The Journal of Microbiology, October 2008, p. 513-518 Vol. 46, No. 5
DOI 10.1007/s12275-007-0115-6

Taxonomic Revision of the Nematode-Trapping Fungus *Arthrobotrys multisecundaria*

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(Received July 11, 2007 / Accepted August 5, 2008)

The gene encoding an extracellular serine protease was cloned from *Arthrobotrys multisecundaria* using degenerate primers. The gene was highly similar (99.26%) to protease Mlx from *Monacrosporium microscaphoides*. To clarify the taxonomic relationship between these species, genes encoding the internal transcribed spacer (ITS) and β-tubulin were also cloned and sequenced from *A. multisecundaria* and *M. microscaphoides*, respectively. Homologous analysis of the nuclear (ITS) and protein (β-tubulin) encoding genes showed that the two species of nematode-trapping fungi also shared extensive identity (99.82 and 99.63%, respectively), although they exhibited obvious differences in secondary conidia morphology. Accordingly, a taxonomic revision is recommended, with *A. multisecundaria* being revised as *A. microscaphoides* var. *multisecundaria*. In addition, the identified mutation may better facilitate the study of the sporulation of nematode-trapping fungi.

***Keywords*:** *Arthrobotrys multisecundaria*, *Monacrosporium microscaphoides*, serine protease, ITS, β-tubulin, taxonomy

Nematode-trapping fungi have been studied for decades. Traditional taxonomic identification of nematode-trapping fungi is based on morphological characteristics of conidia and conidiophores including growth-related morphology, conidial size and shape, and trapping structures (Cooke and Dickinson, 1965). While useful, the development of molecular technology has indicated that morphology-based taxonomic identification is limited. DNA-based molecular taxonomical techniques can aid in the detection and identification of nematode-trapping fungi.

Monacrosporium microscaphoides (designated YMF1.00028) is a familiar species of nematode-trapping fungi, which captures nematodes by means of an adhesive three-dimensional network. The extracellular serine protease has been purified and the gene has been cloned (Wang *et al*., 2006). *Arthrobotrys multisecundaria* (YMF1.01821) was reported as a new nematode-trapping fungus that differs from other related species in the secondary conidia, having a catenulate arrangement of four conidia (Hu *et al*., 2005).

Presently, we cloned and sequenced an extracellular serine protease gene from the nematode-trapping fungus *A. multisecundaria*. The gene shares a high degree of similarity (99.26%) to the reported serine protease Mlx from *M. microscaphoides*. Analysis of the internal transcribed spacer (ITS) and β-tubulin genes indicated the two species also share extensive similarities (more than 99%). Therefore, we propose that *A. multisecundaria* should be revised as a spontaneous mutant of *M. microscaphoides*.

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Materials and Methods

Microorganisms and morphological identification

The nematode-trapping fungi *A. multisecundaria* (YMF 1.01821) and *M. microscaphoides* (YMF1.00028) were originally isolated from field soil samples in Yunnan Province in China were deposited in the Yunnan Microbiological Fermentation Culture Collection Center (Hu *et al*., 2005). The two species were maintained on Cornmeal agar at 28°C for 1 week to observe the morphological characters of their conidia and conidiophores. The free-living nematode *Panagrellus redivivus* was added to induce the development of trapping devices. *P. redivivus* was maintained as described previously (Luo *et al*., 2004). *Escherichia coli* DH5α used in all DNA manipulations was grown in Luria-Bertani medium at 37°C as previously described (Yang *et al*., 2005a).

Cloning of protease, ITS, and β-tubulin genes

A. multisecundaria and *M. microscaphoides* were grown in PL-4 medium (Yang *et al*., 2005b) at 28°C using a rotary shaker operating at 150 rpm for 6 days. The generated mycelia were recovered by filtration using a nylon mesh and genomic DNA was isolated using an E.ZN.A.® fungal DNA Mini Kit (Omega Bio-Tek, USA) according to the manufacturer's instructions.

