The Journal of Microbiology, August 2008, p. 380-389 Vol. 46, No. 4
DOI 10.1007/s12275-007-0122-7


# High Infectivity of an Endoparasitic Fungus Strain, *Esteya vermicola*, against Nematodes

**Chun Yan Wang[1], Zhe Ming Fang[1], Bai Shen Sun[1], Li Juan Gu[1], Ke Qin Zhang[2], and Chang-Keun Sung[1]***

[1]*Department of Food Science and Technology, College of Agriculture and Biotechnology, Chungnam National University, Daejeon 305-764, Republic of Korea*
[2]*Laboratory for Conservation and Utilization of Bio-resources, Yunnan University, Kunming 650091, P. R. China*

(Received July 24, 2007 / Accepted May 2, 2008)

***Esteya vermicola*, as the first recorded endoparasitic fungus of pinewood nematodes, exhibits great potential as a biological agent against nematodes. However, only two strains of this species have been described so far. In this study, we identified a novel endoparasitic fungal strain, CNU 120806, isolated from infected nematodes in forest soil samples during a survey of nematophagous fungi in Korea. This strain showed similar morphological characteristics and infection mode with the two previously described strains of *E. vermicola*. All strains are characterized by the ability to produce two types of conidiogenous cells and conidia, and to parasitize nematodes with lunate adhesive conidia. Moreover, the CNU 120806 strain showed 100% identity with *E. vermicola* CBS 115803 when their partial sequences of 28S rRNA gene were compared. Molecular phylogenetic analysis further identified CNU 120806 as a strain of *E. vermicola*, by clustering CNU 120806 and *E. vermicola* CBS 115803 into a single subclade. Culture medium influenced the proportion of dimorphic CNU 120806 conidia, and further changed the adhesive and mortality rates of nematodes. The CNU 120806 strain exhibits high infection activity against nematodes on nutrient-rich PDA medium. Almost all tested nematodes were killed within 8~10 days after inoculation. This study provides justification for further research of *E. vermicola*, and the application and formulation of this fungus as a bio-control agent against nematodes.**

***Keywords*:** pinewood nematode, *Esteya vermicola*, morphological identification, 28S rRNA, molecular phylogenetic analysis, biological control

The pinewood nematode, *Bursaphelenchus xylophilus*, is the causal pathogen of pine wilting disease. It invades healthy pine trees through the feeding wound of its vector beetle, *Monochamus alternatus*, and can enter a dying or dead tree through the vector's oviposition mark. In 1988, the pathogen pinewood nematode was accidentally introduced from Japan into Republic of Korea, after which the first pine wilting disease broke out in Busan. The disease eventually spread to the southern provinces and eastern coastal regions of Korea, and on to 5,111 hectares of forests in 51 countries, in the process becoming a major ecological catastrophe with serious economic losses to the pine forest industry. As a result, great remedial efforts such as direct felling and burning of wilted trees, fumigation of logs by insecticide, and introduction of natural enemies have been attempted.

Nematophagous fungi are a diverse range of fungi that have the capacity to capture, engage in parasitic relationships, or paralyze different populations and life stages of nematodes (Li *et al*., 2000). The ecology, distribution and systematics of nematophagous fungi, and their potential as biological control agents against plant pathogenic nematodes have been extensively studied (Liu and Zhang, 2003; Li *et al*., 2005; Mo *et al*., 2005; Zhao *et al*., 2005; Wang *et al*., 2007). They are usually classified into four groups depending on their infection modes; nematode-trapping fungi, endoparasitic fungi, egg- and female-parasitic fungi, and toxin-producing fungi (Barron and Thorn, 1987; Dackman *et al*., 1992). Endoparasitic fungi can infect nematodes with encysting spores, adhesive conidia or ingesting conidia. So far, 22 genera and approximately 70 species have been identified. Among them, *Esteya vermicola* is the first recorded endoparasitic fungus of the pinewood nematodes.

*Esteya vermicola* was first isolated from infected pinewood nematodes (*B. xylophilus*) in Taiwan and proposed as a new species within a new genus by Liou *et al*. (1999). Subsequently, another *E. vermicola* strain was isolated from European oak bark beetles, *Scolytus intricatus*, adults, and larvae, including their galleries on three oak species (*Quercus petraea*, *Q. polycarpa*, and *Q. robur*) in the Polabí and Krivoklátsko regions of Czech Republic (Kubátová *et al*., 2000). This was the first time *E. vermicola* was found in European, suggesting that the fungus might be a pathogen of pinewood nematode with broad distribution across the globe. *Esteya vermicola* has been patented in the United States based on its potential as a biological control agent against the pinewood nematode (Tzean *et al*., 2001). Although there are two types of conidiogenous cells and conidia produced by *E. vermicola*, only the lunate conidia were adhe-

* To whom correspondence should be addressed.
(Tel) 82-42-821-6722; (Fax) 82-42-822-2287
(E-mail) kchsung@cnu.ac.kr

sive and could attach to the cuticle of nematodes, causing subsequent infections. *Esteya vermicola* exhibited high infectivity toward the pinewood nematode. *In vitro*, almost 100% of the pinewood nematode population were infected by adhesive lunate conidia and completely killed within 8~10 days (Liou *et al*., 1999). However, so far only two strains of this fungus have been described. There is still a paucity of data on their biology, ecology, infection mechanism against hosts, and potential as bionematicides.

During a survey of nematophagous fungi in Korea, a novel endoparasitic fungal strain, CNU 120806, was isolated from forest soil and identified as a rare hyphomycete, *E. vermicola*, based on phenotypic characteristics and molecular phylogenetic analysis. The morphological and molecular characteristics, infection mode, and other traits of this strain were described and briefly discussed. Furthermore, the infection activity of this strain against two nematode populations, and the factors that influence its infection activity were evaluated in detail. In addition, the exploitation and utilization of nematophagous fungi resources in Korea were also briefly discussed.

## Materials and Methods

### Isolation and morphological identification of endoparasitic fungus

The fungus was isolated from infected nematodes in forest soil samples collected from Choengdo, Korea (Nov. 15, 2006), according to Li's method (Li *et al.*, 2005). It was cultured on Potato Dextrose Agar (PDA) plate at 26°C. Its microscopic features were observed, measured and photographed using an Olympus BX51 microscope.

