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

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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

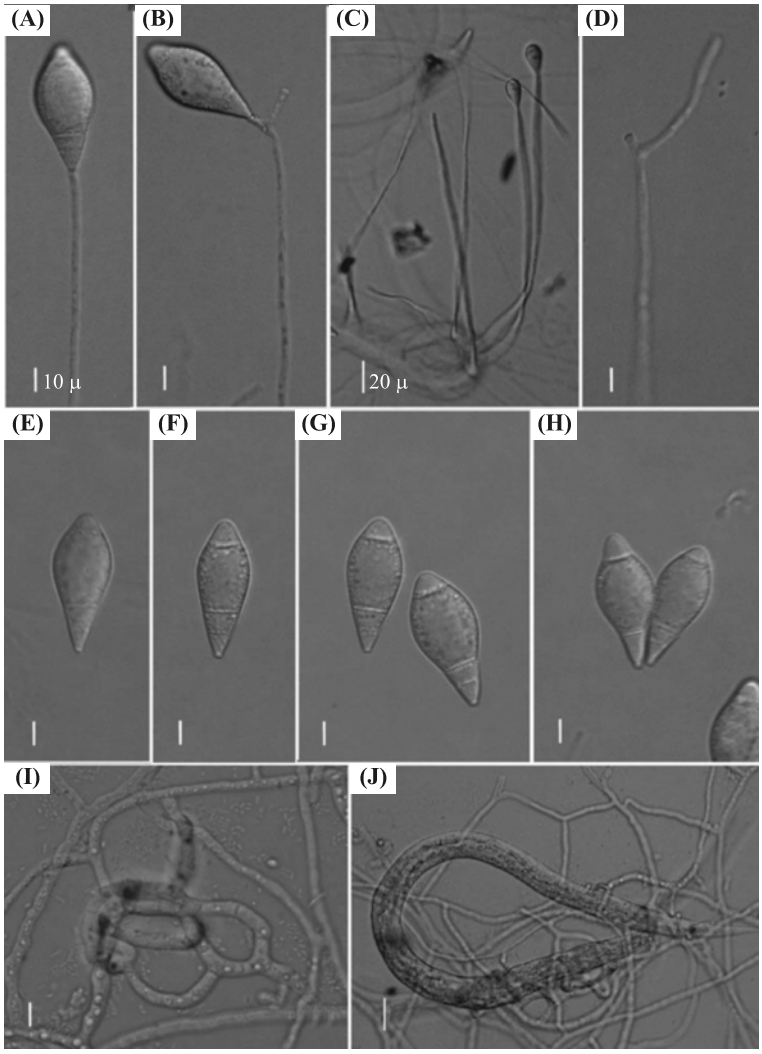

Fig. 1. Appearance of *Monacrosporium microscaphoides* (YMF1.00028). Similar to a previous description (Wang *et al*., 2006), visual examination revealed (A~D) conidiophores, (E~H) conidia with two or three septa, (I) three-dimension networks (traps) and (J) nematode trapped by the traps. Scale bars: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

