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An efficient protocol for DNA extraction from *Meliolales* and the description of *Meliola centellae* sp. nov.

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Abstract — The first black mildew fungus associated with *Apiaceae* is collected from the state of Minas Gerais, Brazil. The new species is described, illustrated, and compared morphologically with species reported on hosts belonging to the related *Araliaceae*. Additionally, two efficient methods for extracting DNA from biotrophic fungi are described. Phylogenetic 28S rDNA sequence analyses confirm *Meliolales* as a distinct order within *Sordariomycetes*.

Key words — *Ascomycetes*, medicinal plant, *Meliolaceae*, tropical fungi, phylogeny

Introduction

Centella asiatica is a native plant of tropical Asia and commonly grows in humid areas in many tropical regions. This plant was introduced in Brazil and grows as a weed in the lawns of gardens in the southern and southeastern regions (Kissmann & Groth 1999). Brazilian researchers have paid relatively little attention to fungi associated with *C. asiatica*. Among the 20 fungal species described on this host worldwide, only three are reported from Brazil: *Capnodium brasiliense* Puttemans, *Septoria asiaticae* Speg., and *Vitalia setofasciculata* Bat. et al. (Farr & Rossman 2012, Mendes & Urben 2012).

During recent surveys of black mildew fungi in Brazil, several species were recorded on representatives of *Anacardiaceae*, *Asteraceae*, *Bignoniaceae*, *Burseraceae*, *Euphorbiaceae*, *Fabaceae* (*Caesalpinioideae* and *Papilionoideae*), *Piperaceae,* and *Sapindaceae* (Pinho et al. 2009, 2012; Macedo et al. 2010; Silva et al. 2012). The present work includes the description of a new species collected on *C. asiatica*.

Black mildew fungi infect plant species from numerous botanical families and are widely distributed in the tropics and subtropics (Hansford 1961, Hosagoudar 1996). As these fungi are strictly obligate biotrophs and must interact with living plant cells for growth and reproduction, they are usually host specific or have a very narrow host range (Hansford 1961; Hosagoudar 1996). Thus, attempts to grow species of *Meliolales* in pure culture have not been successful, making their DNA extraction challenging (Hansford 1961, Hosagoudar 1996, Vitoria et al. 2010). The development of an efficient protocol for DNA extraction from this fungal group would allow the taxonomic value of different morphological characters to be determined and might reveal discriminatory differences previously overlooked (Saenz & Taylor 1999, Lumbsch & Huhndorf 2007, Rodriguez & Piepenbring 2007, Vitoria et al. 2010).

In the present paper we provide a detailed account of the black mildew fungus found on *C. asiatica* and describe it as a new *Meliola* species based on its morphology and purported host specificity. In addition, we present two efficient DNA extraction methods from biotrophic fungi and the phylogenetic relationship of *Meliolales* based on 28S rDNA nucleotide sequences.

Materials & methods

Morphology

Centella asiatica plants were found covered with black colonies on both sides of the leaves during September 2009 in the "Mata da Dendrologia", a fragment of secondary tropical seasonal semi-deciduous montane forest, a component of the Brazilian Atlantic forest in the campus of the Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil. Symptomatic leaves were collected, photographed, and dried in a plant press. Fresh samples examined under an Olympus SZ40 stereomicroscope were promptly recognized as a meliolaceous fungus. Representative structures were either scraped with a scalpel or removed with an adhesive tape and mounted in lactophenol. Slides containing the fungal structures were examined and photographed under an Olympus BX 51 light microscope equipped with an Olympus e-volt 330 digital camera. Illustrations were prepared with a drawing tube and finalized with the method described by Barber & Keane (2007). For scanning electron microscopy, air-dried material was directly mounted and coated by a thin layer of gold in a sputter coater (Balzers® model FDU 010) for 2 min. Photographs were made with a Carl-Zeiss Model LEO VP 1430 scanning electron microscope (SEM). Biometric data was based on 30 measurements of structures. A representative specimen was deposited in the local herbarium at the Universidade Federal de Viçosa (Herbarium VIC).