### Molecular phylogenetic analysis

In order to confidently establish the taxonomic status of CNU 120806 strain, partial sequence of 28S rRNA gene was amplified and sequenced. The reported strain, *E. vermicola* CBS 115803, was also used in molecular analysis.

***DNA extraction***: CNU 120806 and *E. vermicola* CBS 115803 were cultured in liquid PDB (potato dextrose broth) medium at 26°C without shaking for 5 days. Mycelia were collected by filtration in a sterilized filter funnel and ground to a fine powder in liquid $N_2$. Genomic DNA was extracted using the CTAB method as outlined by Zhang *et al*. (1996).

***Amplification and sequence of 28S rRNA*:** The universal primers LROR (Bunyard *et al*., 1994) and LR5 (Vilgalys and Hester, 1990) were synthesized by Genotech (Korea) and used to amplify the partial region of 28S rRNA gene sequence. Polymerase Chain Reaction (PCR) amplification was conducted in a 50 μl reaction volume. Each reaction mixture consisted of 2.5 units of Top DNA polymerase (Bioneer, Korea), 5 μl of 10× PCR buffer with $MgCl_2$, 1 μl of 10 mM dNTPs, 1 μl of 10 μM each primer, 3 μl of DNA template (50~100 ng), and made up to a final volume of 50 μl with double-distilled sterile water. PCR reaction was performed with the following steps; 4 min at 95°C, 35 cycles of 30 sec at 98°C, 40 sec at 48°C, 50 sec at 72°C and a final extension step of 10 min at 72°C. Amplified products were electrophoresed on 1% agarose gels to check for size and purity. A 100 bp DNA ladder (Bioneer, Korea) was included on each gel as molecular size standard.

The PCR products were purified from 1% agarose gel according to the instruction of QIAquick PCR Purification Kit (QIAGEN, Germany) and directly sequenced by using the universal primers LROR and LR5 in Genotech (Korea).

***Nucleotide sequence accession number*:** The partial sequences of 28S rRNA gene of strains CNU 120806 and *E. vermicola* CBS 115803 in this study (862 nucleotides) have been deposited in the GenBank database. The accession number for strains CNU 120806 and *E. vermicola* CBS 115803 are EU627684 and EU668903, respectively.

***Sequence alignment and phylogenetic analysis*:** Sequence searches were performed using Blast X against database NCBI (http://www.ncbi.nlm.nih.gov/BLAST). The partial sequences of 28S rRNA gene of CNU 120806, *E. vermicola* CBS 115803, and corresponding sequences of other most related species obtained from GenBank database were edited and aligned using BioEdit (Hall, 1999) and CLUSTAL X 1.83 (Thompson *et al*., 1997). The phylogenetic tree was conducted using the neighbor joining method (Saitou and Nei, 1987) as outlined by Syed *et al*. (2007), from the evolutionary distance data corrected by Kimura's 2-parameter model (Kimura, 1980).

### Monoxenic culture of Nematodes

***Panagrellus redivivus*:** The saprophytic nematodes were cultured on oatmeal medium (oatmeal: 20 g, water 80 ml) at 26°C for 7 days.

***Bursaphelenchus xylophilus*:** *Botrytis cinerea* was cultured on PDA plate at 26°C, and then inoculated with the pinewood nematodes while the fungus grew fully. Subsequently, the plate was cultured until fungal mycelia had been completely consumed.

The two cultured nematodes were separated from culture medium using the Baerman funnel technique, and an aqueous suspension of nematodes was prepared for use as working stock.

### Influence of nutrition and nematodes on the production of dimorphic conidia

In order to reveal the relationship between nutrition and the production of dimorphic conidia, three kinds of media, PDA, Corn Meal Agar (CMA) and 2% Water Agar (WA), were selected for tests. After being cultured at 26°C for 8 days, the growth condition of strain CNU 120806 was inspected and the proportion of dimorphic conidia was evaluated under light microscope.

The influence of nematodes on the fungus was illustrated by adding several drops of nematode suspension (about 30~50 individuals) onto three kinds of media plates with 8-day-old pure cultures of CNU 120806 strain, and incubated at 26°C for another 7 days. The growth state and production of two types of conidia were observed under light microscope, and pure cultures of CNU 120806, without nematodes, were used as control.

### Germination mode of dimorphic conidia

In order to determine the germination mode of dimorphic conidia, and establish whether the nematodes stimulate their germinations, the germination processes of conidia were

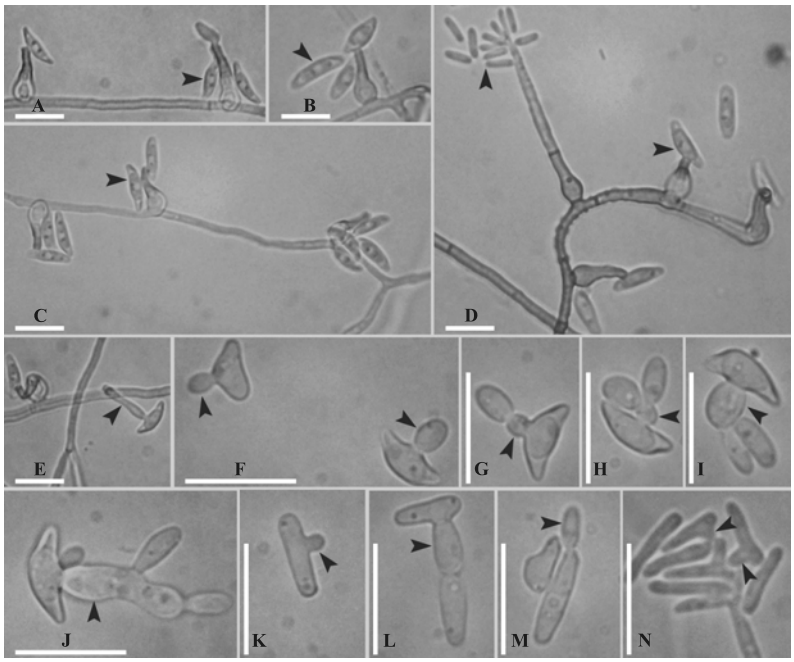


**Fig. 1.** *Esteya vermicola* CNU 120806. (A∼C) Conidiogenous cells with lunate conidia (arrowheads). (D) Two types of conidiogenous cells and conidia (lunate and bacilloid) on the same hypha (arrowheads). (E) A lunate conidia germinating from the centre of concave side to form germination tube (arrowhead). (F) Two lunate conidia germinating respectively from the centre of concave side and convex side (arrowheads). (G) A lunate conidia germinating from the centre of convex side (arrowhead). (H∼J) The germinating lunate conidia (arrowheads). (K, L) Cylindrical conidia producing germination tube from one side (arrowheads). (M) Cylindrical conidia forming germination tube at the end (arrowhead). (N) Cylindrical conidia germinating from one side (arrowheads). Bars=10 μm.

