied among the *Gilbertella* strains and the choanephoraceous strains from 85 to 88%,

Table 2
Homology values (%, upper right) and evolutionary distances (lower left) of the ITS sequences

	Choanephoraceae				Gilbertellaceae		Mucoraceae					
	Blakeslea		*Poitr.*	*Choan.*	*Gilbertella*		*Mucor*			*Rhizomucor*		
	1	2	3	4	5	6	7	8	9	10	11	12
1[a]		99	94	94	88	88	80	81	53	26	26	27
2	0.0036		94	94	88	88	81	81	51	25	25	26
3	0.0555	0.0574		99	85	86	81	81	69	25	25	20
4	0.0536	0.0555	0.0017		86	86	80	82	69	25	25	20
5	0.1185	0.1206	0.1478	0.1457		99	80	80	52	29	29	25
6	0.1209	0.1187	0.1503	0.1481	0.0052		79	79	52	29	29	25
7	0.2075	0.2092	0.2349	0.2324	0.2331	0.2336		96	55	29	29	27
8	0.1934	0.1951	0.2121	0.2097	0.2273	0.2278	0.0363		75	29	29	27
9	0.2862	0.2920	0.3263	0.3237	0.3336	0.3371	0.2960	0.2885		44	25	23
10	0.5294	0.5271	0.5622	0.5582	0.5593	0.5593	0.5631	0.5468	0.5484		98	75
11	0.5308	0.5284	0.5633	0.5593	0.5657	0.5657	0.5643	0.5481	0.5491	0.0018		74
12	0.6077	0.6049	0.6555	0.6513	0.6394	0.6394	0.6344	0.6239	0.6604	0.1869	0.1838	

[a] 1. *B. trispora* CBS 130.59; 2. *B. trispora* CBS 131.59; 3. *P. circinans* NRRL 2545; 4. *C. infundibulifera* NRRL 2560; 5. *G. persicaria* SzMC 1460; 6. *G. persicaria* IMI 101698; 7. *M. rouxii* NRRL 24905; 8. *M. circinelloides* ATCC 1216b; 9. *M. piriformis* NRRL 26211; 10. *R. tauricus* NRRL 3695; 11. *R. pusillus* IBP M.p./1; 12. *R. miehei* NRRL 5901.

among the *Gilbertella* strains and *Mucor* strains from 52 to 80%, among the *Gilbertella* strains and *Rhizomucor* strains from 25 to 29%. Interestingly the homology between the *Rhizomucor* and the other mucoraceous strains was very low (19–29%).

Phylogenetic analysis of the ITS regions

The evolutionary distances calculated by Kimura's formula are presented in Table 2. Strains belonging to the Choanephoraceae are closely related, but the mucoraceous strains used in this study form a heterogeneous group. The genetic distances among the *Mucor* strains and the choanephoraceous strains are two or three times higher than between the two *G. persicaria* strains and the choanephoraceous strains.

The 5.8S rDNA and ITS sequences were compared using the parsimony method and NJ, and trees were obtained by PHYLIP programs. Parsimony analysis gave one most parsimonious tree of 1118 steps (Fig. 1), bootstrap confidence values were calculated from 1000 cycles. Five clusters could be discerned: *M. circinelloides* and *M. rouxii*; strains representing the Choanephoraceae; the two *Gilbertella* strains; *M. piriformis*; and the three *Rhizomucor* strains. The clusters were supported by bootstrap values of 89–100%. The clade of the *G. persicaria* strains has an intermediate position between the clades of *Mucor* strains and the choanephoraceous strains. NJ analysis resulted in a congruent tree supported by similar bootstrap values (Fig. 2). On the basis of both the parsimony and the NJ analysis *Rhizomucor* with *Mucor* appear to be polyphyletic and the branch *M. piriformis* with the other *Mucor* strains studied seem to be paraphyletic. *Gilbertella* strains form a sister-group of the clade of the choanephoraceous strains (*Blakeslea*, *Choanephora*, *Poitrasia*).

DISCUSSION

G. persicaria (Mucorales) is known to be a storage-rot microorganism. This species was originally described as *Choanephora persicaria* and was included in the Choanephoraceae on the basis of the morphology of the sporangia and sporangiospores. Later, the genus *Gilbertella* was introduced and placed into the Mucoraceae, because this fungus forms *Mucor*-type zygospores. Finally, this genus (and *G. persicaria*) was removed to a newly-created monogeneric family (Gilbertellaceae). The present research was undertaken to clarify the phylogenetic relations of the families Gilbertellaceae, Choanephoraceae and Mucoraceae on the basis of the ITS region as phylogenetic marker.

