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Mucor nidicola sp. nov., a fungal species isolated from an invasive paper wasp nest

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A strain of a novel mucoralean fungus was isolated from a nest of the invasive paper wasp, *Polistes dominulus*. Phylogenetic analysis based on the internal transcribed spacer (ITS) regions and 5.8S rRNA gene sequences, along with physiological tests, revealed that this strain represents a novel species within the genus *Mucor*. The novel species also includes a representative that had previously been characterized as part of the *Mucor hiemalis* complex. Unlike the type strain of *M. hiemalis*, these two strains can grow at 37 °C and sporulate at 35 °C. Here, we present a partial resolution of the *M. hiemalis* species complex and propose the novel species *Mucor nidicola* sp. nov. to accommodate the isolate; the type strain of *M. nidicola* is F53^T (=NRRL 54520^T=UAMH 11442^T=CBS 130359^T).

Paper wasps are nearly globally distributed insects that create nests of macerated cellulose pulp, foraged by wasps from leaves, grass, cardboard and decomposing plant matter (Evans & West-Eberhard, 1970). Because of the ubiquity of fungal saprophytes in decomposing plant matter (reviewed by Ribes *et al.*, 2000), it is not surprising that multiple *Mucor* species have been isolated in the few mycobiota assessments conducted on paper wasp nest material (Jayaprakash & Ebenezer, 2010; Fouillaud & Morel, 1995). While ubiquitous in distribution, species of *Mucor* are valued particularly for their fast growth and novel metabolic pathways. Various *Mucor* species are used in applications such as bioremediation (Purnomo *et al.*, 2010; Srinivasan & Viraraghavan, 2010; Jabasingh & Pavithra, 2010), and the production of biofuels (Alam *et al.*, 2009), bioprotein (Jamal *et al.*, 2007), and pharmaceutical and industrial enzymes and chemicals (reviewed by Yazdi *et al.*, 2006). Some *Mucor* species are even used as a model for drug metabolism (Moussa *et al.*, 1997).

An assortment of fungal species was isolated from nests of the invasive paper wasp, *Polistes dominulus*, in Massachusetts, USA, as part of a biodiversity study. Initial sequence analysis of the ITS region, the standard phylogenetic marker used for Mucorales identification (Balajee *et al.*, 2009), and subsequent phylogenetic and phenotypic studies of these strains revealed the presence of a previously uncharacterized strain related to *Mucor hiemalis* and *Mucor irregularis*. Here, a novel species, *Mucor nidicola* sp. nov., is proposed to accommodate this strain.

Active nests of the invasive paper wasp *P. dominulus* were collected aseptically in Medford, MA, USA in August, 2008. Nest material was homogenized in a dilute PBS solution and maintained at 15 °C for 6 months prior to fungal isolation. Samples were plated on potato dextrose agar (PDA) (Difco). After incubating at approximately 24 °C for 72 h under diffuse light, morphologically distinct colonies were further purified on PDA.

DNA was extracted and purified directly from fungal colonies following the Fast DNA kit protocol (Bio 101), with a minor modification: the homogenization step was repeated three times with a FastPrep FP120 instrument (Thermo Savant). DNA was quantified by GeneQuant Pro (Amersham Pharmacia Biotech). The internal transcribed spacer (ITS) region of the nuclear rRNA gene was amplified with the primer pair ITS5 and ITS4 (White *et al.*, 1990). The PCR mix (25 µl) included 10 mM Tris/HCl (pH 8.3), 50 mM KCl and 2.5 mM $MgCl_2$ [10× Perkin-Elmer buffer II plus $MgCl_2$ solution (Roche Molecular Systems)], 100 µM each dNTP (Promega), 1 µM of each primer and 1.5 U Ampli*Taq* DNA polymerase (Roche). The amplification program included an initial denaturation at

Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the ITS1–5.8S rDNA–ITS2 sequences of the strains of *Mucor* species examined in this study are provided in Table S1.

Two supplementary tables are available with the online version of this paper.