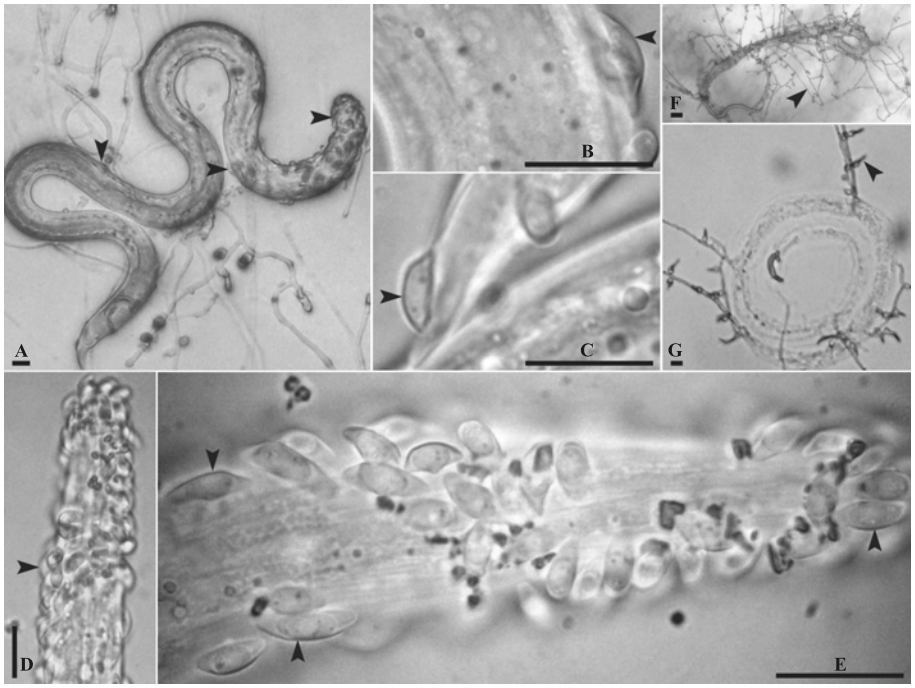


**Fig. 2.** *Esteya vermicola* CNU 120806. (A) The cuticle of a nematode attached by many lunate conidia of *E. vermicola* CNU 120806 (arrowheads). (B) A lunate conidia attached to the middle body of nematode (arrowhead). (C) Lunate conidia attached to the tail of nematode (arrowhead). (D, E) Many lunate conidia attached to the head of nematode (arrowheads). (F, G) Nematode infected by *E. vermicola* with the conidiophores, conidiogenous cells and lunate conidia produced outside of the nematodes (arrowheads). (A∼E) and (G) Bars=10 μm; 20, Bar=20 μm.

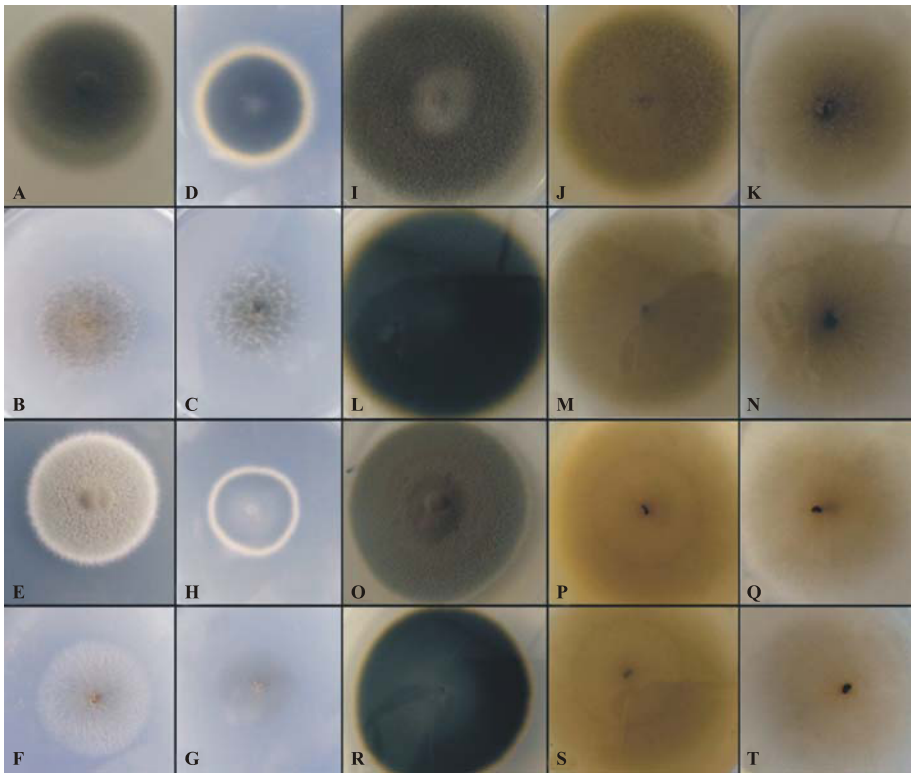


**Fig. 3.** *Esteya vermicola* CNU 120806 and *E. vermicola* CBS 115803. (A~D) The colonies of *E. vermicola* CNU 120806 on PDA, CMA, and WA plates, respectively and the reverse on PDA plate (8 days). (E~H) The colonies of *E. vermicola* CBS 115803 on PDA, CMA, and WA plates, respectively and the reverse on PDA plate (8 days). (I~N) The colonies of *E. vermicola* CNU 120806 on PDA, CMA, and WA plates and their reverses, respectively (13 days). (O~T) The colonies of *E. vermicola* CBS 115803 on PDA, CMA, and WA plates and their reverses, respectively (13 days).

observed under light microscope. Several drops of heavy conidia suspension, collected from the pure culture of CNU 120806 strain, were respectively added into 2% WA plate with some nematodes, 2% WA plate without nematodes, 1 ml nematode suspension, and 1 ml sterile water in 1.5 ml centrifuge tube. Conidia under all the conditions were incubated at 26℃ and inspected under light microscope on the second day.