DNA extraction

To obtain a representative fungal DNA, ca. 50 fertile perithecia of *Asteridiella obesa* (Speg.) Hansf., *Irenopsis heveae* Hansf., *Meliola centellae,* and *M. vernaliae* D.B.Pinho & O.L.Pereira were examined under a stereomicroscope to check for possible contamination by other fungi. They were removed with the aid of a fine glass needle and placed into

a microcentrifuge tube (1.5 ml) containing 5 µl of double distilled water and stored at –20°C for later use. Total genomic DNA was extracted by using Wizard® Genomic DNA Purification Kit (Promega Corporation, WI, U.S.A.) with some adjustments as follows:

Fungal samples were processed by freezing the sample with liquid nitrogen and grinding it into a fine powder using a microcentrifuge tube pestle. The crushing continued after adding 100 µl Nuclei Lysis Solution + 100 mg polyvinylpyrrolidone. After 500 µl of the same solution was added, the sample mixture was vortexed at high speed for 3–5 seconds to wet the tissue. The samples were incubated at 65°C for 15 minutes. After the solution was cooled at –20°C for 5 min, 200 µl Protein Precipitation Solution was added, mixed in a vortex at high speed for 20 seconds, and centrifuged for 10 min at 14000 rpm. The supernatant (about 600 µl) containing the DNA was transferred to a clean 1.5 mL microcentrifuge tube containing 600 µl of cold isopropanol. The solution was mixed by inversion until thread-like DNA strands formed a visible mass and then stored at –20°C for 24 hours. The next day the solution was centrifuged at 14000 rpm for 5 min, and the supernatant was discarded. 600 µl cold ethanol was added to the pellet and centrifuged at 14000 rpm for 5 min. This ethanol step was repeated again to wash the DNA. After the ethanol was removed, the tube was put upside down over clean towel paper and the pellet was air dried for 30 min. As a final step, the pellet was re-suspended in 20 µl DNA Rehydration Solution and 3 µl RNase Solution was added and mixed by inverting the tube, which was incubated at 37°C for 1 hour. The sample was then stored at –20°C for later use.

Direct amplification

For direct amplification of the 28S rDNA, ca. 20 clean (free of mycoparasites) fertile perithecia were removed using a fine glass needle and placed into a 0.2 ml microcentrifuge tube containing 3 µl double distilled water (Jaklitsch & Voglmayr 2012). After soaking in the water, the samples were squashed with a sterile forceps to release their contents and mixed in a vortex at high speed for 1 min. We also tested the protocol from Vitoria et al. (2010), replacing 3 µl double distilled water by 3 µl Cell Lysis Buffer (0.05M NaOH, 0.25% [w/v] SDS). The samples were frozen for 15 min at –20°C and thawed in a water bath at 80°C for 15 min, modifying the protocol in Griffin et al. (2002). This process was repeated three times using crushed dry ice and thawing in a water bath. When the solution cooled, the ingredients for PCR reactions were added.

PCR amplification and DNA sequencing

For each 25 µl PCR reaction we used 12.5 µl Dream Taq TM PCR Master Mix 2X (MBI Fermentas, Vilnius, Lithuania), 1 µl each of 10 µM forward and reverse primers (Invitrogen, Carlsbad, EUA), 1 µl dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, U.S.A.), 5 µl 100× (10 mg/mL) Bovine Serum Albumin (BSA, Sigma-Aldrich, St. Louis, MO, U.S.A.), 2 µl genomic DNA, and nuclease-free water to bring the total volume to 25 µl.

The primers LR0R (5'-ACCCGCTGAACTTAAGC-3') and LR5 (5'-TCCTGAGGGAAACTTCG-3') and ITS1 (5'-TCCGTAGGTGAACCTGCGG–3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify the partial 28S rDNA and ITS, respectively (Vilgalys & Hester 1990, White et al. 1990). Amplifications began with an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 53°C for

Table 1. Genbank accession numbers of 28S rDNA sequences derived from strains used in the phylogenetic analysis.

