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Taxonomic position of *Mucor hiemalis* f. *luteus*

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Abstract – The taxonomic position of isolates described by Schipper in 1973 as *Mucor hiemalis* f. *luteus*, nom. inval., was reevaluated using morphological and molecular data. Based on these data, we propose to validate this taxon at specific rank, as *M. luteus*. A complete taxonomic description is given and a diagnostic signature sequence is indicated.

Key words – *Mucorales*, endophytes, phylogeny, rhizoids

Introduction

Mucor hiemalis Wehmer 1903 is the most common and the most variable species within this genus (Schipper 1973). Representatives of this species are frequent soil-borne fungi but they can also be isolated as saprotrophs or parasites from plant material and animals (Costa et al. 1990). Schipper (1973) reexamined the *M. hiemalis* complex and described *M. hiemalis* as one species with four forms: *M. hiemalis* f. *corticola* (Hagem) Schipper 1973, *M. hiemalis* Wehmer 1903 f. *hiemalis*, *M. hiemalis* f. *luteus* (Linnem.) Schipper 1973, and *M. hiemalis* f. *silvaticus* (Hagem) Schipper 1973. Although f. *luteus* is invalid because it lacks a Latin diagnosis (McNeill et al. 2006: Art. 36.1), this name is commonly used (Costa et al. 1990). The taxon has also been treated at specific rank (e.g. Mehorta et al. 1966, Zycha et al. 1969, Pei 2000), either as *M. luteus* Linnem. 1936 (nom. inval.; McNeill et al. 2006: Art. 36.1) or as *M. luteus* Linnem. ex K.Q. Pei 2000 (nom. inval.; McNeill et al. 2006: Art. 37.1). *Mucor hiemalis* is a representative of the polyphyletic genus *Mucor* (O'Donnell et al. 2001), which comprises about 50 species (Zycha et al. 1969, Schipper 1978a, Mehrotra & Mehrotra 1978, Mirza et al. 1979, Subrahamanyam 1983, Chen & Zheng 1986, Schipper & Samson 1994, Watanabe 1994, Zalar et al. 1997, Kirk et al. 2008). Furthermore, *M. hiemalis* does not form a monophyletic clade with *M. mucedo,* the type species of the genus, which suggests that *M. hiemalis* should not be classified within the genus *Mucor* (O'Donnell et al. 2001).

Moreover, some studies employing molecular data (Voigt et al. 1999) revealed that some *Rhizomucor* species form a clade with *M. hiemalis*. The morphological traits diagnostic for representatives of *Rhizomucor* genus are: presence of irregular rhizoids and stolons as in the fungi of genus *Rhizopus,* a sympodially branched sporangiophore, and a well visible collar as in some members of *Mucor racemosus* group (Lucet & Costantin 1900). The genus *Rhizomucor* as monographed by Schipper (1978b) comprised three thermophilic species, all pathogenic to humans: *R. miehei* (Cooney & R. Emers.) Schipper 1978*, R. tauricus* (Milko & Schkur.) Schipper 1978, and *R. pusillus* (Lindt) Schipper 1978. Four new *Rhizomucor* taxa have been added since 1978: *R. pakistanicus* M. Qureshi & J.H. Mirza 1979*, R. endophyticus* R.Y. Zheng & H. Jiang 1995, *R. variabilis* var. *regularior* R.Y. Zheng & G.Q. Chen 1993, and *R. variabilis* R.Y. Zheng & G.Q. Chen 1991 var. *variabilis.* Among them *R. endophyticus* and *R. variabilis* are not thermophilic, which is an exception in the genus (Zheng & Jiang 1995). *Rhizomucor endophyticus* was isolated as an endophyte from leaves of *Triticum aestivum* L., and its ITS sequence is available in GenBank (EF583635). Although both *R. variabilis* varieties were described as human primary cutaneous mucormycosis-causing species (Zheng & Cheng 1991, 1993), descriptions of sequences recorded in GenBank suggest that they could also be found in soil (EU327189) or in plants (EU196747). Voigt et al. (1999) have already demonstrated the polyphyly of *Rhizomucor.* The two thermophilic species — *R. pusillus* and *R. miehei* — form a clade closely related to *Thermomucor indicae-seudaticae* Subrahm. et al. 1977, *Mycocladus blakesleeanus* (Lendn.) J.H. Mirza 1979, and *Mycocladus corymbifer* (Cohn) Váňová 1990, while the mesophilic *R. variabilis* and *R. endophyticus* form a clade with *M. hiemalis* (Voigt et al. 1999).