## Infection effectiveness of CNU 120806 strain against nematodes

The saprophytic nematode, *P. redivivus*, and plant-parasitic nematode, *B. xylophilus*, were selected as the tested targets.

Two kinds of tests were carried out.

In test one, the CNU 120806 strain was respectively inoculated onto the center of PDA, CMA, and 2% WA plates and incubated at 26℃ for 8 days. Numerous lunate conidia were produced and readily adhered to nematodes by adhesive mucilage on the concave side as the nematodes migrated and came into contact with the conidiogenous cells and conidia. Subsequently, the plates were infested with 10 μl suspension of each nematode containing 250~300 individuals.

In test two, 1 ml (about 400~500 individuals) heavy suspension of each tested nematode was respectively added onto PDA plates with 8-day-old pure cultures of CNU 120806 strain, and subsequently mixed gently with a glass rod. Several drops of the mixture were spread onto the 2% WA plates.

Nematodes in both tests were examined at intervals of 1, 2, 4, 8, 16, 24 h and 2, 3, 4, 5, 6, 7, 8, 10 days under light microscope. Nematodes were considered dead if they showed no response to physical stimulation. The adhesive rate and proofreading mortality rate (%) were estimated based on the percentage of nematodes adhered by lunate conidia and dead nematodes. Plates containing only medium and nematodes were used as control and each treatment was replicated three times.

Nematodes at different infection stages were randomly picked up with a metal filament under dissecting microscope and mounted in a drop of water on a slide with a cover slip. Subsequently, they were examined under light microscopy to observe the infection process and determine the mode of parasitism.

## Infection effectiveness of CNU 120806 strain against beetle larvae

The beetle larvae of *M. alternatus*, collected from the dead pine trees were used for the infection test. The CNU 120806 strain firstly was cultured on PDA plates at 26℃ for 8 days. Subsequently, beetle larvae were placed into

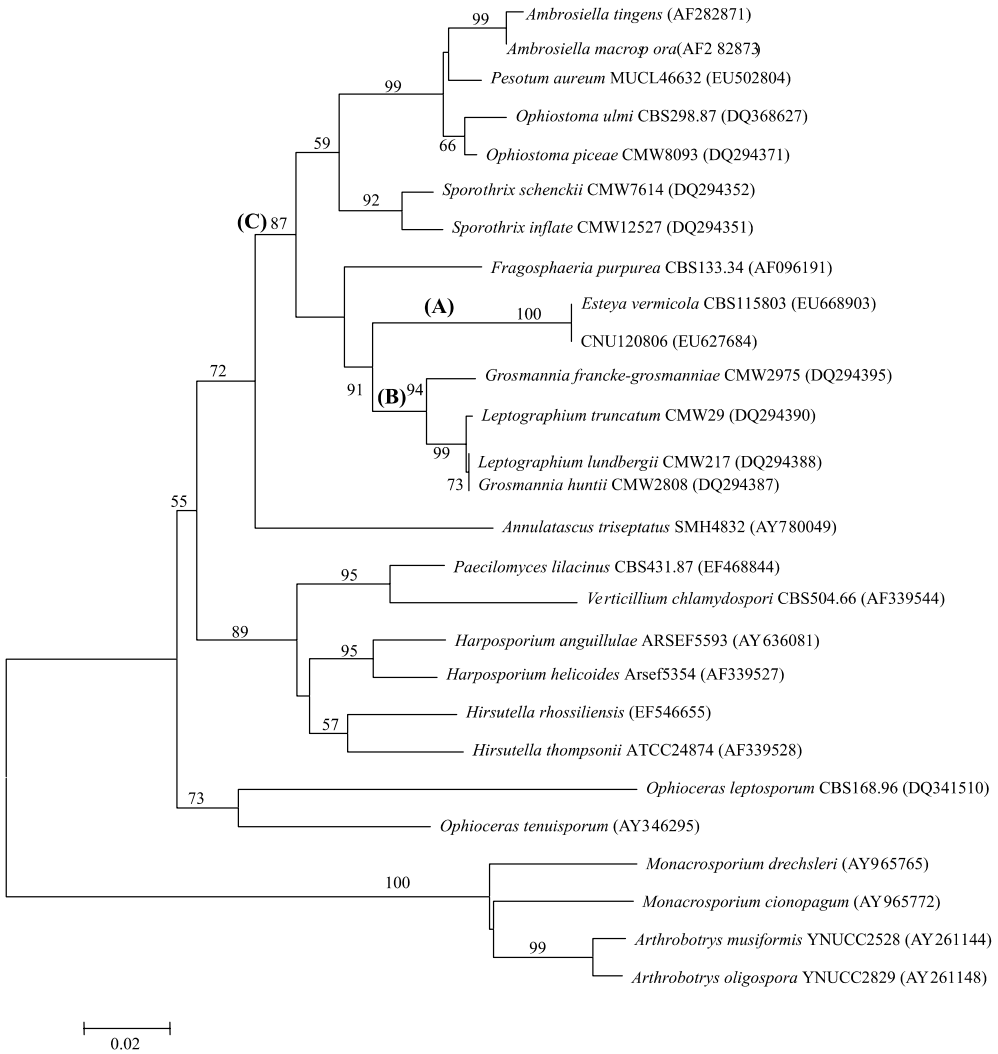


**Fig. 4.** Phylogenetic dendrogram obtained by distance matrix analysis of partial 28S rRNA gene sequence, showing the position of strains CNU 120806 and *E. vermicola* CBS 115803 among their phylogenetic neighbours. Numbers at the branch nodes are bootstrap values, expressed as a percentage of 1,000 replicates (only values above 55% are shown). The sequences of *Arthrobotrys* spp. and *Monacrosporium* spp. were used as root. Bar, 1% sequence divergence.

these plates and continuously cultured at 26°C. At interval of 2 days, beetle larvae were inspected under light and dissecting microscope.