Species	Voucher	28S rDNA
Aphysiostroma stercorarium	ATCC 62321	AF543792
Apiospora bambusae	ICMP 6889	DQ368630
A. setosa	ICMP 4207	DQ368631
Appendiculella lozanellae	MP3432	DQ508302
Asteridiella sp. 1	PPMP 796	EF094839
Asteridiella obesa	VIC31239	JX096809
Balansia henningsiana	AEG 96-27A	AY545727
Camarops petersii	JM1655	AY346265
C. tubulina	SMH4614	AY346266
C. ustulinoides	SMH1988	AY346267
Ceratocystiopsis manitobensis	CW13792	DQ294358
C. minima	CMW162	DQ294361
Ceratocystis fimbriata	CBS 374.83	AF221009
Chaetosphaeria ovoidea	SMH 2605	AF064641
Claviceps paspali	CBS 110.22	U47826
Coniochaeta leucoplaca	JONG 54	FJ167399
C. ostrea	AFTOL-ID 915	DQ470959
C. velutina	UAMH 10912	EU999180
Coniochaetidium savoryi	TRTC 51980	AY346276
Cornipulvina ellipsoides	—	DQ231441
Cryphonectria parasitica	CMW 13749	AF277132
Diaporthe phaseolorum	CBS 435.87	U47830
Diatrype disciformis	CBS 197.49	U47829
Discostroma botan	HHUF 4642	DQ368629
Endomeliola dingleyae	PDD 98304	GU138866
Faurelina elongata	CBS 126.78	DQ368625
Gelasinospora tetrasperma	ATCC 96230	AY346281
Gnomonia ribicola	CBS 115443	DQ368626
Grosmannia grandifoliae	CMW703	DQ294399
G. penicillata	CMW470	DQ294385
Halosarpheia fibrosa	JK5132C	U46886
Halosphaeria appendiculata	CBS 197.60	U46885
Hypocrea pallida	GJS89-83	U00740
H. schweinitzii	CBS 243.63	U47833
Hypomyces polyporinus	CBS 168.89	AF543793
Irenopsis sp.	VIC31752	JX096807
Melanochaeta hemipsila	SMH 2125	AY346292
Meliola centellae	VIC31244	JQ734545
M. variaseta	DRJ 54	EF094840
M. vernaliae	VIC31240	JX096808
Nectria cinnabarina	IAM 14568	AF193237
Neurospora crassa	CBS 709.71	AY681158
Ophiostoma lunatum	CMW10564	DQ294355
O. nigrocarpum	CMW651	DQ294356
O. piliferum	CBS129.32	AY281094

Table 1, concluded

Species	Voucher	28S rDNA
O. ulmi	CBS 298.87	DQ368627
Plagiostoma euphorbiae	CBS 340.78	AF277131
Poroconiochaeta discoidea	SANK 12878	AY346297
Pyxidiophora arvernensis	AFTOL-ID 2197	FJ176894
Seiridium cardinale	ATCC52521	AF382377
Sordaria fimicola	CBS 723.96	AF132330
S. macrospora	ATCC 36709	AY346301
Valetoniellopsis laxa	CBS 191.97	AY015635
Valsa ceratosperma	AR3426	AF408387
Valsonectria pulchella	SMH1193	AY346304
Xylaria acuta	AFTOL-ID 63	AY544676
X. hypoxylon	CBS 499.80	U47841

45s, extension at 72°C for 2 min and a final extension of 7 min at 72°C. PCR products were analyzed on 2% agarose electrophoresis gels stained with GelRed™ (Biotium Inc., Hayward, CA, U.S.A.) in a 1× TAE buffer and visualized under UV light to check for amplification size and purity. PCR products were purified and sequenced by Macrogen Inc., South Korea (http://www.macrogen.com). The nucleotide sequences were edited with the DNA Dragon software (Hepperle 2011). All sequences were checked manually and nucleotides with ambiguous positions were clarified using primer sequences in both directions. Obtained sequences were deposited in GenBank (www.ncbi.nlm.nih.gov).

Phylogenetic analysis

For phylogenetic analysis, 28S rDNA sequences from additional species were retrieved from GenBank (Table 1). Consensus regions were compared against GenBank's database using Mega BLAST program. The closest hit sequences and representatives of selected *Sordariomycetes* (*Boliniales*, *Chaetosphaeriales*, *Coniochaetales*, *Diaporthales*, *Hypocreales*, *Meliolales*, *Microascales*, *Ophiostomatales*, *Sordariales*, and *Xylariales*) were obtained from Genbank (www.ncbi.nlm.gov) to help clarify the phylogenetic relationship of *Meliolales* within the class. All sequences were downloaded in FASTA format and aligned using the multiple sequence alignment program MUSCLE® (Edgar 2004) built in MEGA v. 5 software (Tamura et al. 2011). Alignments were checked and necessary manual adjustments were made. All ambiguously aligned regions within dataset were excluded from the analysis. Gaps (insertions/deletions) were treated as missing data. The resulting alignment was deposited in TreeBASE (http://www.treebase.org/) (accession number S12768).