A pair of degenerate primers (NP & NR) (Table 1) was designed according to the conservative sequences of the serine proteases PII, Aoz1, and Mlx from the nematode-trapping fungi *Arthrobotrys oligospora* and *M. microscaphoides* (GenBank accession no. X94121, AF516146, and

Table 1. Primers used in this study

Primer	Primer DNA sequence (5'→3')	Annealing tempterature (°C)	Target gene
NP	AATG(A/C)T(G/T)(A/T)(C/T)GAACGGCCT(C/T)A	51	ESP[a]
NR	TTAAGC(G/A)(G/T)(A/T/C)(G/T)CC(G/A)TTGTAG		
ITS4	TCCTCCGCTTATTGATATGC	55	ITS
ITS5	GGAAGTAAAAGTCGTAACAAGG		
Bt1a	TTCCCCCGTCTCCACTTCTTCATG	58	β-Tubulin
Bt1b	GACGAGATCGTTCATGTTGAACTC		

[a] ESP=extracellular serine protease

AY841167, respectively) to amplify the serine protease encoding gene of *A. multisecundaria*. The polymerase chain reaction (PCR) reaction mixture consisted of 0.5 μl of 1.5 units of *Taq* DNA polymerase, 5 μl of reaction mixture buffer, 3 μl of 25 mM $MgCl_2$, 1 μl of 2.5 mM dNTPs, 1 μl of 100 mM degenerate primer and 3 μl of DNA template, which was prepared to a final volume of 50 μl with double-distilled sterile water. Amplification was accomplished at 95°C for 5 min, followed by 35 cycles with 95°C for 40 sec, 51°C for 40 sec, and 72°C for 1.5 min. After cycling, the reaction mixture was maintained at 72°C for 10 min.