## Results

### Taxonomy

Colonies of CNU 120806 strain grew moderately on PDA medium, reaching 4.0~4.5 cm in diameter after 8 days at 26°C. They were white at first, compact, somewhat floccose in the outer regions, but gradually turned grayish green and eventually dark green, producing dark-brown pigments (Fig. 3A, D, I, and L). Assimilative hyphae were moderately branched, septate, hyaline or grayish green, smooth to roughened, 2.8~4.0 μm wide on PDA plate and mostly 1.8~3.5 μm wide when developing within free-living nematodes. Conidiophores were formed outside of the dead nematode body, erect or procumbent, colorless, septate, commonly unbranch, and mostly 50~280 μm long, and 1.4~2.5 μm wide (Fig. 2F and G). In pure culture, CNU 120806 strain produced two types of conidiogenous cells and conidia. One type of conidiogenous cells were singly borne on the conidiophores, integrated, globose or flask-shaped inflated base, 3~5 μm in diameters, tapering upward into a thin neck with varying length, 14~25×1.5~1.9 μm, often crooked, and sometimes percurrent. Conidia were one-celled, asymmetrically ellipsoidal, lunate, concave, ends moderately apiculate, hyaline, smooth-walled, 7.7~12.1×3.0~3.8 μm, containing an endospore-like structure, and adhesive with a droplet of mucus on the concave. 1~4 lunate conidia were successively produced by the same conidiogenous cell (Fig. 1A~D). Conidiogenous cells of another type were also borne singly on the conidiophores, integrated, enteroblastic, 30.4~45.0 μm long, mostly with a swollen base, and 3.0~4.8 μm in diameter. Conidia were one-celled, bacilloid to cylindrical, hyaline, smooth, 4.3~8.4×1.4~1.9 μm, and non-

adhesive. Between 1∼15 bacilloid conidia often aggregate at the apex forming a false head (Fig. 1D).

Culture of this species was deposited and maintained at Agriculture Bioscience Biotech Centre, Chungnam National University, Korea. The identification number was *E. vermicola* CNU 120806.

## Molecular phylogenetic analysis

The partial sequences 28S rDNA gene (862 bp) of CNU 120806 strain and *E. vermicola* CBS 115803 were determined in this study. The sequences alignment showed that CNU 120806 shared 100% identity with *E. vermicola* CBS 115803 (no nucleotide differences out of 862). Preliminary blast against the GenBank database using 28S rRNA gene sequence of CNU 120806 strain indicated that members of the *Ophiostomataceae* family were the closest phylogenetic relatives. Among them, CNU 120806 shared the highest binary similarity value of 93% with *Leptographium* spp. and *Grosmannia* spp..

Subsequently, molecular phylogenetic analysis was performed among the members of the *Ophiostomataceae* family and other typical nematode-endoparasitic fungi to determine the taxonomic status of CNU 120806 and *E. vermicola* CBS 115803. Related 28S rRNA gene sequences were downloaded from GenBank database and used for phylogenetic tree construction (Fig. 4).

From the phylogenetic tree it can be seen that clade C was comprised within a different genera. CNU 120806 and *E. vermicola* CBS 115803 were clustered into the subclade A, which was closed to subclade B (two *Leptographium* species and two *Grosmannia* species). However, clade A and B only shared 91% similarity. Therefore, CNU 120806 and *E. vermicola* CBS 115803 differed from currently identified genera within the *Ophiostomataceae* family. The results of sequence alignment and phylogenetic analysis, based on 28S rRNA gene sequence, provided evidence to support the taxonomic identification of CNU 120806 and CBS 115803 as two strains of *E. vermicola*, which is the only species within the *Esteya* genus.

## Influence of nutrition and nematodes on the production of dimorphic conidia

In the nutrient-rich PDA culture medium, CNU 120806 strain showed the most rapid growth rate compared to CMA and WA medium plates (Table 1). The colonies were compact, olive or dark green (Fig. 3A and I). The strain almost exclusively produced globose or flask-shaped conidiogenous cells and lunate adhesive conidia on PDA plates, coupled with very few cylindrical or bacilloid non-adhesive conidia. On the contrary, in nutrient-poor CMA and WA plates, CNU 120806 strain grew much slower and produced sparse mycelium. The colonies were grey, rising at the center, and growing upward on CMA plates, while arachnoid greyish green on WA plates (Fig. 3B, C and Fig. 3J, K). The strain mainly produced flask-shaped conidiogenous cells, lunate adhesive conidia on CMA and WA plates. At the same time, many cylindrical, non-adhesive conidia also were produced. It seemed that rich nutrition in the medium leads to the production of more adhesive conidia. In addition, CNU 120806 strain developed conidiophores outside of infected nematodes cadavers, and only produced discrete, flask-shaped conidiogenous cells, and lunate adhesive conidia. No cylindrical conidia have been found on the nematodes cadavers.

On plates containing nematodes, the assimilative hyphae of CNU 120806 strain were vigorous and fresh with more lunate adhesive conidia and less bacilloid conidia. Nevertheless, on control plates, the assimilative hyphae were aged with only a small quantity of the two conidia types present. Therefore, nematodes could stimulate the growth of CNU 120806 strain and induce the production of adhesive conidia.

**Table 1.** The growth speed of *E. vermicola* CNU 120806 on three kinds of media plates at 26°C for 8 days

| Media plates | Colonies diameter (cm) |
|---|---|
| PDA | 4.0∼4.6 |
| CMA | 3.8∼4.1 |
| WA | 2.8∼3.0 |

## Germination of dimorphic conidia

Regardless of nematodes presence, dimorphic conidia of CNU 120806 strain germinated after inoculation both in water suspension and WA plates on the second day.

The lunate conidia germinated to form a bulb bulge from the concave side of spores around the central point, then grew and developed one or more germ tubes approximately at right angle from the conidia (Fig. 1E, F and Fig. 1H, I). Later, the germ tubes branched and formed vegetative hyphae (Fig. 1J). Occasionally, lunate conidia germinated from the convex side at the central point (Fig. 1F and G). Almost 90% of the cylindrical or bacilloid conidia produced germination tubes from one side of conidia at about the 1/3 or 1/2 point, and only 10% produced germination tubes at the end (Fig. 1K∼N).

## Infection effectiveness of CNU 120806 strain against nematodes

With regards to the first test on three kinds of media plates, both tested nematode populations were parasitized by the lunate adhesive conidia of CNU 120806 strain. While exposed to the fungus on pure culture plates, nematodes were attached by lunate conidia through adhesive mucilage on the concave side (Fig. 2A). Although adhesive conidia appeared randomly attached to the cuticle of nematodes, the head, in particular, and also tail regions appeared to be the preferred sites (Fig. 2B∼E).