Bayesian inference (BI) analysis employing a Markov Chain Monte Carlo method (MCMC) was performed. Before launching BI, the best nucleotide substitution models were determined with MrModeltest 2.3 (Posada & Buckley 2004). After calculation of likelihood scores, models were selected according to the Akaike Information Criterion (AIC). The general time-reversible evolution model (Rodriguez et al. 1990) was used, including estimation of invariable sites and assuming a discrete gamma distribution with six rate categories (GTR+I+G). The alignment was phylogenetically analyzed on CIPRES webportal (Miller et al. 2010) using MrBayes v.3.1.1 (Ronquist & Huelsenbeck

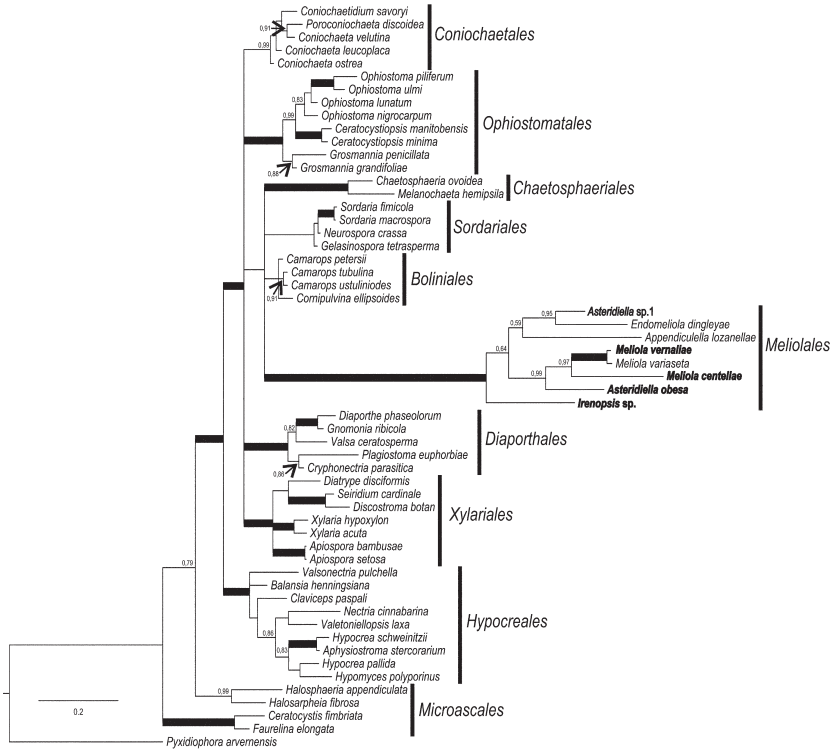


Fig. 1. Phylogenetic tree inferred from Bayesian Analysis of 28S rDNA *Sordariomycetes* sequences. Bayesian posterior probability of >70% are indicated above the nodes and bold lines indicate posterior probabilities of 1.00. The species in study are in bold. The tree is rooted with *Pyxidiophora arvernensis*.

2003). Four MCMC chains were run simultaneously, starting from random trees for 10,000,000 generations. Trees were sampled every 1000th generation for a total of 10,000 trees. The first 2500 trees were discarded as the burn-in phase of each analysis. Posterior probabilities (Rannala & Yang 1996) were determined from a majority-rule consensus tree generated with the remaining 7500 trees. Convergence of the log likelihoods was analyzed with TRACER v. 1.4.1 (Rambaut & Drummond 2003); no lack of convergence was detected. Trees were visualized in FigTree (Rambaut 2009) and exported to graphics programs. *Pyxidiophora arvernensis* (Breton & Faurel) N. Lundq. was used as outgroup in these analyses.

Results

Meliola centellae Pinho & O.L. Pereira, **sp. nov.** Figs 2–3

Mycobank MB519037

Differs from *Meliola pectinata* by its smaller ascospores and longer mycelial setae.

Type: Brazil, Minas Gerais, Mata da Dendrologia, on living leaves of *Centella asiatica* (L.) Urb. (*Apiaceae*), 16 September 2009, D.B. Pinho (**Holotype**, VIC 31244).

