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First record of *Bursaphelenchus rainulfi* on pine trees from eastern China and its phylogenetic relationship with intro-genus species*

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Abstract: *Bursaphelenchus rainulfi* isolated from dead pine trees in Zhejiang, China, is described and illustrated. It also provided some molecular characters of the Chinese population, including the PCR-RFLP and sequences of ITS region and D2-D3 expansion region of the large subunit (LSU) rRNA gene. Both the morphological characters and ITS-RFLP patterns match with the original description. The phylogenetic trees based on the 13 sequences of D2-D3 expansion region of the LSU rRNA gene and ITS region of *Bursaphelenchus* species were constructed, respectively, with the results showing the similar clades. The phylogenetic relationship based on the molecular data is similar to that with morphological characters. This is the first report of the species on pine wood in eastern China.

Key words: *Bursaphelenchus rainulfi*, Pine wood, Morphology, Morphometrics, ITS-RFLP, Phylogeny
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INTRODUCTION

Species within the genus *Bursaphelenchus*, were regarded only as fungivorous organisms or associated with bark beetles until the insidious pine wood nematode *B. xylophilus* started to devastate entire coniferous forests in Japan (Mamiya and Enda, 1979). The pathogenic effects of this species were discovered later also in other East Asian countries with the nematode being found distributed in North America and in Europe on imported wood (Evans *et al*., 1996). More than 75 species have been described in the genus with most of them being tree-inhabiting and insect-vectored, and mainly living in conifers and transmitted by longhorn beetles (Cerambycidae) or bark beetles (Scolytidae) (Hunt, 1993; Ryss *et al*., 2005).

In China, the pine wood nematode was first detected in Nanjing, Jiangsu Province in 1982, afterwards the nematode was found distributed in east and south region of China, causing serious problem to conifers. In addition to *B. xylophilus*, there are other 11 species in the genus reported as occurring in China, i.e. *B. aberrans*, *B. dongguanensis*, *B. doui*, *B. hellenicus*, *B. hylobianum*, *B. hofmanni*, *B. hunanensis*, *B. leoni*, *B. lini*, *B. mucronatus*, and *B. sinensis*. *B. mucronatus* is non-pathogenic to conifers but often detected in dead conifers and *B. hunanensis*, *B. aberrans*, *B. dongguanensis*, *B. doui*, *B. lini* and *B. sinensis* are new species described from the conifers or packaging wood of conifers from China (Yin *et al*., 1988; Yin and Fang, 1995; Fang *et al*., 2002a; 2002b; Dan *et al*., 2003; Braasch, 2004; Braasch *et al*., 2005; Wang *et al*., 2004; Palmisano *et al*., 2004).

Studies of phylogenetic relationships among nematodes are not essential to taxonomy, but allow more complete understanding of the biology of nematodes as agricultural pests. Comparative analysis of coding and noncoding regions of ribosomal DNA has become a popular tool for construction of phy-

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logenetic trees of many organisms including nematodes. Phylogenetic analysis based on the sequences of ITS region of rDNA and D2-D3 expansion region of the large subunit (LSU) rRNA gene were used to examine evolutionary relationships among many group of nematodes including the genus *Bursaphelenchus* (Ferris *et al*., 1993; Hoyer *et al*., 1998; Iwahori *et al*., 1998; Maafi *et al*., 2003; Zheng *et al*., 2003; Madani *et al*., 2004; Subbotin and Sturhan, 2004; Burgermeister *et al*., 2005).

During a survey of the pine wilt disease caused by *B*. *xylophilus* in Zhejiang, a province in the east coast of China, *B*. *rainulfi* (Braasch and Burgermeister, 2002) was first found in dead pine trees of this area. This species was isolated for the first time in the Malaysia from pine wood (Braasch and Burgermeister, 2002), and then on Chinese packaging wood of *Callitris columellaris* (Ambrogioni *et al*., 2003). This paper presents the morphology, molecular characters, including the ITS region of rDNA and D2-D3 expansion region of the LSU rRNA gene, of *B*. *rainulfi* from Zhejiang, and the phylogenetic relationships of the species with some other intro-genus species.