The primer pair Bt1a and Bt1b (Table 1) was used to

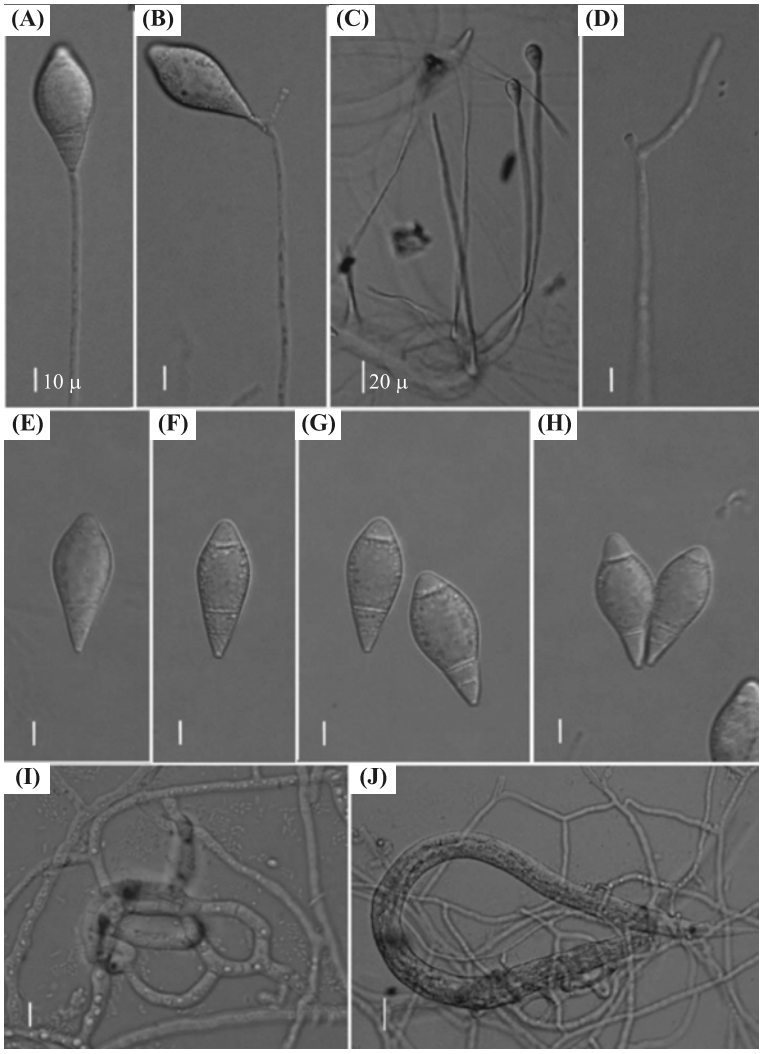

Fig. 1. Appearance of *Monacrosporium microscaphoides* (YMF1.00028). Similar to a previous description (Wang *et al*., 2006), visual examination revealed (A~D) conidiophores, (E~H) conidia with two or three septa, (I) three-dimension networks (traps) and (J) nematode trapped by the traps. Scale bars: Fig. A, B, and D~I=10 μm; C=20 μm; J=50 μm

amplify the β-tubulin gene (Li *et al*., 2005), and the primer pair ITS4 and ITS5 (Table 1) was used to amplify the complete ITS (White *et al*., 1990) of *A. multisecundaria* and *M. microscaphoides*, respectively. PCR conditions used have been previously described (Li *et al*., 2005).

Sequencing and analysis

The PCR products were electrophoresed on 1% agarose gels to check for size and purity. The 100 bp DNA ladder (Sangon, China) was used as a size marker. All PCR products were purified using a DNA Fragment Purification Kit (version 2.0; TaKaRa-Bio, Japan). ITS fragment PCR products were directly sequenced using the ITS4 and ITS5 primers. PCR products of serine protease and β-tubulin were subcloned into vector pMD18-T (TaKaRa-Bio) and several positive clones were selected randomly. Plasmid DNA was sequenced using an ABI 3730 autosequencer (Perkin-Elmer, USA) with four fluorescent dyes. The sequencing primers were M13 universal primers.

Database searches and conservative analyses were performed using BlastX (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al*., 1990). Sequence assemblies were performed with using SeqMan software (DNASTAR, USA) and DNAman software package (Version 5.2.2; Lynnon, Pointe-Claire). Signal sequence prediction was performed using Signal P (http://www.cbs.dtu.dk/services/signalP/) (Bendtsen *et al*., 2004). Protein molecular masses and isoelectric points were determined online using ProtParam tools (http://us.expasy.org/tools/protparam.html).

Results

Morphological identification and capturing devices

Mycelia of *M. microscaphoides* were composed of hyaline, septate, branched, and prostrate hyphae. Conidiophores were erect, hyaline, septate, unbranched, 230~460 μm long, 3~5 μm wide at the base, gradually tapered upward to a width of 2.5 μm at the apex and bore one or two conidia. Conidia