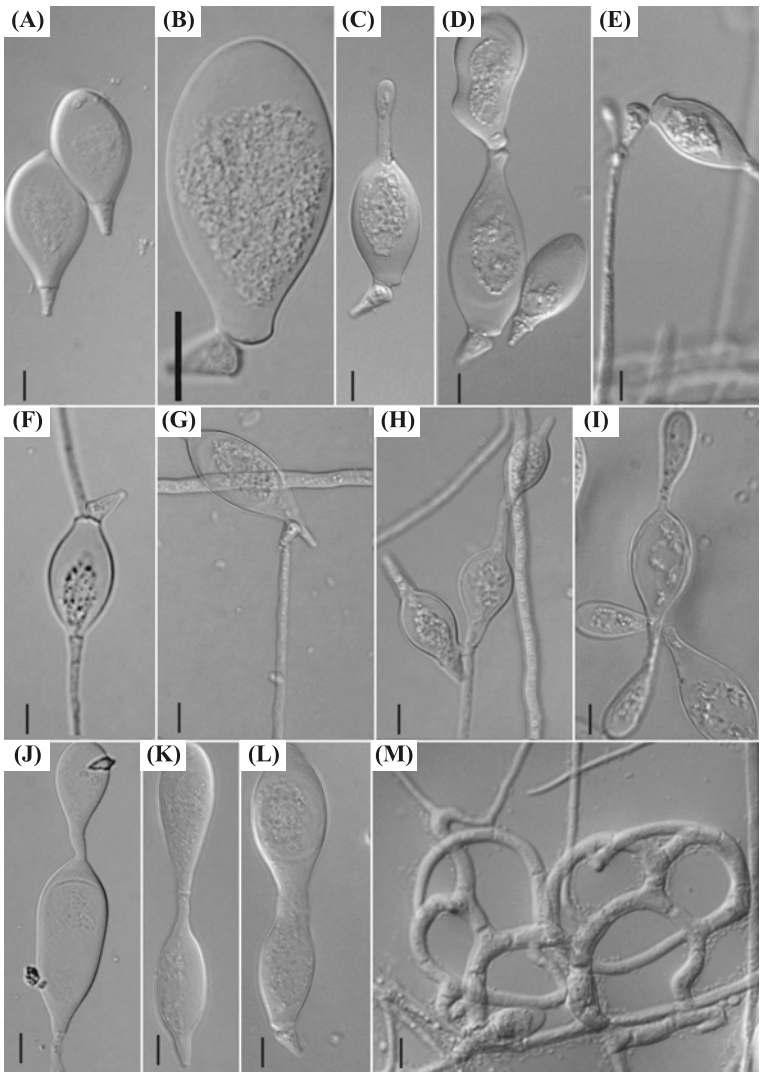

Fig. 2. Appearance of *Arthrobotrys multisecundaria* (YMF1.01821). (A~D) Conidia, (E~G) conidia on conidiophores, (H~L) primary conidia with secondary conidia, (M) adhesive three-dimensional networks. Scale bar: A~M=10 μm.

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,

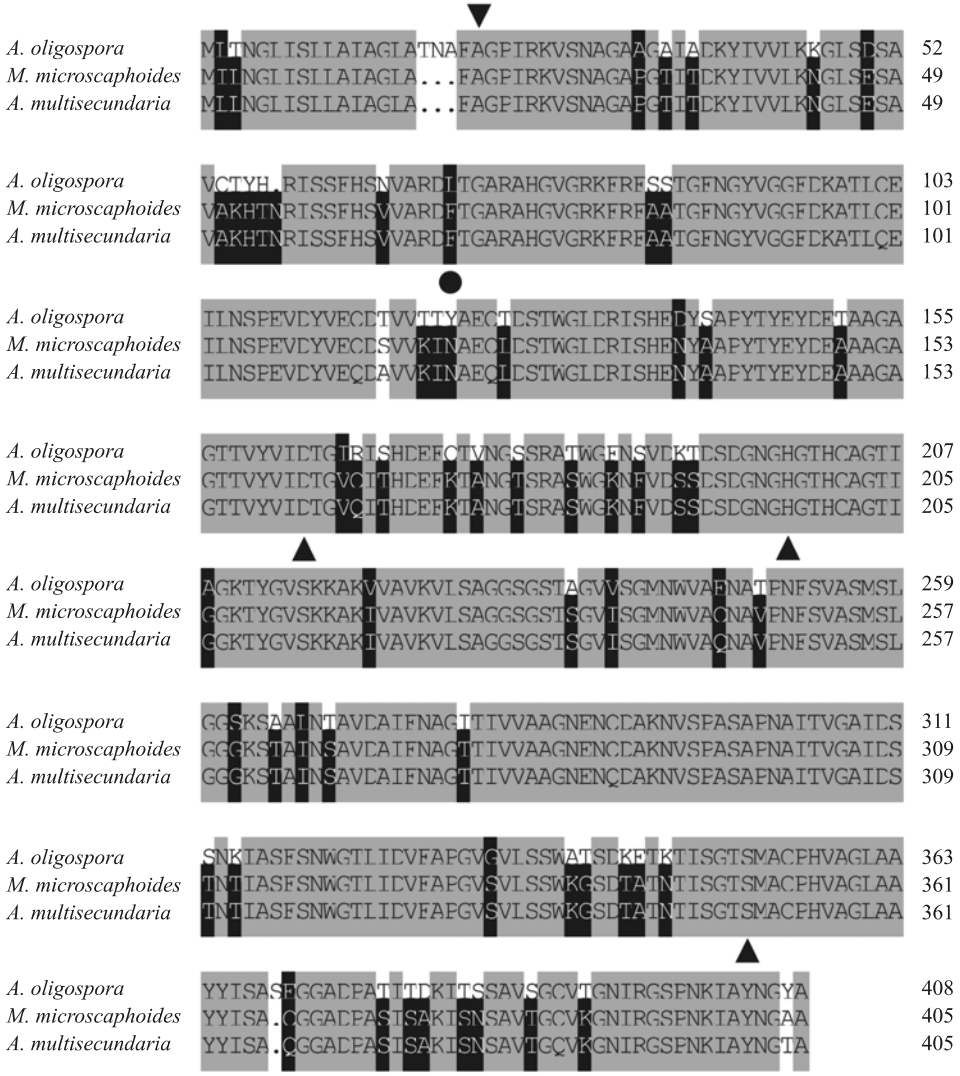

Fig. 3. Comparison of the amino acid sequences of serine proteases from *A. multisecundaria*, *M. microscaphoides*, and *A. oligospora*. GenBank accession no. of the encoding genes are EF055263, AY841167, and X94121, respectively. Areas shaded in grey are conserved regions (100% similarity), areas shaded 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.

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