The anamorph of *G. persicaria* shows the characteristic choanephoraceous shape, but the formation of their zygospores are unambiguously of the *Mucor*-type [2, 8]. Taxonomical significance was attributed to these morphological structures: in the beginning *G. persicaria* was included in the Choanephoraceae as *C. persicaria* [4], but when it was found to produce zygospores it was placed in the Mucoraceae [8]. Finally it was moved to the monogeneric family Gilbertellaceae [2].

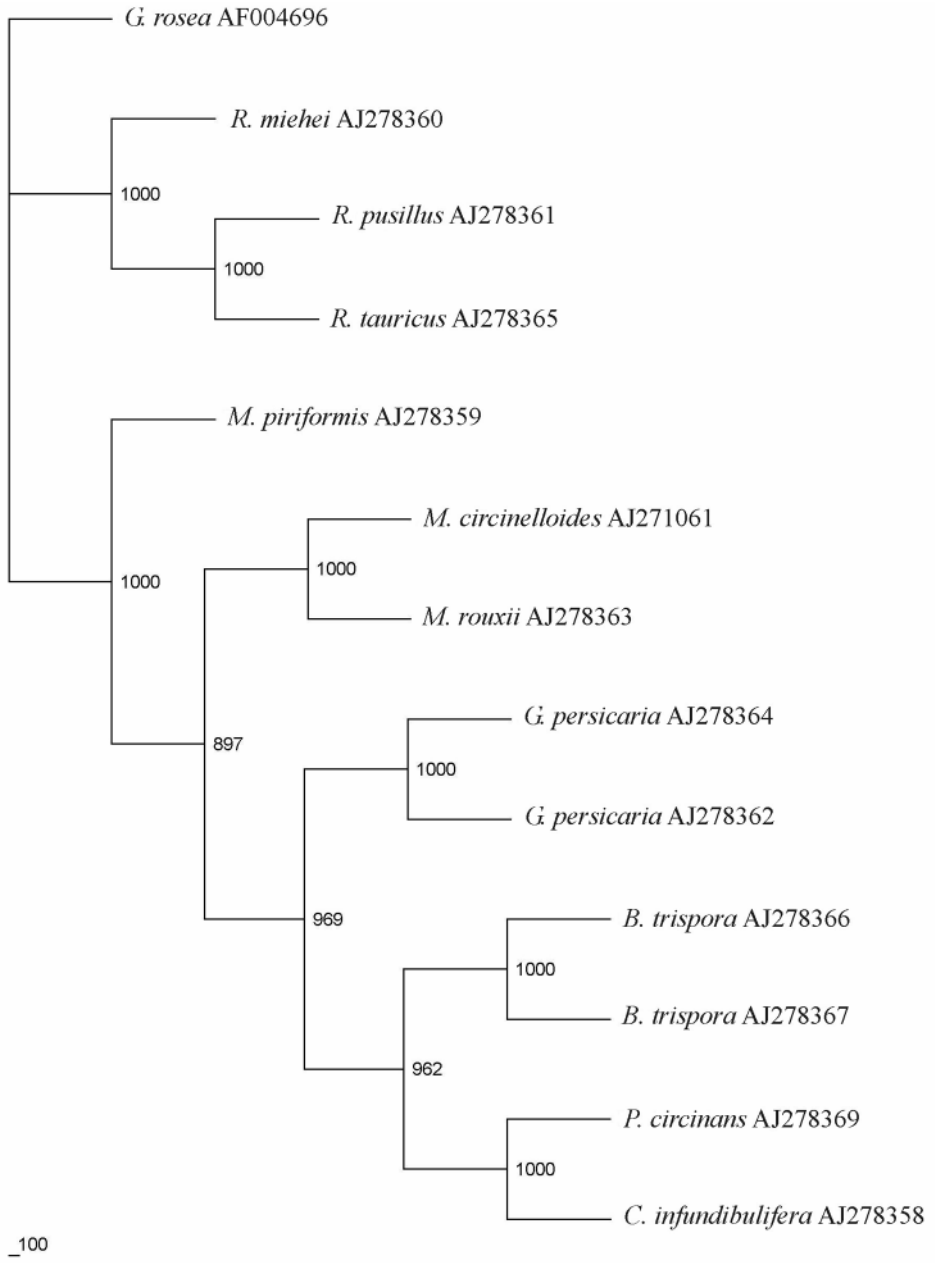

Fig. 1. The most parsimonious phylogenetic tree obtained with the program DNAPARS in the PHYLIP package. Bootstrap confidence values were calculated from 1 000 cycles. The scale for branch lengths is indicated

Phylogenetic analyses based on nuclear rDNA sequences are used at various taxonomic levels [3, 9, 10, 14, 15, 20]. In two earlier studies, 18 and 28S rDNA sequences were used to reveal evolutionary relationships within the Mucorales [17, 27]. Although the sequence comparison of the ITS-5.8 rDNA region is considered as applicable for phylogenetic studies on closely related species or even isolates of a same species, we found this approach valuable to elucidate the phylogenetic position of Gilbertellaceae.