MATERIALS AND METHODS

The nematodes were extracted from pine wood fragments with the modified Baermann funnel technique over 48 h. *B*. *rainulfi* was isolated and multiplied on *Botrytis cinerea* grown on maize culture in Petri dishes at 25 °C. Nematode specimens reared on *B*. *cinerea* were collected, heat killed, fixed in TAF and processed to glycerine. The specimens mounted in permanent slides were used for morphological and morphometric observations under a Zeiss Axioskop 2 plus microscope with an Olympus DP-10 digital camera. For each character mean, standard deviation, and range were calculated.

DNA extraction

Four nematode specimens were transferred into 20 μl of double distilled water on a clear glass slide, cut into several fragments. Ten microlitres of which were transferred into a 0.5 ml eppendorf tube, containing 8 μl nematode lysis buffer (125 mmol/L KCl, 25 mmol/L Tris-Cl pH 8.3, 3.75 mmol/L $MgCl_2$, 2.5 mmol/L DTT, and 1.125% Tween 20) on ice, and 2 μl of proteinase K (600 μg/ml) were added. The tube was incubated at 65 °C (1 h) and 95 °C (10 min) consecutively and finally centrifuged (1.5 min, 12000 r/min). The DNA suspension was used for PCR, or stored at −20 °C and used for further study.

DNA amplification

Twenty-five microlitres PCR reaction system was selected, including 2.5 μl 10×PCR buffer, 1.5 μl 25 mmol/L $MgCl_2$, 0.5 μl 10 mmol/L dNTP (Shanghai, China), 1 μl 40 μmol/L each primer of the two pairs primers (synthesized by Shanghai Sangon Biological Engineering Technology and Service Company, Shanghai, China), 1.5 U Taq polymerase (Shanghai, China), 2 μl template DNA suspension and 16.2 μl double distilled water. DNA amplification was carried out in a PTC-0150 MiniCycler (MJ Research Inc., Waltham, MA, USA).

Primers F1 (5′-CGT AAC AAG GTA GCT GTA G-3′) (Ferris *et al*., 1993) and V2 (5′-TTT CAC TCG CCG TTA CTA AGG-3′) (Vrain, 1993) were used to amplify the ITS region of rDNA. DNA thermal cycle consisted of 4 min at 94 °C, 10 cycles of 30 s at 94 °C, 1 min at 54 °C, and 1 min at 72 °C, and another 25 cycles of 15 s at 94 °C, 1 min at 54 °C, and 1 min at 72 °C, followed by a final elongation step of 10 min at 72 °C. After DNA amplification, 2 μl of PCR product was run on a 1% agarose gel (100 V, 50 min). The remainder was stored at −20 °C and used for further study.

Primers D2A (5′-ACA AGT ACC GTG AGG GAA AGT TG-3′) and D3B (5′-TCG GAA GGA ACC AGC TAC TA-3′) (de Ley *et al*., 1999) were used for amplification of D2-D3 expansion region of the LSU rRNA gene. DNA thermal cycle consisted of 4 min at 94 °C, 37 cycles of 30 s at 94 °C, 40 s at 55 °C, and 1 min at 72 °C, final elongation step of 8 min at 72 °C. After DNA amplification, 2 μl of PCR product was run on a 1% agarose gel. To verify the results, the experiments were repeated several times. The remainder was stored at −20 °C and used for further study.

Cloning and sequencing

Purified PCR products were cloned into pGEM-T vector and transformed into JM109 high efficiency competent cells. Several clones of the nematode were isolated by blue/white selection,

subjected to PCR, and then cycle-sequenced by Shanghai Sangon Biological Engineering Technology and Service Co., Shanghai, China.

RFLP procedures for ITS region and D2-D3 expansion region of the LSU rRNA gene

PCR products of both ITS region of rDNA and D2-D3 expansion region of the LSU rRNA gene were used for PCR-RFLP directly. Eight restriction enzymes, *Rsa*I, *Hae*III, *Msp*I, *Hin*fI, *Alu*I, *Bsh*1236I, *Hha*I and *Mbo*I were used for the digestion of 5 μl D2-D3 expansion region of the LSU rRNA gene and ITS region of rDNA PCR product. The digested DNA was loaded on a 1.5% agarose gel, separated by electrophoresis, stained with ethidium bromide, visualized and photographed under UV-light.