94 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing for 1 min at 55 °C, and extension for 1 min at 72 °C. Subsequent products were purified with an Illustra GFX PCR DNA and gel band purification kit (General Electric Healthcare), and stored at −20 °C until they were used in sequencing. PCR products were sequenced by using the same primers used for amplification and following the *Taq* DyeDeoxy Terminator Cycle Sequencing kit protocol (Applied Biosystems). Reactions were run on a 310 DNA sequencer (Applied Biosystems). Consensus sequences were obtained using the Autoassembler program (Perkin Elmer/Applied Biosystems) and Seqman software (Lasergene package, DNASTAR).

CLUSTAL_X v.1.8 was used to align the sequences, followed by manual adjustments with a text editor (Microsoft Office Word 2003, version SP3). Once the sequences were aligned and adjusted, the similarities (%) were calculated manually as the difference in the number of nucleotides between sequences A and B divided by the number of total bases of these sequences × 100. For additional analysis of the genes, we used the software program MEGA 4.0. The maximum composite likelihood algorithm was used to determine the evolutionary distances between sequences. Phylogenetic trees were generated using the neighbour-joining method. Gaps were treated by the pairwise deletion option of MEGA. Support for internal branches was assessed by a search of 1000 bootstrap replications.

Isolates were subcultured on PDA (Pronadisa) and malt extract agar (MEA: 10 g malt extract, 20 g agar, 1000 ml distilled water), and incubated at room temperature (25 °C) for 2–5 days. Microscopic features were determined in mounts in lactic acid. Photomicrographs were taken using a Zeiss Axio Imager M1 light microscope. All isolates were characterized morphologically following traditional criteria (Schipper, 1973). Colour notations in parentheses are from Kornerup & Wanscher (1978). Growth rates of the isolates at different temperatures (4, 7, 15, 25, 30, 35, 37, 40, 42, 45 and 50 °C) were determined on 90 mm diameter PDA Petri dishes that had been inoculated in the centre. Colony diameters (in mm) were measured daily for up to 10 days.

Using methods outlined above, the length of the amplicon of the ITS region of the strain designated F53^T was determined to be 583 bp. A strain of the type species of the genus *Mucor*, *Mucor mucedo*, was used as a natural outgroup to root the phylogenetic tree (Fig. 1). In the resulting phylogram, isolate F53^T formed a clade with a strain of the unnamed species currently known as *M. hiemalis* f. *corticola*, with several strains of related *M. hiemalis* f. *hiemalis*, and with the type strains of *M. hiemalis* (var. *hiemalis*), *M. irregularis* (previously *Rhizomucor variabilis* var. *variabilis*), and *Mucor luteus*. However, isolate F53^T was genetically distinct from all of these strains; its ITS region nucleotide sequence differed by more than 2 % (Alvarez *et al.*, 2009), with the exception of strain *M. hiemalis* f. *hiemalis* CBS 638.67, with which it formed a terminal branch supported by a 100 % bootstrap value (Fig. 1). Strain CBS 638.67, isolated from a greenhouse soil sample from the Netherlands, had been previously reported by Schipper (1973) as '*Mucor* species 2'. Isolates F53^T and CBS 638.67 exhibited similar morphological and physiological features and were able to grow at 37 °C and sporulate at 35 °C. The subclade in which strains F53^T and CBS 638.67 were placed (bootstrap value of 73 %), includes two other branches. The first branch, in a basal position, includes only the type strain of *M. irregularis* (CBS 103.93^T), whereas the second is a sister branch of F53^T and CBS 638.67 (bootstrap lower than 70 %). This sister branch (bootstrap 100 %) includes strains CBS 975.68A, CBS 975.68B and CBS 976.68, all deposited in the CBS as *M. hiemalis* f. *hiemalis*. These three isolates were also designated '*Mucor* species 2' by Schipper (1973), but their ITS rRNA sequences, and those of strains F53^T and CBS 638.67 differed by 29 bp (6 %). Table S2 shows the similarities (%) between the ITS region nucleotide sequences of these strains. On the basis of our results, it is clear that *M. hiemalis sensu lato* represents a species complex.

The neotype strain of *M. hiemalis* f. *hiemalis* (CBS 201.65^{NT}) clustered with strain CBS 106.09, which was deposited as *M. hiemalis* f. *corticola* (Fig. 1). Strain CBS 412.71, representing *M. hiemalis* f. *silvaticus*, was placed outside of the *M. hiemalis* complex. Surprisingly, the type strain of *Mucor genevensis* (CBS 114.08^T), a species morphologically related to the *M. hiemalis* species group, was only distantly related to the species within this group (Fig. 1).