Etymology: The epithet refers to the host genus, *Centella*.

Beeli formula 3112.3232. Colonies black, minute, amphigenous, mostly epiphyllous, initially scattered but becoming confluent with age, dense, 1–3 mm diameter. Hyphae dark brown, septate, straight to sub-straight, branching usually opposite at acute angles, bearing appressoria and conidiogenous cells. Hyphal cells 11–21 × 5–9 µm. Appressoria opposite or alternate, antrorse, straight to curved; stalk cells dark brown, cylindrical to cuneate, 2.5–5 × 5–7.5 µm, head

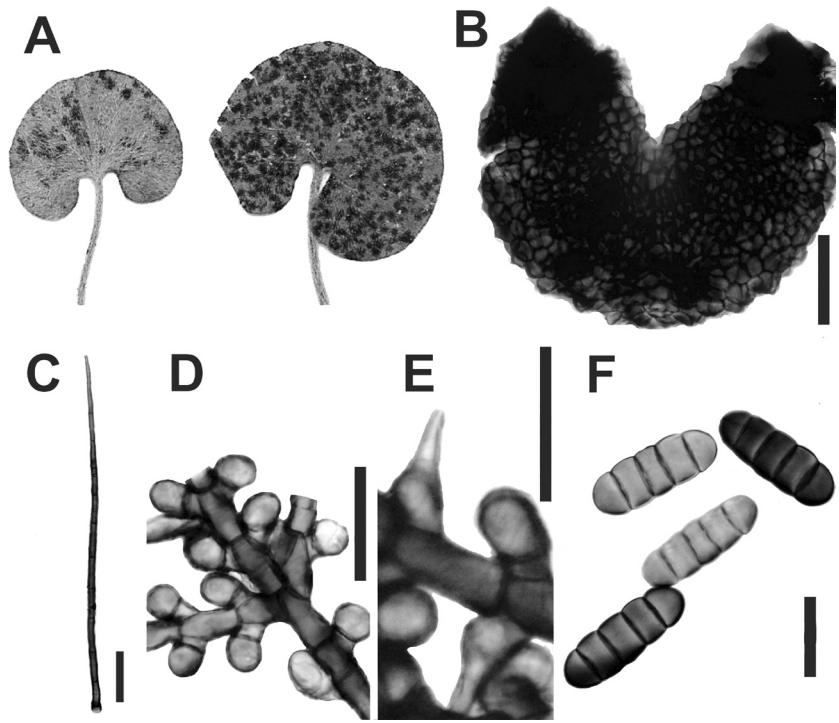


Fig 2. *Meliola centellae* on *Centella asiatica*. A. General view of abaxial (left) and adaxial (right) colonies on leaf surface. B. Perithecium. C. Mycelial setae. D. Appressoria. E. Mixed appressoria and phialides. F. Ascospores. Bars: B, C = 50 µm; D–F = 25 µm.

cells dark brown, globose to ovate, slightly attenuated at the apex, rounded, entire, 7.5–12.5 × 5–10 µm. Phialides light brown, mixed with appressoria, opposite, ampulliform, 12.5–25 × 6–9 µm. Mycelial setae dark brown, simple, straight, apex acute, 5–12 septate, 218–430 × 7.5–10 µm. Perithecia brown, globose, scattered, black, 152–247 µm diameter. Asci evanescent. Ascospores hyaline inside the ascus, becoming grey or brown with age, dark brown or grey at maturity, cylindrical to ellipsoid, rounded at the tips, 4-septate, constricted at the septa, 32–39.5 × 9.5–12.5 µm.