Phylogenetic analysis

The two sequences, ITS region of rDNA and D2-D3 expansion region of the LSU rRNA gene from the specimens of *B*. *rainulfi*, were submitted to GenBank, with the sequence Nos. DQ257621 and DQ257624, respectively. The sequences of *Bursaphelenchus* species were obtained with BLAST (basic local alignment search tool) from GenBank (Http://www.ncbi.nlm.nih.gov) according to the sequence of *B*. *rainulfi*. In order to compare ITS region with D2-D3 region, the sequences of common species were selected. Sequences were compared by Clustal X 1.8 using MEGA 2.1 software and the phylogentic trees were constructed based on both ITS region of rDNA and D2-D3 expansion region of the LSU rRNA gene sequences available on the GenBank. Kimura 2-parameter model was selected and UPGMA analysis was applied. Bootstrap analysis with 1000 replicates was performed to assess the degree of support for each clade on the trees.

RESULTS

Morphometrics and morphological characters of *Bursaphelenchus rainulfi* (Table 1, Fig.1)

Nematodes were collected from Shangyu, Zhejiang, east coast of China. The specimens display generally all the features of *B*. *rainulfi* according to the original description (Braasch and Burgermeister, 2002). Both females and males were slim nematodes with elongate-ovaled median bulb and distinct offset lips, and slightly ventrally curved when killed by heat. Stylet short (9~11 μm), with very slight basal swellings on the shaft. Excretory pore was situated *ca* level with posterior region of median bulb, only two lateral lines in the lateral field. There is a small vulval flap

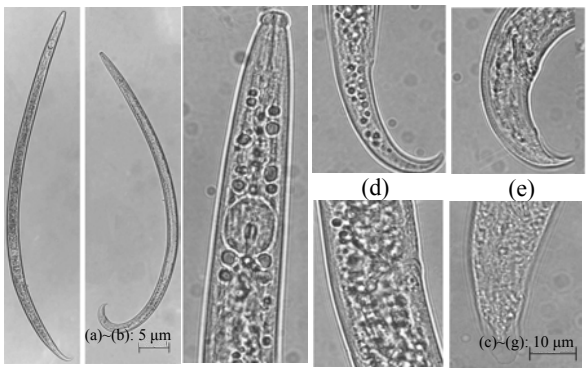

Fig.1 Photomicrographs of *Bursaphelenchus rainulfi* from China. (a) Female; (b) Male; (c) Female head region; (d) Female tail; (e) Male tail; (f) Female vulva region; (g) Bursa of male

Table 1 Morphometric comparison of *Bursaphelenchus rainulfi* from China with lectotypes

	B. *rainulfi* from Zhejiang Province		*B*. *rainulfi* lectotype*	
	Females	Males	Females	Males
n	11	11	15	15
L	611.0±43.0 (550~647)	611.0±26.5 (550~646)	661.0±61.0 (525~750)	584.0±74.0 (475~750)
a	31±0.82 (29~33)	32±3.00 (27~39)	32±5.10 (23~40)	35±5.00 (25~44)
b	8.4±0.98 (7.4~9.3)	7.0±0.79 (6.4~7.5)	9.1±0.70 (7.5~10.0)	8.0±0.90 (6.8~9.2)
c	16.9±0.98 (15.2~18.9)	18.6±1.23 (16.8~21.0)	17.0±1.50 (15.0~20.0)	25.0±4.40 (19.0~38.0)
c'	4.0±0.21 (3.5~4.4)	2.6±0.14 (2.3~2.8)	4.2±0.50 (3.4~4.9)	2.5±0.20 (2.2~2.9)
V	73.9±0.81 (67.0~72.7)	−	74.0±1.30 (72.0~76.0)	−
Stylet	10.3±0.43 (9.4~11.0)	11.1±0.79 (10.1~12.5)	12.0±0.80 (11.0~14.0)	12.0±0.60 (11.0~13.0)
Spicule	−	12.8±0.67 (11.5~13.6)	−	13.0±1.10 (12.0~15.0)

Note: All measurements in μm; * refer to Braasch and Burgermeister (2002)

and a tail with a rounded ventrally bent terminus in the female.

Male body J-shaped when killed by gentle heat. Spicules paired, dorsally arcuate, ventrally straighter, with a more or less blunt, prominent rostrum located almost in the middle of spicules, and distal tips of spicules without distinct cucullus. Tail with a pointed talon-like terminus and a small dorsoventral terminal bursa. Two subventral pairs of caudal papillae are present, including an adanal pair and a postanal pair in posterior half of the tail.