Isolate F53^T, described here as the type strain of *M. nidicola*, is morphologically similar to other species of the *M. hiemalis* complex. All produce yellowish to orange colonies and tall, mostly unbranched sporangiophores, rarely with a single branch or slightly branched sympodially, ending with a yellowish, brownish or brownish-black sporangium with mostly ellipsoidal-shaped sporangiospores (Fig. 2). However, *M. nidicola* grows and sporulates at 30 °C, whereas the type strain of *M. hiemalis* does not. Moreover, *M. nidicola* differs from *M. hiemalis* f. *corticola*, *M. luteus*, *M. hiemalis* f. *silvaticus* and *M. genevensis* in its ability to sporulate at 35 °C and to grow at 37 °C. This fact was previously noticed by Schipper (1973) in several strains of *M. hiemalis* f. *hiemalis* that were designated '*Mucor* species 2'. The maximum temperature of growth and sporulation are important taxonomic tools because they permit the separation of phylogenetically different, but morphologically similar mucoralean fungi, such as certain species belonging to the genera *Mucor* and *Rhizomucor* (Alvarez *et al.*, 2009).

Although the differences among the species within the *M. hiemalis* complex are not great, as they are all members of the same complex, *M. nidicola* can be morphologically and physiologically differentiated from the closely related species or varieties of the complex. *M. hiemalis* f. *corticola* produces cylindrical–ellipsoidal sporangiospores, which are narrow ellipsoidal to nearly fusiform in *M. luteus*, and cylindrical in *M. hiemalis* f. *silvaticus*, whereas they are mostly ellipsoidal,

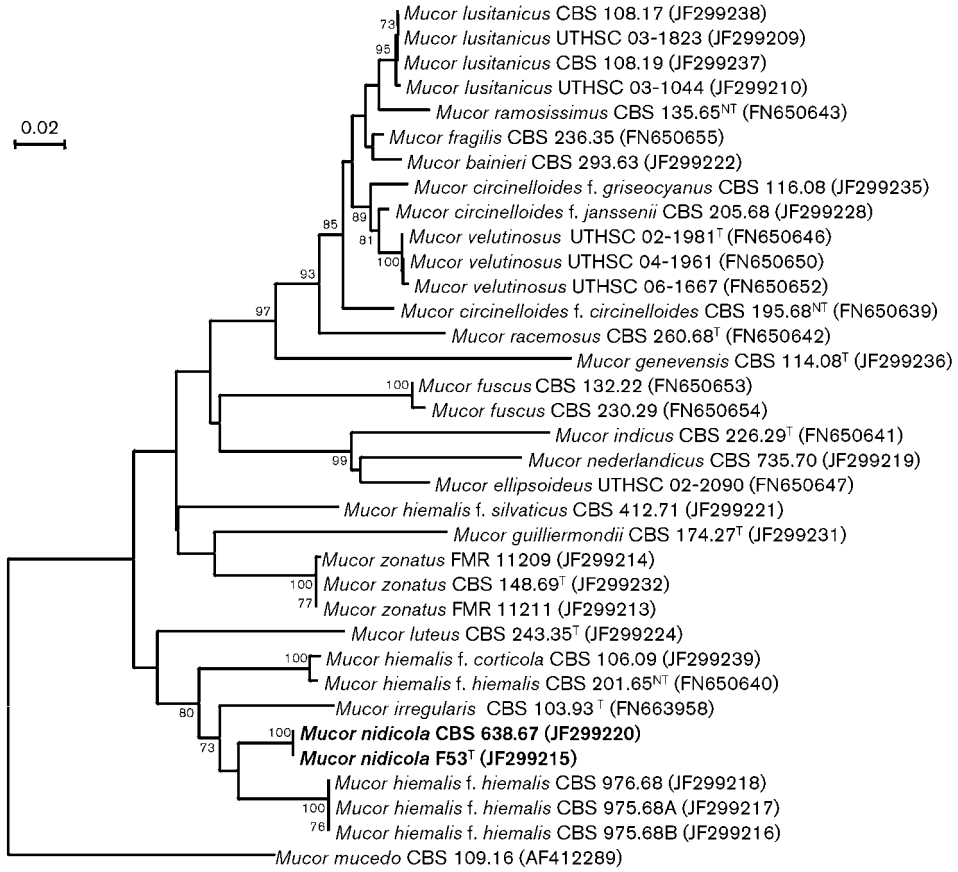

Fig. 1. Neighbour-joining tree based on maximum composite likelihood corrected nucleotide distances among the ribosomal internal transcribed spacer (ITS) regions and 5.8S rRNA gene sequences of the strains of the *Mucor* species studied. Bootstrap support values above 70 % are indicated at the nodes. Bar, genetic distance.