In the second test on 2% water agar plates containing mixture of nematodes and conidia suspension, no nematodes were found attached or infected by the conidia of CNU 120806 strain. It seems that once the lunate conidia shed from their conidiogenous cells, they immediately lost adhesive ability and infectivity against nematodes.

Culture medium played a significantly role on the infection ability of CNU 120806 strain, both against *B. xylophilus* and *P. redivivus*. On PDA plates, the adhesive rates were comparatively higher than CMA and WA plates (Fig. 5). Within 24 h, most tested nematodes (89% and 90.2%) were found with numerous conidia on their cuticles. Furthermore, proofreading death rates were also the highest on PDA

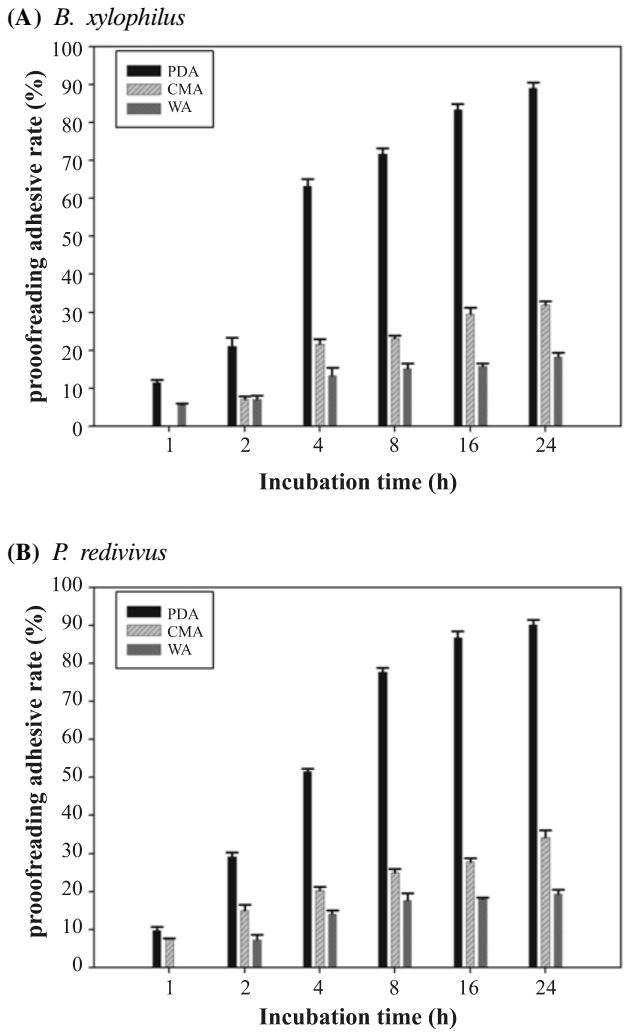


**Fig. 5.** The adhesive rates (%) of *E. vermicola* CNU 120806 toward *B. xylophilus* and *P. redivivus* on three kinds of plates. (Data shown are combined results from three independent experiments. Error bars represent the Mean±SE.)

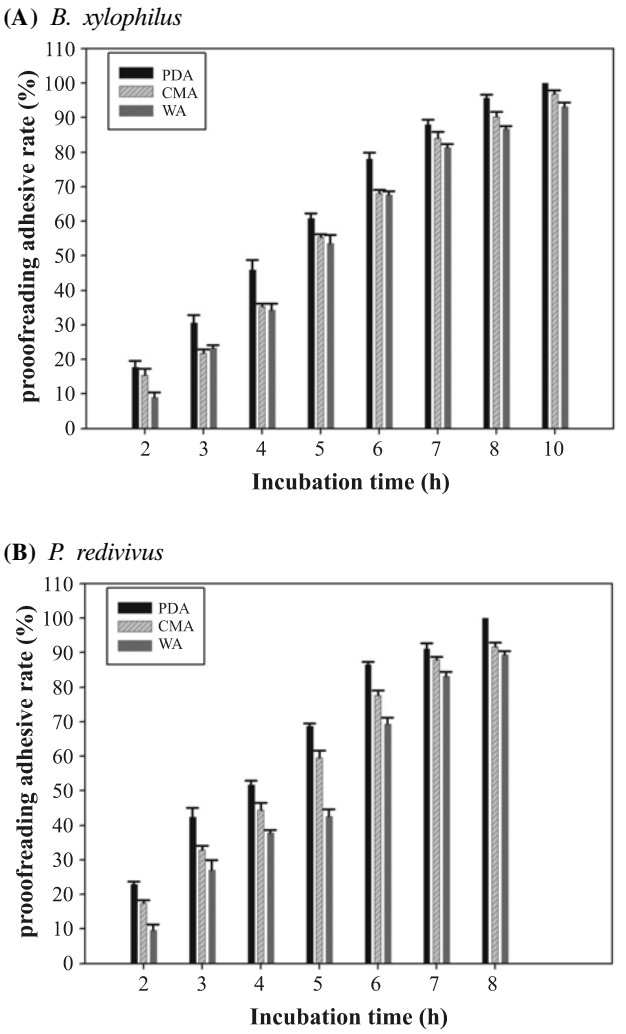


**Fig. 6.** The proofreading death rates (%) of *E. vermicola* CNU 120806 toward *B. xylophilus* and *P. redivivus* on three kinds of plates. (Data shown are combined results from three independent experiments. Error bars represent the Mean±SE.)

plates, among three kinds of tested plates within 8~10 days (Fig. 6). In addition, results showed that adhesive rates and proofreading death rates of CNU 120806 strain against *P. redivivus* were slightly higher than *B. xylophilus*.

The infection process of CNU 120806 strain against two types of nematodes was also documented in detail. After attachment to nematodes for 18~24 h, the conidia germinated and penetrated the cuticle using the fine infection peg, after which a subcutaneous infection bulb was produced, from which two or three trophic hyphae initiated. In most examined nematodes, assimilative hyphae were septate, sparingly branched, and 1.8~3.5 μm wide. Hyphal growth greatly reduced the motility of nematodes. When the bodies of infected nematodes were filled with tangled mass of hyphae, their organs and tissues were destroyed. While the nutrition of host was used up, the fungal hyphae penetrated directly through the cuticle and grew out from the nematode cadavers to produce conidiophores, flask-shaped conidiogenous cells and lunate adhesive conidia (Fig. 2F and G). The whole infection process was usually completed within 8~10 days after inoculation, until almost all of the tested nematodes were killed (Fig. 3C and D).