Recently, new strains forming rhizoid-like structures were isolated from healthy gametophytes of *Sphagnum magellanicum* Brid. and sporophytes of *Huperzia selago* (L.) Bernh. ex Schrank & Mart. in Poland. The aim of the present study was to evaluate the taxonomic position of these isolates, using morphological observations and sequences of ITS and SSU rDNA.

Materials and methods

Fungal strains and culture condition

Fungal strains were isolated from healthy sporophytes of *H. selago* and gametophytes of *S. magellanicum.* Plants were subsequently surface sterilized according to the protocols of Szypuła et al. (2005). Plant explants were incubated on potato-dextrose agar (PDA) for 2 weeks from which pure cultures were established. Reference strains of isolated fungi are maintained in the Herbarium Generale Universitatis Varsoviensis (WA00000017113 and WA0000009410), Warsaw, Poland and in the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (CBS 124075).

Light microscopy observations

Strains were studied on 2% PDA medium. The hyphae and sporulating structures were mounted in lactophenol mounting medium (Amann's fluid; Russell 1974) and measured using a light microscope (Nikon Eclipse – 600, Tokyo, Japan). Digital images were recorded with a Nikon DX 1200 camera.

DNA isolation, amplification and sequencing

Total genomic DNA was extracted from fresh mycelium grown on PDA plates using a Plant DNasy Extraction Kit (Qiagen, Inc. Valencia, California). The internal transcribed spacer region (ITS; ca. 0.5 kb) and 18S rDNA (SSU rDNA; ca. 1.8 kb) were amplified via PCR. Forward primers ITS1-f, ITS5 and reverse primers ITS4, LR3 were used to amplify the ITS region (Gardens & Bruns 1993). Forward primers nssu97a, nssu131 and reverse primer nssu1088 were used to amplify SSU rDNA (Kauff & Lutzoni 2002). PCR and sequencing protocols followed Korniłłowicz-Kowalska et al. (2006). Forward and reversed sequences were using BioEdit Sequence Alignment Editor v. 7.0.0 (Hall 1999).

Phylogenetic analysis

Pairwise and global alignments of the ITS and 18S rDNA regions were performed in BioEdit Sequence Alignment Editor v. 7.0.0 (Hall 1999). Phylogenetic trees were obtained from the data using maximum parsimony (MP) in PAUP* v. 4.0b10 (Swofford 2002) and Bayesian analysis (BA) in MrBayes v. 3.1.2 (Huelsenbeck & Ronquist 2001). Tree robustness was evaluated by 10000 replicate bootstrap analysis. The Akaike Information Criterion (AIC) implemented in Modeltest 3.7 (Posada & Crandall 1998) was used to select the model that best fit each data set. BLAST (Basic Local Alignment Search Tool) searches in GenBank with ITS region sequences were performed using the blastn algorithm. For phylogenetic analysis *Mortierella alpina* (EF519911, EF519912) for the ITS data set and *Mortierella verticillata* (AF157145) for the 18S rDNA data set were used as outgroups. GenBank accession numbers used in these studies are indicated on the phylogenetic trees.

DNA barcoding

The hairpin loop 2 (L2) of the ITS2 fragment is a variable and specific fragment of DNA that can be used for species identification in fungi (Landis & Gargas 2007). The ITS2 fragment of isolate CBS 124075 was found using the ITS2-Databese (Selig et al. 2008; Eddy 1998). The RNA folding structure was determined using Mfold program (Zuker & Stiegler 1981) on the DINAMelt server (Markham & Zuker 2005) and RNAfold web server (Hofacker et al. 1994). The output files were aligned and the sequence of L2 was determined using the BioEdit Sequence Alignment Editor v. 7.0.0 (Hall 1999). The accuracy of characteristic sequence identification was verified using the BLAST algorithm against the whole GenBank database.