The topologies of the trees obtained with the analysis of the ITS region (Figs 1–2) put *G. persicaria* in an intermediate position between the Choanephoraceae and the Mucoraceae and support the establishment of the new family (Gilbertellaceae) for *G. persicaria*, which was originally postulated from morphological and theoretical considerations [2]. The clade corresponding to the monogeneric family Gilbertellaceae differs significantly from both the clades and branches of the mucoraceous genera and the clade of the Choanephoraceae. At the same time molecular phylogenetic analysis reinforces the earlier suggestion that *Gilbertella* could be considered an intermediate form between Mucoraceae and Choanephoraceae [8]. Kirk, who also placed the fungus into the Mucoraceae on the basis of the *Mucor*-type zygospores, did not take into consideration the structure of the sporangia and the sporangiospores at the family level as taxonomically important characters [12]. He and others emphasized the taxonomic role of the nature of the sexual spores [1, 7, 8, 12]. However, *Gilbertella* strains are a sister-group to the choanephoraceous clade, but they appear to be paraphyletic to the mucoraceous genera (Figs 1–2). Sequence homology values and evolutionary distances also indicate that *Gilbertella* is phylogenetically closer to the Choanephoraceae than the Mucoraceae (Table 2).

Choanephoraceae seems to be a very homogenous family. The strains representing the three genera of the family show a high level of homology (94 to 99%) and they have very close evolutionary distance values (Table 2). Concerning the parsimony and NJ trees based on ITS sequences, *C. conjuncta* and *P. circinans* form a sister-group to the *Blakeslea* strains (Figs 1–2). *Choanephora infundibulifera* and *P. circinans* appear more similar than the congeneric strains, since the evolutionary distance between these strains, calculated on the basis of the ITS sequences, is less than the distances between the strains belonging to the same species (see the distance values of the *Blakeslea* or the *Gilbertella* strains; Table 2). *P. circinans* was originally described as *C. circinans* [12], but then renamed, because it does not produce sporangiola in contrast with *Choanephora* and *Blakeslea*. Sequence comparison of the ITS regions does not support separating *Poitrasia* from *Choanephora*. The lack of the sporangiola may be not a relevant taxonomical marker in this case, however, the verification of this statement requires further investigation.

The taxonomy of the species involved in this study was established primarily on morphological data. The results of the phylogenetic analysis of the ITS region (Figs 1–2) are in agreement with the generic-level morphological classifications. However, Mucoraceae (especially the genus *Mucor*) does not appear as homogenous as the Choanephoraceae. On the basis of the comparison of the ITS sequences *M. piriformis* proved to be polyphyletic with the other two *Mucor* species (*M. circinelloides* and