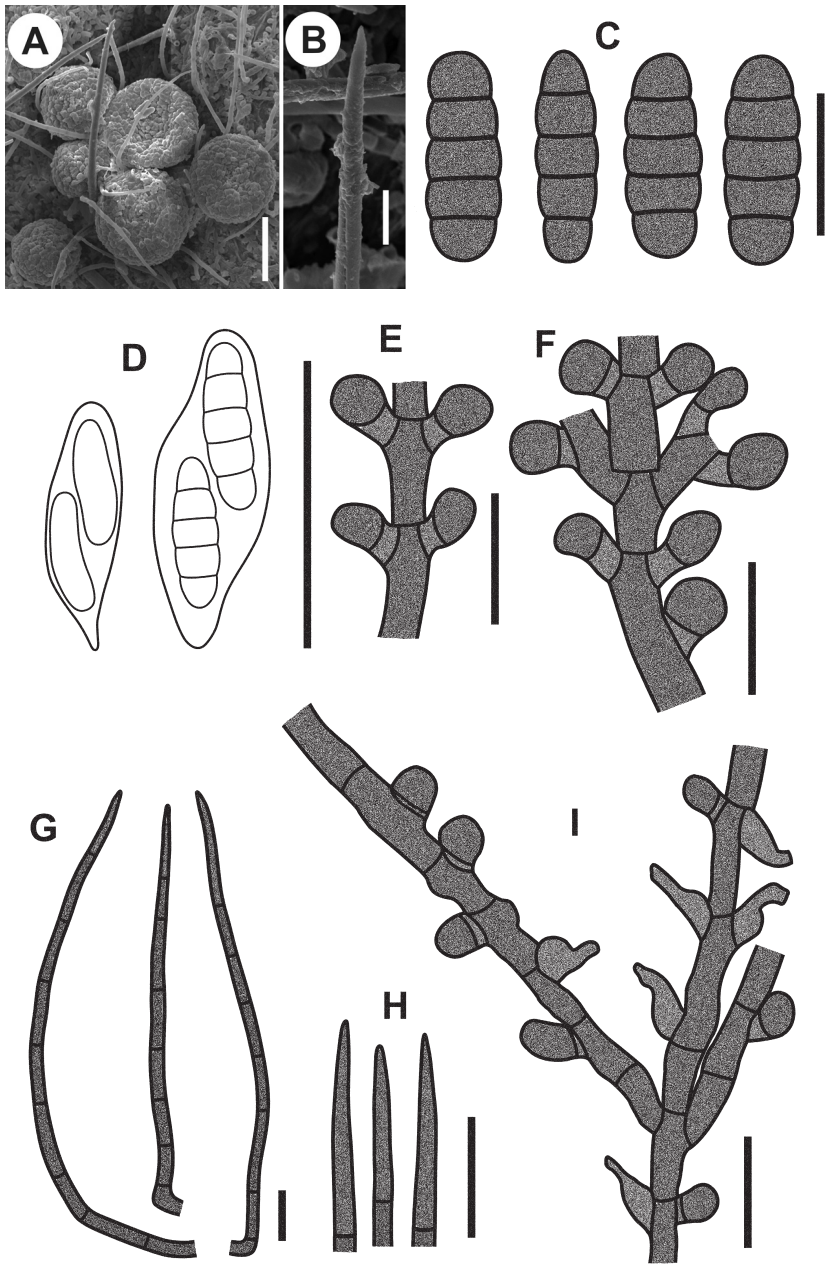
Molecular analyses: The PCR-amplified fragments were obtained by direct amplification and extraction with Wizard® Genomic DNA Purification. Both protocols were found suitable for DNA extraction of meliolaceous fungi. On the other hand, the protocol from Vitoria et al. (2010) was not appropriate for *Meliola* spp. The amplification of the partial 28S rDNA revealed sequences of ca. 700 bp. Although the ITS sequences were not used in phylogenetic analysis, they were lodged in GenBank (Accession No. KC252606–KC252608) for future studies and identification purposes. The manually adjusted alignment contained 57 strains (including the new species described here). Of the 467 characters used in the phylogenetic analysis, 209 were parsimony-informative, 252 were variable, and 207 were conserved. The Bayesian Inference tree confirms *Meliolales* as a distinct order within *Sordariomycetes*.

Discussion

Centella asiatica belongs to *Apiaceae* subfam. *Mackinlayoideae*, which is somewhat intermediate between *Apiaceae* sensu lato and *Araliaceae* (Plunkett et al. 2004, Oskolski & Wyk 2010). Because no other black mildew fungus has been reported on hosts in the *Apiaceae*, we compared the *C. asiatica* fungus with *Meliola* spp. occurring on taxa in *Araliaceae*.