Results

Morphological observations

Morphological characters are presented in Table 1 and are compared with other closely related species. They are also presented in the taxonomic description.

Table 1. Comparison of morphological characters between *Mucor hiemalis* and *Rhizomucor* species.

		Thermo-philic	Rhizoids	Colony color	Sporangiophore branching	Sporangia color	Sporangial diameters (µm)	Columellae	Sporangiospore shape	Sporangiospore dimensions (µm)
Mucor hiemalis* f. *luteus	**CBS 124075**	–	+	**marguerite yellow**	**sympodial**	**yellowish**	**31.5–50.5** (SD 9.5 µm)	**globose**	**narrow ellipsoidal**	**4.6–7.4** (SD 1.4) × **1.1–2.9** (SD 0.9)
Mucor hiemalis* f. *luteus	**CBS 243.35**	–	+	**marguerite yellow**	**sympodial**	**yellowish**	**26.5–53.5** (SD 13.5 µm)	**globose**	**narrow ellipsoidal**	**4.4–8.2** (SD 1.9) × **1.9–3.5** (SD 0.8)
Rhizomucor endophyticus	Zheng & Jiang 1995	–	+	dark gray to blackish	sympodial	dark brown	38–80	globose, subglobose	variable	3–16 × 2–8
Rhizomucor variabilis	Hoog et al. 2000	–	+	whitish to ochraceous	simple (once branched)	nd	≤ 100	spherical, ellipsoidal to cylindrical	variable	3–11 × 2–7
Rhizomucor pusillus	Schipper 1978b	+	+	brownish	combined monopodial-sympodial	gray	≤ 80	obovoidal to slightly pyriform	subglobose	3–4
Rhizomucor miehei	Schipper 1978b	+	+	pale olive gray	sympodial	brownish	≤ 60	spherical to subspherical	subspherical to ellipsoidal	3–4
Rhizomucor tauricus	Schipper 1978b	+	+	pale olive gray	unbranched or weakly sympodial	gray	≤ 125	globose to obovoid	subglobose	3–4
Mucor hiemalis f. *hiemalis*	Schipper 1973	–	–	pale olive gray	sympodial	brownish	≤ 70	globose	ellipsoidal	≤ 9.5
Mucor hiemalis f. *corticola*	Schipper 1973	–	–	pale olive gray	sympodial	brownish	≤ 70	globose	cylindrical-ellipsoidal	≤ 9.5
Mucor hiemalis f. *silvaticus*	Schipper 1973	–	–	pale olive gray	sympodial	gray	≤ 70	globose	cylindrical	≤ 9.5

Phylogenetic analysis

The SSU rDNA dataset contained 32 taxa and 1829 characters, including gaps. 631 characters were parsimony informative. The ITS rDNA dataset contained 43 taxa and 657 characters, including gaps. 473 characters were parsimony informative. The topologies of trees obtained using MP and BA were very similar or even identical in respect to all *Rhizomucor* branches. The highest support values were obtained using BA (Figs 1 and 2).