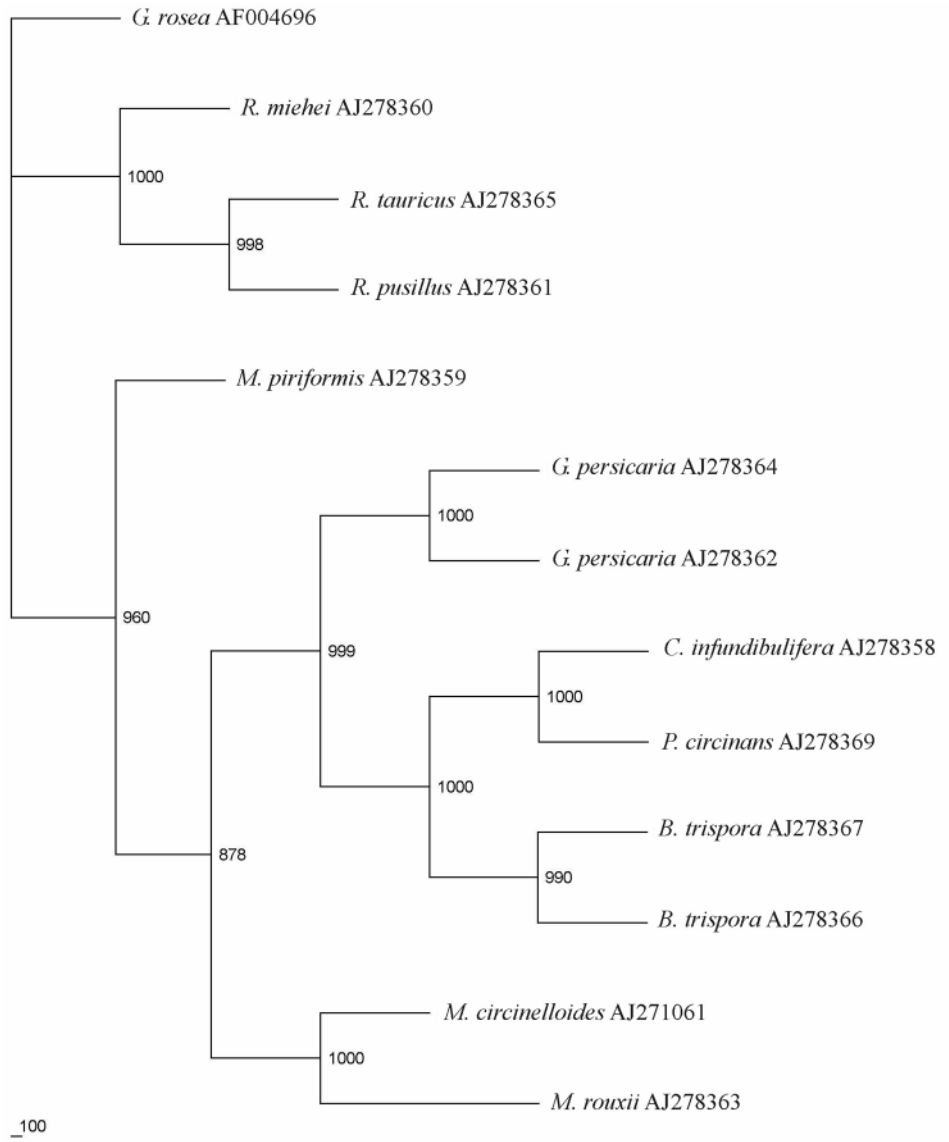

Fig. 2. Neighbor-joining tree obtained with the program Neighbor in the PHYLIP package. Bootstrap confidence values were calculated from 1 000 cycles. The scale for branch lengths is indicated

M. rouxii). In other studies, where the 18 and 28S rDNA of several zygomycetes were analyzed, the genus *Mucor* was also found polyphyletic on the 28S rDNA tree [17, 27]. Analysis of the actin and translation elongation factor EF-1α also gave similar results [28]. *M. circinelloides* and *M. mucedo* were in different groups in this tree,

which may be in agreement with our results since *M. piriformis* is closely related to *M. mucedo* according to the system elaborated by Schipper [21]. The analysis of the ITS region of *M. circinelloides* and *M. rouxii* indicated that they are closely related.

Rhizomucor strains form a monophyletic group in the trees (Figs 1–2). The similarity and the low evolutionary distance of 0.0018 (Table 2) between the ITS sequences of the *R. pusillus* and the *R. tauricus* are concordant with the results of other studies based on isoenzyme and ITS-RFLP analysis. This study questioned the necessity of the differentiation of *M. tauricus* at the species-level [22] and suggested that the single known strain of this species was really a heterothallic mutant strain of *R. pusillus* [25]. A relatively low level of homology was detected in the ITS regions of *R. pusillus* and *R. miehei* (Table 2) supporting the results of an earlier work, where cluster analysis based on the data of isoenzyme and carbon source utilization studies also detected a great distance between the clusters representing the two species [26].

Surprisingly, ITS sequences of the thermophilic *Rhizomucor* strains do not show similarity with the sequences of the other mucoraceous strains. The ITS regions of *Mucor* strains are more similar to those of the choanephoraceous and *Gilbertella* strains than to the *Rhizomucor* strains (Table 2). In parallel with these results, the topologies of 18 and 28S rDNA trees show that *Mucor* and *Rhizomucor* also are polyphyletic [27]. The Mucoraceae does not seem to be as homogenous a family as could be presumed on the basis of morphological and physiological characters after the reduction of the number of the genera by von Arx [1].

The three families investigated in this study, especially the Gilbertellaceae and the Choanephoraceae, appear to be closely related. The distinction of *Gilbertella* at the family level seems to be justified.

AKNOWLEDGEMENTS

This research was supported by the Hungarian Scientific Research Fund (OTKA) T032738 and F035053.

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