During the study, cylindrical or bacilloid conidia of CNU 120806 strain were not observed on the conidiophores which grew out from the host cadavers even in the prolonged culture and no infested nematodes were found attached or invaded by this type of conidia (Fig. 2F and G). In addition, CNU 120806 never produced chlamydospores within cadavers of infected nematodes, nor in the pure culture (Fig. 2F).

## Infection effectiveness of CNU 120806 strain against beetle larvae

In this test, both types of CNU 120806 strain conidia could neither attach to the cuticle of beetle larvae nor infect them. All tested larvae were alive 15 days post-inoculation.

## Discussion

The endoparasitic fungus strain, CNU 120806, possesses similar traits in terms of morphological characteristics and infection mode against nematodes, with two known *E. vermicola* strains reported by Liou *et al*. (1999) and Kubátová *et al*. (2000), respectively. The CNU 120806 strain shared 100% similarity of partial 28S rRNA gene sequence with *E. vermicola* CBS 115803. Phylogenetic analysis further showed that they were clustered into subclade A, below the *Ophiostomataceae* family and differed from other described genera. This result supported the identification of CNU 120806 as a strain of *E. vermicola* within the *Ophiostomataceae* family. Moreover, there were also some differences among CNU 120806 strain and the two reported *E. vermicola* strains. A comparative phenotypic analysis was performed in order to clearly illustrate the main differences among the three *E. vermicola* strains (Table 2).

Based on the above analysis, we proposed CNU 120806 as a new strain of *E. vermicola* in Korea. Our investigation significantly added to the knowledge-base of *E. vermicola*. In this study, the CNU 120806 strain also exhibited high activity both toward pinewood nematodes and saprophytic nematodes. Almost 100% of the tested nematodes were killed by the strain within 8~10 days after inoculation. Furthermore, the adhesive rates and mortalities varied on different nutrient media. Among three kinds of tested media, CNU 120806 showed the highest adhesive rate and death rate on nutrient-rich PDA medium. This may be due to the fact that adhesive and death rates were directly related to the amount of lunate adhesive conidia. This varied on different media, as more adhesive conidia were produced among richer media.

The concave side of *E. vermicola* lunate conidia, with adhesive mucilage, was extroversive, while the convex side connected to conidiogenous cells. This inserted model is very delicate and in favor of attachment to the cuticle of hosts as nematodes come into contact with the conidia. However, as lunate conidia are shed from their conidiogenous cells, they immediately lose adhesive ability and infectivity against the hosts. This phenomenon was also observed in the genus *Hirsutella*. The conidia of *Hirsutella* spp. were also coated by a mucous sheath and could not infect nematodes after departing from their conidiogenous cells (Chen *et al*., 2000). However, the underlying reasons are currently unknown. As for *E. vermicola*, we suspected that conidia are floating when they depart from conidiogenous cells, and the adhesive mucilage only exists on the concave side. Therefore, the

**Table 2.** Morphological comparison among of three strains of *E. vermicola*

| Strain no. | ATCC 74485 | CBS 115803 | CNU 120806 |
|---|---|---|---|
| Reference | Liou *et al*. (1999) | Kubátová *et al*. (2000) | |
| Location | Taiwan, China | Czech Republic | Korea |
| Isolation material | Pinewood nematodes, *B. xylophilus* | Larvae, adult beetles of *S. intricatus* and their galleries in oak | Soil, saprophytic nematodes |
| Growing speed (PDA, 7~8 days) | Moderately, 3.0~4.5 cm in diam. | More slowly, 3.0~3.7 cm in diam. | More quickly, 4.0~4.5 cm in diam. |
| Colonies color (Fig. 3) PDA | Grey, grayish green to dark green; green to dark green in reverse | Grey to grayish green; dark green in reverse | Dark green; dark green in reverse |
| Colonies color (Fig. 3) CMA and WA | | Light grayish green to chartreuse; light chartreuse in reverse | Grey to grayish green; light grayish green in reverse |
| Lunate conidia | 8.2~11.1×3.5~3.7 μm, conidia solitary | 9.3~12.4×3.0~3.2 μm, 1~4 conidia produced by the same conidiogenous cell | 7.7~12.1×3.0~3.8 μm, 1~4 conidia produced by the same conidiogenous cell |
| Bacilloid conidia | 4.4~7.4×1.5~1.9 μm | 4.8~6.2×1.3~1.5 μm | 4.3~8.4×1.4~1.9 μm |
| Nutrition influence on production of dimorphic conidia | Mainly produced bacilloid conidia on enriched media and produced numerous lunate conidia on nutrient-poor medium | Both types of conidia on different nutrient media were produced very early, sporulation seemed to be nearly the same under different light conditions | Mainly produced lunate conidia on enriched nutrient medium |
| Sporulation from the nematode cadavers | Almost exclusively produced lunate conidia | | Only lunate conidia were produced and bacilloid conidia never were observed |
| Germination model of lunate conidia | | Lunate conidia formed one or more germ tubes in the centre of concave side | Mainly germinated from the centre of the concave side, occasionally from the convex side |
| Germination model of bacilloid conidia | | Bacilloid conidia formed germ tubes at the end of conidia | Almost 90% germinated from one side of conidia at the 1/3 or 1/2 point, and only 10% from the end |

opportunity for mucilage to come into contact with nematodes decreased sharply. The adhesive rate consequently declined enough that they lost infectivity.