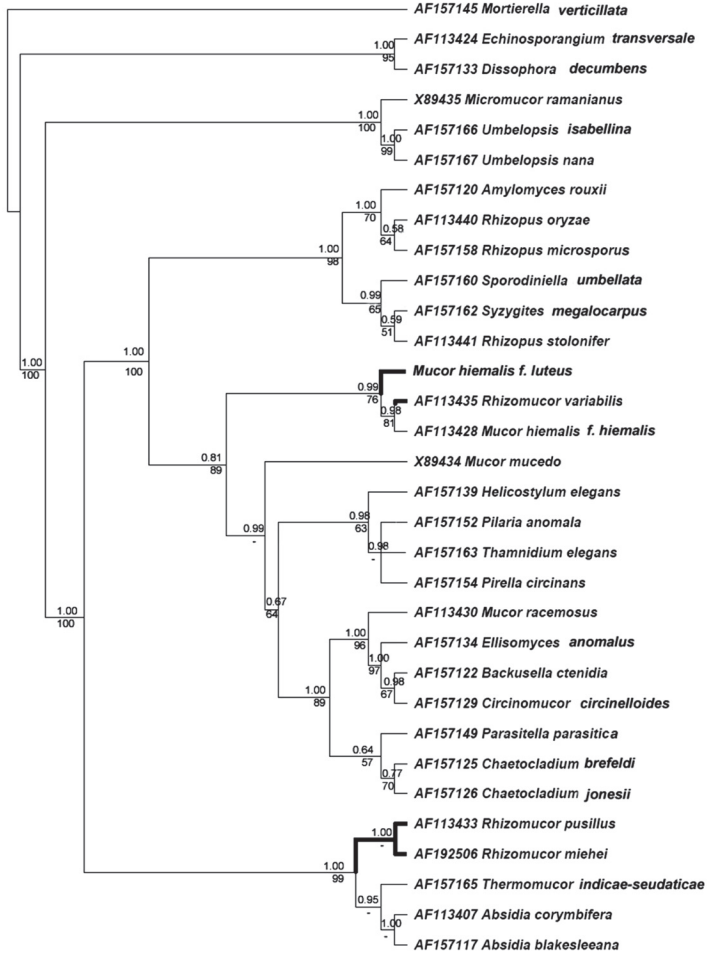

Fig. 1. Majority rule consensus tree based on Bayesian analysis of SSU rDNA data for *Mucorales*. Numbers above branches indicate Bayesian posterior probability values; numbers under branches indicate bootstrap values inferred by maximum parsimony analysis. Branches shown with black, bold lines indicate rhizoid-forming species.

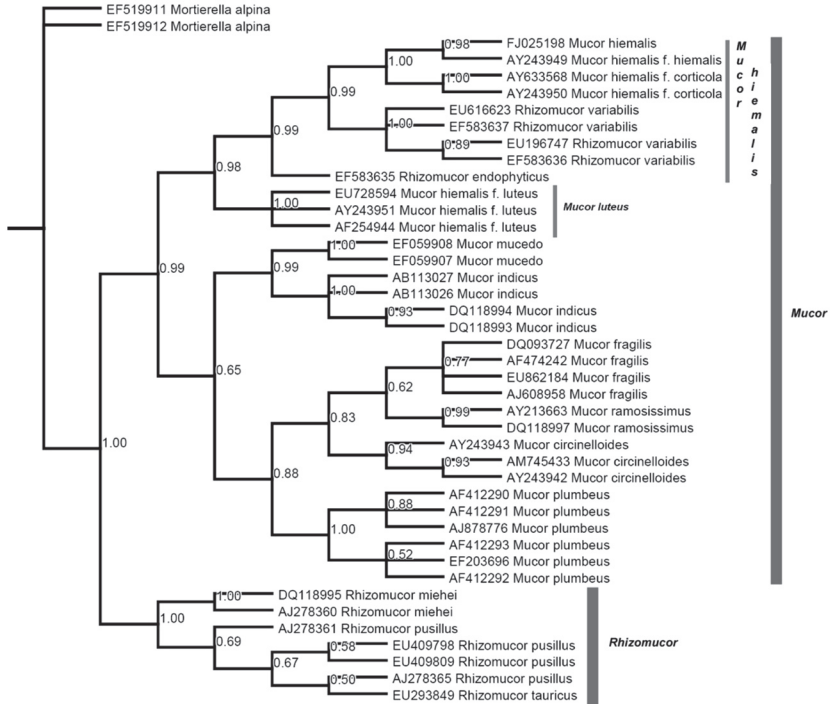

Fig. 2. Majority rule consensus tree based on Bayesian analysis of ITS1, 5.8S rDNA, ITS2 data for *Mucor* and *Rhizomucor* genus. Numbers at nodes indicate Bayesian posterior probability values.

The ITS sequence of CBS 124075 strain revealed the highest similarity to *Mucor hiemalis* f. *luteus* (AY243951; e value = 0.0; maximum identity = 99%). And the SSU sequence of this isolate revealed the highest similarity to *M. hiemalis* f. *hiemalis* (AF113428 and *Rhizomucor variabilis* AF113435; e value = 0.0; maximum identity = 98% both). It is the second record of this taxon in Poland (Kwaśna 1997).