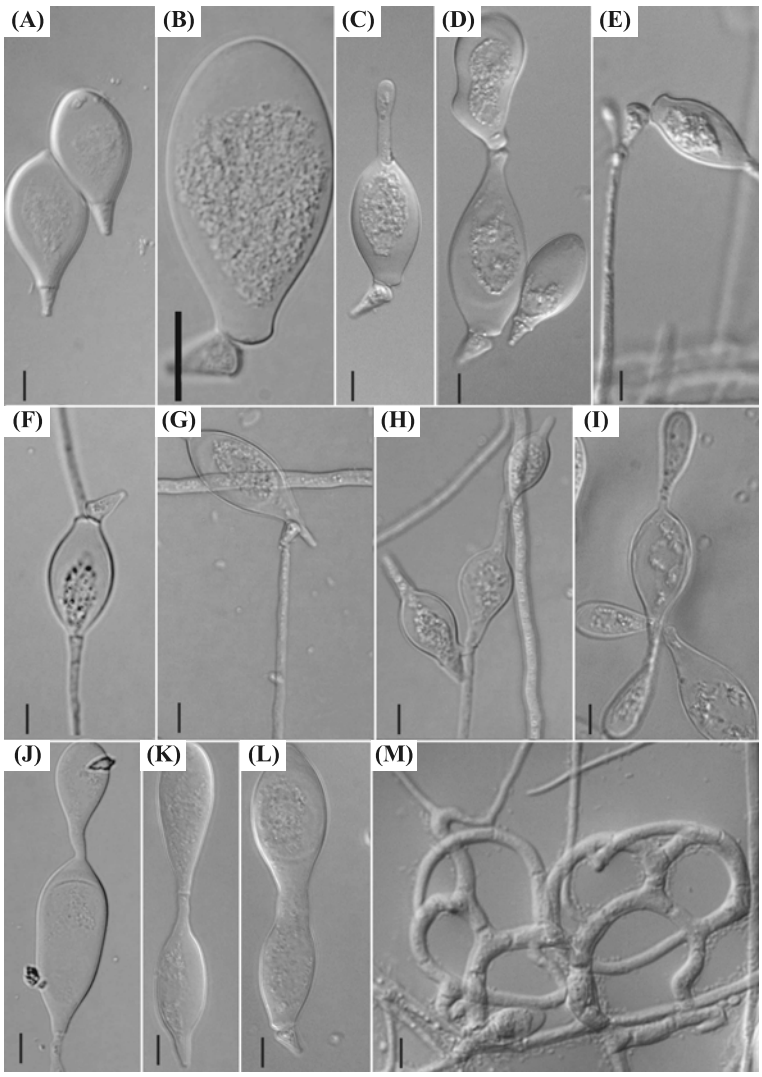

Fig. 2. Appearance of *Arthrobotrys multisecundaria* (YMF1.01821). (A~D) Conidia, (E~G) conidia on conidiophores, (H~L) primary conidia with secondary conidia, (M) adhesive three-dimensional networks. Scale bar: A~M=10 μm.

were hyaline, ellipsoidal, 22.5~45.0×10~20 μm, 0~3 septate (Fig. 1).

Morphological characters of *A. multisecundaria* (Fig. 2) were the same as previously described (Hu *et al*., 2005). The mycelia were hyaline, septate, branched, and scanty. Conidiophores (Fig. 2) were erect, hyaline, septate, unbranched, 200~365 μm long, 5 μm wide at the base, gradually tapered upward to a width of 2.5 μm at the apex and bore one or two conidia. Conidia were hyaline, ellipsoidal, 32.5~55.0×15.0~22.5 μm, contained a single septum septate (75%) or were non-septate (25%). Secondary conidia that could be produced from both distal and basal ends of primary conidia were non-septate with dimensions of 32.5~55.0×15.0~22.5 μm.

After induction using the free-living nematode *P. redivivus*, *M. microscaphoides*, and *A. multisecundaria* produced adhesive three-dimension networks to capture nematodes (Fig. 1 and 2).

Cloning of serine protease, β-tubulin, and ITS genes

Three pairs of primers (Table 1) were designed and synthesized to amplify the serine protease, β-tubulin, and ITS genes. A 1,271-bp PCR fragment of the serine protease gene was amplified from *A. multisecundaria* by using the degenerate primers NP and NR. Using primers Bt1a and Bt1b, two 543-bp PCR products were obtained from *A. multisecundaria* and *M. microscaphoides*, respectively. Two PCR fragments of approximately 547-bp were amplified from *A. multisecundaria* and *M. microscaphoides*, respectively, using primers ITS4 and ITS5. The PCR products of serine protease, β-tubulin and ITS were sequenced. These genes have been submitted to the GenBank database (accession numbers EF055263,