The production of dimorphic conidia in *E. vermicola* was reminiscent of the accessory conidia produced by some *Harposporium* species. The conidiogenous cells of accessory conidia also differ from that of typical *Harposporium*-like conidia (Wang *et al*., 2007). Hodge *et al*. (1997) suggested that the accessory conidia of *Harposporium* are synanamorph of *Hirsutella* species, which contains many insect pathogens. *Harposporium janus* (Shimazu and Glockling, 1997) was isolated from a beetle larva and proved to produce dimorphic conidia on pure culture, curved infected conidia and obovoid accessory conidia. It possesses two host organisms associated with two different spore types; beetle larvae were infected by obovoid conidia which are the only conidial type produced on the host cadavers, while bacterial feeding nematodes were parasitized by curved conidia which did not result in oboviod conidia from infected nematodes. *Harposporium janus* was the only species which has been reported to infect insects by obovoid conidia. It seems that the role of accessory conidia might be to parasitize other hosts, such as insects. However, as to the other species of *Harposporium*, the explanations are different. *Harposporium lilliputanum*, *H. cycloids*, and *H. cerberi* also produce obovoid accessory conidia in pure culture. However, their oboviod conidia were never observed to infect nematodes nor any other insects (Glockling and Shimazu, 1997; Wang *et al*., 2007). Therefore the function of accessory conidia in *Harposporium* is still unclear. Kubátová *et al.* (2000) reported that a strain of *E. vermicola* was isolated from the surface of larvae and adult bark beetles, *S. intricatus*, in Czech Pepublic. In order to ascertain the relationships between the beetle larvae and *E. vermicola*, and the role of cylindrical conidia, we used the beetle larvae of *M. alternatus* as the infected case-study target. However, it is not observed that the beetle larvae were attached or infected by the conidia of CNU 120806 strain, as they were still alive 15 days post-inoculation. Therefore, more research is required to ascertain the function of cylindrical conidia in *E. vermicola*.

*E. vermicola* is the first reported endoparasitic fungus of pinewood nematode and exhibits high infectivity both toward pinewood nematodes and saprophytic nematodes. As such, it shows potential as a bio-control agent against pinewood nematode and a possible solution to combat the pine wilting disease. The results of this study revealed some new characteristics, offered more information about *E. vermicola*, and added to the knowledge-base currently present in published literature. Our isolation of CNU 120806 was an attempt to prove that *E. vermicola* is not only restricted to pinewood nematodes, but also associated with other saprophytic nematodes. In Korea, reports of nematophagous fungi, especially the endoparasitic fungi, are rare. However, there is no room for complacency, as application of fungal resources to control pinewood nematodes provides a practical opportunity to resolve the devastating pine wilting disease.

## Acknowledgements

The authors would like to thank colleagues X.M. Zhu, J.F. Bao, Z. Wang, and H.F. Xu for assisting with research activities. Dr. B.Y. Tian is thanked for his helpful comments on preparing the manuscript. This work was supported by the project from Ministry of Agriculture and Forestry, Republic of Korea.

## References

Barron, G.L. and R.G. Thorn. 1987. Destruction of nematodes by species of *Pleurotus. Can. J. Bot*. 65, 774-778.

Bunyard, B.A., M.S. Nicholson, and D.J. Royse. 1994. A systematic assessment of *Morchella* using RFLP analysis of the 28S rRNA gene. *Mycologia* 86, 762-772.

Chen, S.Y., X.Z. Liu, and F.J. Chen. 2000. *Hirsutella minnesotensis* sp. nov.-a new parasites of soybean cyst nematode. *Mycologia* 92, 819-824.

Dackman, C., H.B. Jansson, and B. Nordbring-Hertz. 1992. Nematophagous fungi and their activities in soil, p. 95-103. *In* G. Stotzky and J.M. Bollag (eds.). Soil Biochemistry, Marcel Dekker, New York, N.Y., USA.

Glockling, S.L. and M. Shimazu. 1997. Culturing of three species of endoparasitic fungi infecting nematodes. *Mycol. Res*. 101, 55-60.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser*. 41, 95-98.

Hodge, K.T., N.M. Viaene, and W. Gams. 1997. Two *Harposporium* species with *Hirsutella* synanamorphs. *Mycol. Res*. 101, 1377-1382.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequence. *J. Mol. Evol*. 16, 111-120.

Kubátová, A., D. Novotný, K. Práil, and Z. Mrácek. 2000. The nematophagous hyphomycete *Esteya vermicola* found in the Czech Republic. *Czech Mycology* 52, 227-235.

Li, X., H. Luo, and K.Q. Zhang. 2005. A new species of *Harposporium* parasitic on nematodes. *Can. J. Bot*. 83, 1-5.

Li, T.F., K.Q. Zhang, and X.Z. Liu. 2000. Taxonomy of nematophagous fungi. Chinese Scientific and Technological Publication, China.

Liou, J.Y., J.Y. Shih, and S.S. Tzena. 1999. *Esteya*, a new nematophagous genus from Taiwan, attacking the pinewood nematode (*Bursaphelenchus xylophilus*). *Mycol. Res*. 103, 242-248.

Liu, X.F. and K.Q. Zhang. 2003. *Dactylella shizishanna* sp. nov., from Shizu Mountain, China. *Fungal Diversity* 14, 103-107.

Mo, M.H., X.W. Huang, W. Zhou, Y. Huang, Y.E. Hao, and K.Q. Zhang. 2005. *Arthrobotrys yunnanensis* sp. nov., the fourth anamorph of *Orbilia auricolor*. *Fungal Diversity* 18, 107-115.

Saitou, N. and M. Nei. 1987. The neighbour joining method; a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*. 4, 406-425.

Shimazu, M. and S.L. Glockling. 1997. A new species of *Harposporium* with two spore types isolated from the larva of a cerambycid beetle. *Mycol. Res*. 101, 1371-1376.

Syed, G.D., W.J. Li, A. Dayanand, B.S. Mudgulkar, S.K. Tang, X.P. Tian, and X.Y. Zhi. 2007. *Streptomyces gulbargensis* sp. nov., isolated from soil in Karnataka, India. *Antonie Van Leeuwenhoek* 91, 99-104.

Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin, and D.G. Higgins. 1997. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*. 24, 4876-4882.

Tzean, S.S., J.Y. Liou, and J.Y. Shih. 2001. Nematophagous fungus *Esteya vermicol*a. United States Patent. US 006168947B1.

Vilgalys, R. and M. Hester. 1990. Rapid genetic identification and

mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *J. Bacteriol*. 172, 4238-4246.

Wang, C.Y., M.H. Mo, X. Li, B.Y. Tian, and K.Q. Zhang. 2007. Morphological characteristics and infection processes of nematophagous *Harposporium* with reference to two new species. *Fungal Diversity* 26, 287-304.

Zhang, D., Y. Yang, L.A. Castlebury, and C.E. Cerniglia. 1996. A method for the large scale isolation of high transformation efficiency fungal genomic DNA. *FEMS Microbiol. Lett*. 145, 261-265.

Zhao, M.L., J.S. Huang, M.H. Mo, and K.Q. Zhang. 2005. A potential virulence factor involved in fungal pathogenicity: serine-like protease activity of nematophagous fungus *Clonostachys rosea*. *Fungal Diversity* 19, 217-234.