Our results confirmed the polyphyly of the *Rhizomucor* genus (Voigt 1999, O'Donnell et al. 2001) and placing *Rhizomucor variabilis* among the different *Mucor hiemalis* formae (Fig. 1). Thus, we decided to see whether other rhizoid-forming strains could be found within the *M. hiemalis* clade. The ITS fragment analysis confirmed that two species described after critical revision of *Rhizomucor* genus (Schipper 1978b) are in fact located among representatives of *Mucor hiemalis*.

CBS strain 124075 is placed within the *M. hiemalis* f. *luteus* clade, outgroup to all other isolates within the *M. hiemalis* clade in SSU and ITS analyses. Taking into account the presence of a specific, well-defined signature sequence within the ITS2 L2, low ITS sequence similarity to other *M. hiemalis* representatives (less than 90%) and distinct morphological characters, this taxon should be treated as a separate species, *Mucor luteus.*

Taxonomic description

Mucor luteus Linnem. ex Wrzosek, **sp. nov.** Plate 1

MycoBank MB 515300

"*Mucor luteus*" Linnem., Flora 130: 195. 1936, nom. inval. (ICBN [Vienna] Art. 36.1).

"*Mucor hiemalis* f. *luteus*" Schipper, Stud. Mycol. 4: 33. 1973, nom. inval. (ICBN [Vienna] Art. 36.1).

"*Mucor luteus*" Linnem. ex K.Q. Pei, Mycosystema 19(1): 10. 2000, nom. inval. (ICBN [Vienna] Art. 37.1).

Coloniae in PDA ad temp 17°C lutae vel subalbae, reverso simile colorato; hyphis in substrata radicularibus, in hyphis sterylibus fasciculis minoris cum ramis singularis, sporangiophora (100–)500–2000(–3000) µm alta, erecta, (3–)5–11(–15) µm diam., symplicia, raro sympodice ramosa; sporangia globosa, lutea, (10–)30–50(–70) µm diam.; parietibus deliquescentibus, columellae globosae vel obovoideae, collaribus plerumque parvis sed distinctis; sporangiosporae hyalinae, ellipsoideae, variabiles in magnitudine, (3–)4–7(–13) × (0.5–)1–3(–5) µm. A species differret a ordinatione L2 ITS2 rDNA sequenti: GAGAAGTTCCACCTTGGTGGATTTCTT.

Type: mating type (-), Marburg, Germany, G. Linnemann, Centraalbureau voor Schimmelcultures **CBS 243.35** (**holotype:** lyophylised culture)

Signature sequence: ITS2 L2: 5′ GAGAAGTTCCACCTTGGTGGAT-TTCTT 3′

Etymology: from colony color

Colonies grow rapidly on PDA medium with an optimum growth temperature of 17°C. Colonies marguerite yellow (Ridgway 1912). Colony reverse is baryta yellow (Ridgway, 1912). Vegetative hyphae is nonseptate and (3–)5–11(–15) µm in diameter. Stolons and abundant variously shaped rhizoids may be present. Most rhizoids were found on vegetative hyphae or stolons, but they were also present on sporangiophores. Sporangiophores (100–)500–2000(–3000) µm in length, rarely singly sympodially branched. Sporangia globose, yellowish, (10–)30–50(–70) µm in diameter, transparent walls that leave a visible collar. Columellae regularly globose. Sporangiospores narrow ellipsoidal, smooth walled, colorless, relatively small and variable in dimensions (3–)4–7(–13) × (0.5–)1–3(–5) µm.

Specimens examined: – **Germany**, Hessen: Marburg, *G. Linnemann*, ex-holotype Malt Extract Agar culture CBS 243.35 – **Poland**, Podlaskie: Mikaszówka, Augustów Primeval Forest (53°53'18"N, 23°24'45"E; WGS84 system) from healthy gametophytes of *S. magellanicum*, 22 Oct 2008, *J. Budziszewska*, CBS 124075.