Of the 19 species and four infraspecific taxa of *Meliola* known on *Araliaceae* (Hansford 1961, Hosagoudar et al. 1995, Hosagoudar 1996, Hosagoudar & Archana 2009), none matched *M. centellae*. Six taxa (*M. acanthopanacis* W. Yamam., *M. araliicola* W. Yamam., *M. didymopanacis* var. *stevensii* Hansf., *M. schefflerae* Hansf., *M. payakii* Hosag., and *M. pectinata* Höhn.) have straight simple mycelial setae (like *M. centellae*) but differ in several other traits (Hansford 1961, Hosagoudar 1996). *Meliola pectinata* matches the first four characters of the *M. centellae* Beeli formula but has larger ascospores and shorter mycelial setae. *Meliola acanthopanacis*, *M. araliicola*, *M. didymopanacis*

Fig 3. *Meliola centellae* on *Centella asiatica*. A. SEM photographs of the perithecia and mycelial setae (B). C. Ascospores. D. Asci with two immature ascospores. E, F. Superficial hyphae with two-celled oppositely and alternately positioned lateral appressoria. G. Mycelial setae. H. Detail of mycelial setae with acute apex. I. Superficial hyphae with phialides and appressoria. Bars: A= 100 µm; B= 20 µm; C–I = 30 µm.


A
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var. *stevensii*, and *M. payakii* also have larger ascospores than *M. centellae*. *Meliola centellae* is also very similar to *M. schefflerae* but also produces larger ascospores (45–51 × 17–19 µm; Hansford 1961, Hosagoudar 1996). In addition, the similar *M. heteroseta* Höhn. and *M. dichotoma* var. *kusanoi* (Henn.) Hansf. are distinguished from the new species by larger ascospores and dichotomously branched mycelial setae (Hansford 1961). We therefore propose *Meliola centellae* for the first *Meliola* species (and the first *Meliolaceae*) recorded on a member of the *Apiaceae*.

Although mycologists in several laboratories tried to isolate DNA from meliolalean fungi, only a few attempts have been successful (Rodriguez & Piepenbring 2007). The *Meliolales* are a non-culturable group frequently contaminated by numerous other fungi. It is thought that the dark pigment of their thick cell walls might also interfere with DNA isolation (Rodriguez & Piepenbring 2007). Although the Vitoria et al. (2010) method has been reported as suitable for extracting DNA from ascomata, we found it inappropriate for *Meliola* spp. Here we successfully extracted DNA from *Meliolales* through direct amplification and extraction with Wizard® Genomic DNA Purification kit. Successful direct amplification of fungal DNA have been published previously for *Xylariales* and *Meliolales* (Saenz & Taylor 1999, Rodriguez & Piepenbring 2007, Jaklitsch & Voglmayr 2012), but adding the freezing/thawing steps probably improved the process. This is commonly used for most species that possess rigid cell walls and resist lysis techniques by compromising the integrity of the cell wall (Griffin et al. 2002). The extraction with Wizard® Genomic DNA Purification kit is described herein as the first successful protocol for isolating and purifying DNA from *Meliolales*. This procedure allows amplification of multiple gene regions.

Of the several papers revising the systematics of the *Sordariomycetes* (Spatafora et al. 2006, Zhang et al. 2006, Lumbsch & Huhndorf 2007, Tang et al. 2007, Schoch et al. 2009), only Saenz & Taylor (1999) included members of the *Meliolales*. Their phylogeny grouped *Meliolales* in a distinct clade close to *Sordariales* and thus showed that phylogenetic analysis of molecular characters confirmed previous morphologically based classifications placing *Meliolales* in the *Sordariomycetes*. The most widely used gene sequences for phylogenetic studies have been 28S rDNA genes because of their easy amplification due their high copy number and the availability of numerous universal primers (Miller & Huhndorf 2005, Tang et al. 2007). The present paper confirms that *Meliolales* comprises a monophyletic order within *Sordariomycetes* (Fig. 1). Although the presence of dark perithecia and inamyloid asci indicate that *Meliolales* belong to the subclass *Sordariomycetidae* (Zhang et al. 2006), the phylogenetic position of the order is still uncertain because the phylogeny inferred from the 28S rDNA datasets are the least supported (Tang et al. 2007). Miller & Huhndorf

(2005) propose that increased taxon sampling will improve resolution in many clades and that phylogenetic support will increase through the incorporation of additional genes. This clearly indicates the need for extensive taxon sampling and further investigation of DNA sequences in order to better clarify the phylogeny of the *Meliolales*.

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