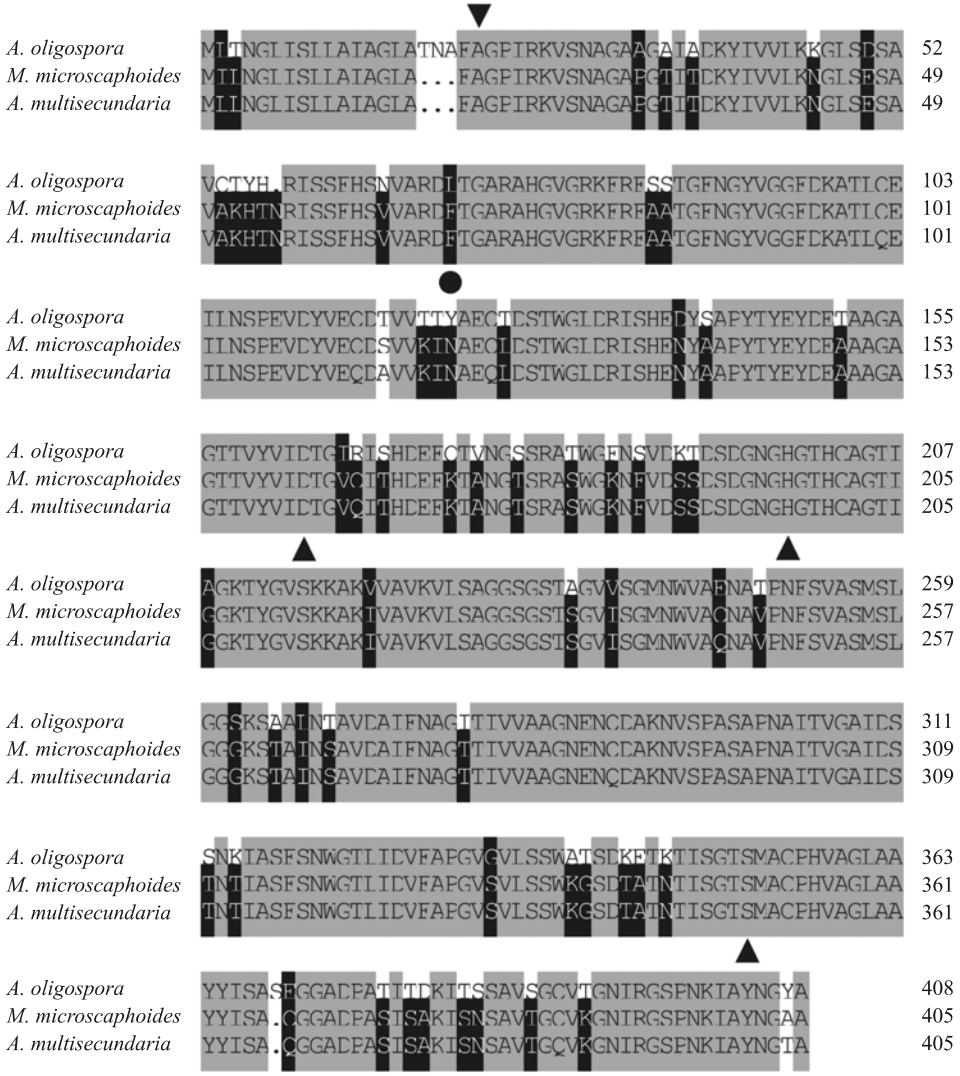

Fig. 3. Comparison of the amino acid sequences of serine proteases from *A. multisecundaria*, *M. microscaphoides*, and *A. oligospora*. GenBank accession no. of the encoding genes are EF055263, AY841167, and X94121, respectively. Areas shaded in grey are conserved regions (100% similarity), areas shaded in black are variable regions. (▼) indicates the putative signal-sequence cleavage site. (●) indicates the proregion cleavage site, (▲) indicates the aspartic acid-histine-serine catalytic triad.

EF059815, EF059816, EF059817, EF059818, respectively).