kidney-shaped, and irregular in *M. nidicola*. Moreover, *M. hiemalis* f. *silvaticus* produces blackish brown sporangia, which are much paler in all other *formae* of *M. hiemalis*. Morphologically, the closest taxon to *M. nidicola* is *M. hiemalis* f. *hiemalis*, although the sporangiospores of this species are regularly ellipsoidal with a flattened side, whereas in *M. nidicola* they are ellipsoidal, reniform and irregularly shaped. The most significant difference between these species is the ability of *M. nidicola* to grow and sporulate at higher temperatures, i.e. 37 °C and 35 °C, respectively; the corresponding temperatures for *M. hiemalis* f. *hiemalis* are 30 °C and 25 °C, respectively.

M. nidicola differs morphologically from *M. irregularis*, as the latter produces sporangiospores that are highly variable in shape and larger in size (2.5–16.5 × 2.0–7.0 μm). Moreover, *M. irregularis* produces rhizoids and profusely branched sporangiophores, whereas rhizoids are absent and the sporangiophores are mostly unbranched in *M. nidicola*.

M. nidicola and all the other species of the *M. hiemalis* complex differ from *M. genevensis* in that the latter species is homothallic, producing zygospores from colonies derived from single sporangiospores.

Based on phylogenetic and phenotypic assessments, the species *Mucor nidicola* is proposed. This species includes the type strain F53^T and the strain currently designated *M. hiemalis* f. *hiemalis* CBS 638.67. Sequence comparisons with those available in GenBank suggest that at least one strain isolated by Pan & May (2009) as an endophyte of corn (*Zea mays*) (accession no. FJ210517), represents an additional member of this species. The varied locations from which members of this species have been isolated, i.e. a paper wasp nest in Massachusetts, USA, as a corn endophyte in Minnesota, USA (Pan & May, 2009), and on a glass-walled herbarium in the Netherlands (Schipper 1973), suggest that, in keeping with many *Mucor* species, *M. nidicola* has a cosmopolitan distribution.

Latin diagnosis of *Mucor nidicola* Madden, Stchigel, Guarro, Sutton *et* Starks sp. nov.

Ad 25 °C *in agaro cum decocto malturorum* (MEA) *coloniae Petri-patellas in die quarto, luteola vel aurantio–grisea* (M. 5A4 to 5B4), *adversum aurantium vel auratio–brunneum. Mycelium* 5–10 mm *altum, primo hyalinum cito aurantium ex guttulis oleosis praesens. Sporangiophora erecta, simplicia*

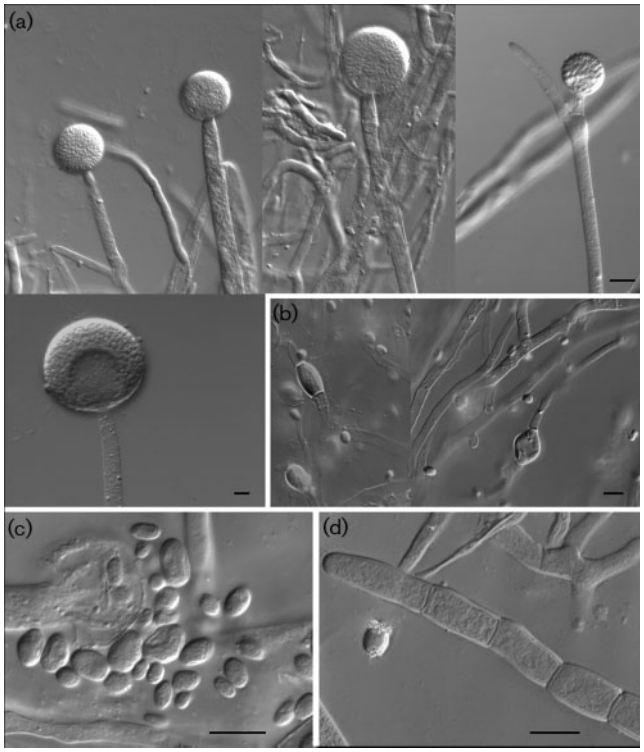

Fig. 2. *Mucor nidicola* F53^T. (a) Sporangiophores with sporangia (b and d) chlamydospores. (c) Sporangiospores. Bars, 20 μm.