Molecular characterization of *Bursaphelenchus* species from Zhejiang, China

The amplification of the ITS region of the *Bursaphelenchus* species yielded one fragment with length of 1029 bp (DQ257621). The results of direct sequencing corresponded well with the sequence of *B. rainulfi* (AM157744) in the GenBank. Seven restriction enzymes except *Mbo*I (no digestion sites) were selected for RFLPs (Fig.2). Identical DNA restriction fragment patterns were obtained for Chinese population with the original Malay populations (Braasch and Burgermeister, 2002); general morphological observation and ITS-PCR-RFLP analysis of the *Bursaphelenchus* Shangyu population match well with the original description of *B. rainulfi*.

The length of fragment amplified from D2-D3 expansion region of the LSU rRNA gene of *B. rainulfi* was 773 bp. Eight of restriction enzymes can digest the amplified DNA fragment (Fig.3). These enzymes and the size of sub-fragments obtained for individual species are listed in Table 2.

Twelve sequences of D2-D3 expansion region of LSU rRNA gene and thirteen sequences of ITS region of *Bursaphelenchus* species and one *Ektaphelenchoides* species, obtained from GenBank, were compared with the *B. rainulfi* from China. UPGMA analysis of the ITS and D2-D3 sequences alignment yielded single parsimonious trees (Figs.4 and 5), in which, the sequence of ITS region of *B. rainulfi* from China shared 99.4% identity with the sequence of *B. rainulfi* (AM157744). The two phylogenetic trees based on the sequences of D2-D3 expansion region of LSU rRNA gene and of ITS region of *Bursaphelenchus* species showed similar clade trends, in which

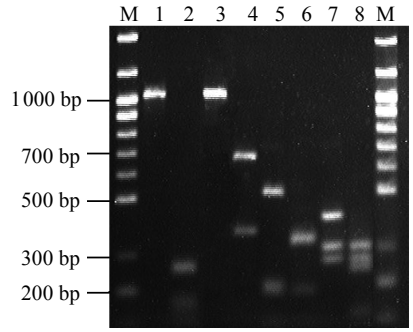

Fig.2 ITS-RFLP patterns of *B. rainulfi* from China
M: 100 bp marker; 1: Unrestricted PCR product; 2: *Rsa*I; 3: *Hae*III; 4: *Msp*I; 5: *Hin*fI; 6: *Alu*I; 7: *Hha*I; 8: *Bsh*1236I

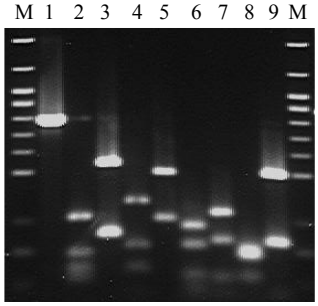

Fig.3 D2-D3 expansion region of LSU rRNA gene-RFLP patterns of *B. rainulfi* from China
M: 100 bp marker; 1: Unrestricted PCR product; 2: *Rsa*I; 3: *Hae*III; 4: *Msp*I ; 5: *Hin*fI; 6: *Alu*I; 7: *Bsh*1236I; 8: *Hha*I; 9: *Mbo*I

Table 2 Restriction fragment of amplified rDNA from *Bursaphelenchus rainulfi*

	PCR product (bp)	Restriction fragments (bp)							
		*Rsa*I	*Hae*III	*Msp*I	*Hin*fI	*Alu*I	*Hha*I	*Bsh*1236I	*Mbo*I
ITS of rDNA	1029	280	1029	700	520	380	480	310	−
		190	−	330	205	200	340	280	−
		180	−	−	200	100	295	270	−
		−	−	−	100	−	−	130	−
LSU rRNA gene	773	310	540	390	500	290	210	360	510
		200	240	220	300	230	205	240	240
		150	−	150	−	150	200	150	−
		110	−	−	−	100	150	−	−

the genetic divergence between rDNA of *B. rainulfi* and the other species is close, with *B. rainulfi* not having formed one obvious clade with others. *B. doui*, *B. xylophilus*, *B. mucronatus* and *B. fraudulentus* formed *B. xylophilus* group; *B. seani*, *B. fungivorus*, *B. hofmanni* and *B. thailandae* grouped together; *B. poligraphi*, *B. borealis*, *B. sexdentati*, *B. eggersi* formed another group.