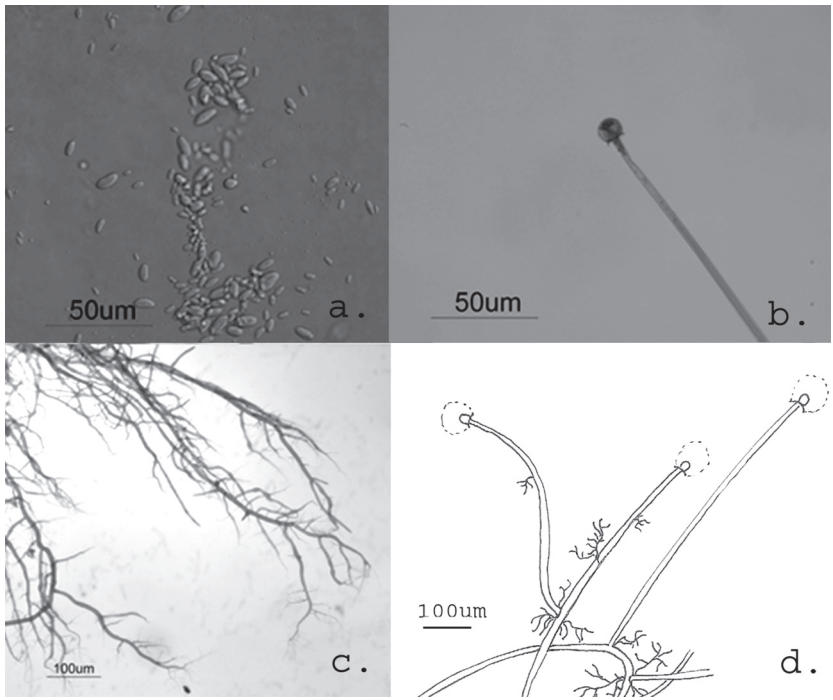

Plate 1. *Mucor luteus:*
a. sporangiospores; b. globose columellae; c. rhizoid-like structures; d. general aspect.

Discussion

Schipper (1973) reported that *Mucor hiemalis* f. *luteus* formed short horizontal sterile branches (spine-like) on the aerial hyphae. Thus the rhizoids observed in strain CBS 124075 (but also in *Rhizomucor endophyticus* and *R. variabilis*) could represent a kind of such excessively well-developed sterile branches. Moreover our observations on the designated type of *M. hiemalis* f. *luteus* (CBS 243.35) revealed that it also produced rhizoids. Our results indicate that the presence of these rhizoids is not a good character for delimiting *Rhizomucor* and *Mucor*, as rhizoids appear independently in these taxa and may be related with a pathogenic or endophytic life style. The *M. luteus* and *R. endophyticus* rhizoids may be well adapted to invade plants whereas those of *R. miehei* and *R. pusillus,* although similar morphologically, may be better adapted to colonize animals. Temperature preference, however, seems to be a good character for distinguishing between *Mucor* and *Rhizomucor.* The higher growth temperature optimum has been also shown to be a character allowing segregation of a new family *Mycocladiaceae* from the mesophilic family *Absidiaceae* (Hoffmann et al.

2007). Interestingly, the thermotolerant *R. pusillus* and *R. miehei* appear closely related to *Mycocladiaceae*.

After careful phylogenetic studies based on ITS and SSU rDNA data, we propose to validate the name *M. luteus* that was in use before the reexamination of the *M. hiemalis* group (Schipper 1973, Schipper 1978a). This taxon was originally included in *M. hiemalis* solely on the basis of mating experiments. However, it is worth noting that not all strains of *M. hiemalis* f. *luteus* formed zygospores with other strains of *M. hiemalis* (Schipper 1973). Although it had been shown that fungi in *Mucor* can form sterile zygospores (Gauger 1965), those capacities were not examined in studies by Schipper (1973). Moreover, all mucoralean fungi (as well as *Mortierellaceae*) form zygospores through interaction of trisporic acid cycle products. This phenomenon could be interspecific (Schimek et al. 2003). Morphological and molecular data confirm the legitimacy of delimiting this taxon as a separate species. However, one should note that *M. mucedo,* type species of the genus, does not form a monophyletic clade with the *M. hiemalis* group. Therefore, species within the *M. hiemalis* group ultimately should be transferred from *Mucor* to a separate genus. However, additional phylogenetic studies within *Mucor* are required in order to elucidate the relationship of the type, *M. mucedo*, to the *M. hiemalis* clade.

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