Sequence analyses

The protease-encoding gene contained an intron and two exons, and encoded a polypeptide of 405 amino acid residues with a *Mr* of 41.3 kDa. Comparison with serine proteases from other nematode-trapping fungi revealed the gene to be typical of fungal serine proteases, containing the conservative aspartic acid-histine-serine catalytic traid (Fig. 3). The protease possessed a pre-pro-peptide structure, which possessed a signal peptide consisting of 18 amino acid residues, a pro-region consisting of 102 amino acid residues and a mature peptide consisting of 285 amino acid residues. The deduced amino acid sequence of the *A. multisecundaria* serine protease showed 99.26 and 85% identity, respectively, to Mlx (*M. microscaphoides*) and PII (*A. oligospora*). The encoding genes of the β-tubulin and ITS from *A. multisecundaria* and *M. microscaphoides*, respectively, were aligned using the DNAman software package. The β-tubulin genes (EF059817 and EF059818) shared 99.63% identify and the ITS genes (EF059815 and EF059816) shared 99.82% identity.

Discussion

Based on morphological characteristics (Fig. 1 and 2), *A. multisecundaria* can be distinguished from *M. microscaphoides*. Although the two species both produce adhesive three-dimensional networks as a predacious organ, they differ in their styles of conidial germination. *A. multisecundaria* has a distinctive catenulate arrangement of conidia, which germinate to produce unicellular secondary conidia from both the distal and basal ends, with the secondary conidia being arranged in a catenulate arrangement of up to four (Hu *et al*., 2005). *M. microscaphoides* does not produce unicellular secondary conidia.

Comparison of the genes for serine protease, β-tubulin and ITS revealed that the nuclear and protein genes from the *A. multisecundaria* and *M. microscaphoides* share a high degree of similarities (>99%). There were differences of only three amino acids in the sequence of serine proteases, one base in the ITS gene sequence and two bases in the β-tubulin gene sequence. Such variation may be intraspecific, as has been described for the entomopathogenic fungus *Pochonia chlamydosporia* (Morton *et al*., 2003) and the nematophagous fungus *Lecanicillium psalliotae* (Yang *et al*., 2005a). Cloning of the gene encoding VCP1 from six *P. chlamydosporia* isolates and comparison of their translated cDNA sequences has revealed two amino acid polymorphisms at positions 65 and 99 (Morton *et al*., 2003). Similarly, one amino polymorphism at position 230 was found in the encoding gene of protease Ver112 from three isolates of *L. psalliotae* (Yang *et al*., 2005a). These results suggest that the two nematode-trapping fungi presently examined represent the same species, and that *A. multisecundaria* may be a spontaneous mutant of *M. microscaphoides.*

Nematode-trapping fungi previously were classified based on morphological characteristics of the conidia and were described as being comprised of three genera: *Arthrobotrys*, *Dactylella*, and *Monoacrosporium* (Subramanian, 1963). Recent studies with ITS and 18S rDNA sequences have indicated that trapping devices are more informative than other morphological structures in delimiting genera (Liou and Tzean, 1997; Pfister, 1997; Ahrén *et al*., 1998; Scholler *et al*., 1999). The refined systematic classification of nematode-trapping fungi based on phylogenies inferred from sequence analyses of 28S rDNA, 5.8S rDNA, and β-tubulin genes and three genera (*Arthrobotrys*, *Dactylellina*, and *Drechslerella*) has been proposed (Li *et al*., 2005). According to this new systematic classification, *Arthrobotrys* is characterized by the production of an adhesive three-dimension network. Therefore, we suggest that *M. microscaphoides* should be revised as *Arthrobotrys microscaphoides*, and *A. multisecundaria* should be revised as *Arthrobotrys microscaphoides* var. *multisecundaria*. Moreover, the morphology differences of *A. multisecundaria* and *M. microscaphoides* remind us that some genes involved in their sporulation of them may have been altered. Therefore, the two species should be suitable for studying the sporulation of nematode-trapping fungi.

Acknowledgements

We thank Drs. Xiaowei Huang, Chenggang Zou and associate researcher Wei Zhou for their help and advice in our experiment. This work was funded by National Basic Research Program of China (approved no. 2007CB411600), and by the Department of Science and Technology of Yunnan Province (approved Nos. 2005NG03, 2005NG05) and the Science & Engineering fund of Yunnan University (2005Q008B).

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