vel 1–2 *ramosa*; *rami sporangiophoris* 500–2000 μm *longi*, 10–15 μm *lati*, *cum septum unicus ad basim*, *hyalini vel lutei*, *cum sporangio terminatibus nonapophysati. Sporangia globosa*, *cum parietibus subpersistentibus*, *lente tabidis vel disruptis*, *levitunicatis vel verrucosi*, *ad* 30–70 μm *in diam*, *luteola vel aurantio–brunnea. Columellae globosae vel subglobosae*, *collaria distincta*, 15−40 × 20−45 μm, *hyalina vel pallide brunnea. Sporangiosporae praecipue ellipsoideae*, *sed reniformis vel irregulares*, 3−10 × 2−6 μm, *pallide griseo–brunnea*, *levae et tenui vel crassitunicatae. Zygosporae ignotae.*

Typus: F53^T (=NRRL 54520^T=UAMH 11442^T=CBS 130359^T) *Holotypus conservatur in collectiones curturorum* USDA Agricultural Research Service (NRRL), University of Alberta Microfungus Collection and Herbarium (UAMH), *et* Centraalbureau voor Schimmelcultures (CBS).

Description of *Mucor nidicola* Madden, Stchigel, Guarro, Sutton & Starks sp. nov.

Mucor nidicola [ni.di′co.la. L. *nidus* nest; L. suff. *-cola* (from L. n. *incola*) inhabitant, dweller; N.L. n. *nidicola* nest-dweller, referring to the location from which the type strain of this species was isolated, a nest of the paper wasp, *Polistes dominulus*].

Colonies are cottony, filling a Petri dish after 4 days incubation at 25 °C on MEA, light orange to greyish-orange (M. 5A4 to 5B4); reverse is orange to brownish-orange (M. 5A6 to 5C5). Colonies are about 5−10 mm high, at first white, becoming yellowish-orange due to the presence of numerous cytoplasmic oil droplets. Hyphae are branched and non-septate when young, becoming septate with age, and 5−20 μm in diameter. Sporangiophores are erect, simple or 1–2 branched, arising directly from superficial and aerial hyphae; branches are 500–2000 μm long, 10–15 μm wide, one septate at base, colourless to yellowish, simple and terminating in a non-apophysate sporangium. Sporangia are multisporate, globose, wall slowly dissolving or broken, 30−70 μm in diameter, yellowish to brownish, smooth-walled to warty. Columellae are globose to subglobose, non-collapsing, 15–40 × 20–45 μm, hyaline to pale brown, collar evident. Sporangiospores are mostly ellipsoidal, but also kidney-shaped or irregular, 3–10 × 2–6 μm (mean=5.5 × 3.0 μm), pale greyish-brown, smooth- and thin- to thick-walled. Chlamydospores are abundant, terminal and intercalary, single or in chains up to 14 chlamydospores, hyaline, globose, barrel-shaped to cylindrical or irregular, 10–30 μm long, 5–15 μm wide, thick-walled, formed on vegetative hyphae. Zygospores unknown.

The optimal growth temperature is 25 °C, but it grows and sporulates well between 15 and 35 °C. At 35 °C, it also grows and sporulates (45–50 mm after 4 days), but produces sporangiophores with shorter branches and broadly ellipsoidal to subglobose sporangiospores. It displays poor growth at 37 °C and no growth at 7 or 40 °C.

Holotype: UAMH 11442^T, a dried culture isolated in February, 2009, from a *P. dominulus* nest in Medford, MA, USA. Ex-holotype culture, F53^T (=NRRL 54520^T=UAMH 11442^T=CBS 130359^T).

The MycoBank accession number for *M. nidicola* is MB 5619980.

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