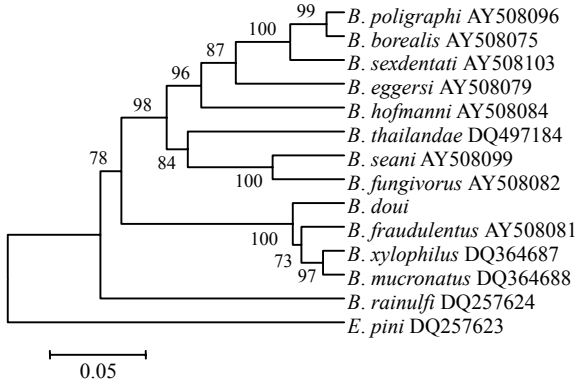

Fig.4 Phylogenetic tree constructed from the elision alignment of the D2-D3 expansion region of LSU rRNA gene sequence of 13 *Bursaphelenchus* species. Bootstrap values are based on 1000 replicates

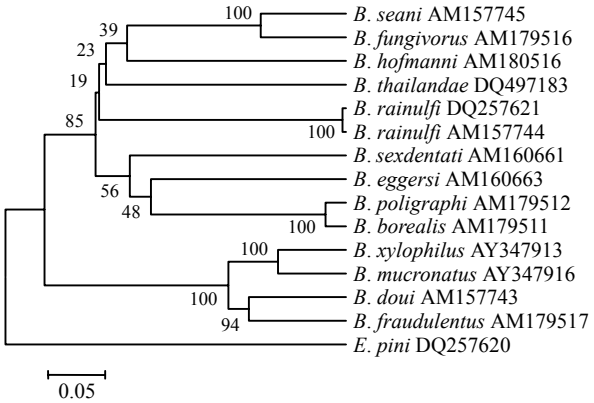

Fig.5 Phylogenetic tree constructed from the elision alignment of the ITS sequence of 13 *Bursaphelenchus* species. Bootstrap values are based on 1000 replicates

DISCUSSION

B. rainulfi, first recorded on coniferous wood in east China, matches consistently well the original description of Malay population for both morphological and molecular characters (Braasch and Burgermeister, 2002; Burgermeister *et al*., 2005).

Phylogenetic analysis showed that the homogeneity between *B. rainulfi* (DQ257621) from Zhejiang and *B. rainulfi* (AM157744) was high. All the results showed that the two populations belong to the same species. The original population of *B. rainulfi* was taken in a region close to the equator and was in the tropical area. This is the first report about the species distributed in eastern China, where the climate has subtropical characters.

B. rainulfi was found in an area where *B. xylophilus* is present, but where *B. rainulfi* was isolated on a large scale from dead trees, only a few *B. xylophilus* were detected in the same sample. We cannot confirm whether the nematode is fungivorous or pathogenic, and if the nematode in this area is indigenous or is introduced. All these need further studies.

LSU RNA gene, relatively conserved compared to the ITS region of rDNA, is more reliable and was used to constructed the phylogenetic relationship of different group of nematodes, such as *Longidorus* and *Xiphinema* species (He *et al*., 2005; de Ley *et al*., 1999). Braasch (2001) and Ambrogioni and Caroppo (2002) grouped *B. poligraphi*, *B. borealis*, *B. sexdentati* together named as *B. sexdentati* group according to the number of incisures, arrangement of the male caudal papillae, presence of a vulva flap and the shape of the female tail; meanwhile, *B. eggersi* formed another group due to its three lateral incisures. Ryss *et al.*(2005) based on the spicule structure, put *B. poligraphi*, *B. sexdentati* and *B. eggersi* into piniperdae-group. The morphological characterizations among xylophilus-group are similar. As a result, the two phylogenetic trees constructed based on ITS region of rDNA and the D2-D3 expansion of LSU rRNA gene show the same phylogenetic relationship trend within the species in the genus *Bursaphelenchus*, most of which corresponded well with the results from their morphological characterization, and supported the D2-D3 expansion region is an ideal region for phylogenetic studies of nematodes.

Compared to more than 75 species described in the genus *Bursaphelenchus*, only few sequences of D2-D3 expansion of LSU rRNA gene and ITS region were available in the GenBank, the phylogenetic trees constructed in this study only provide the trend of phylogenetic relationship of the species. It is better to sequence all the D2-D3 expansion of LSU rRNA gene

and ITS region of the species in genus *Bursaphelenchus* and conduct phylogenic analysis to estimate the molecular phylogenic relationship between species and groups of the species.

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