MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF THE NEMATODES PARASITIZING THE ROOTS AND THE STEMS OF *ASTRAGALAS MEMBRANACEUS* AND *DIOSCOREA OPPOSITE* FROM CHINA

C. Xu[1,2], H. Xie[1,*], J. Wang[2], Y. Wu[1], C. Zhang[1] and X. Jin[1]

[1] *Laboratory of Plant Nematology, Department of Plant Pathology, College of Natural Resources and Environment, South China Agricultural University, Guangzhou 510642, China*
[2]*Present address: Laboratory of Microbial Genetics, Department of Biochemistry, School of Life Sciences, Sun Yat-sen University, No. 135, West Xingang Rd., Guangzhou 510275, China*

Summary. Two nematode populations were extracted from infested roots of *Astragalas membranaceus* and stems of *Dioscorea opposite* from China. The two nematode populations shared morphological and morphometric characteristics and were similar to *Ditylenchus destructor*. Amplification of DNA sequences from the ITS-rDNA region of the two populations yielded products of 891 bp and 751 bp, with both of the sequences bearing more than 98% identity with the sequences of *D. destructor* deposited in the GenBank database. Partial 18S rRNA sequence amplification yielded a 1225 bp length product from both populations, with only 1.2% variance between the two populations and both having more than 98% identity with sequences deposited in the GenBank database for *D. destructor*. Therefore, the two nematode populations were identified as *D. destructor* and the two plant species must be considered new hosts for this nematode.

Keywords: *Ditylenchus destructor*, morphometrics, molecular characterization, new hosts.

Ditylenchus destructor Thorne, 1945, the potato rot nematode, had been mainly reported from temperate regions, where it is an important pest parasitizing potato tubers, bulbous iris, and 90~120 other species of plants (Sturhan and Brzeski, 1991). The nematode is listed among the quarantine pests for Europe (Plowright *et al.*, 2002; Smith *et al.*, 1992) and, since 2006, also for China (Ministry of Agriculture of the People's Republic of China, 2006).

Before 1980, the nematode populations parasitizing sweet potato in China were identified as *D. dipsaci* (Kühn) Filipjev, but Ding and Lin (1982) and Yin and Zhang (1983) identified the pathogen as *D. destructor* by morphological characteristics. Many nematologists in China have expressed doubt about the identity of the pathogen parasitizing sweet potato and other bulb or stem plants in the country, therefore, many surveys and identification studies have been undertaken by many research institutions. In 2004-2007, we also carried out a survey to identify the species of *Ditylenchus* infesting some bulb, stem and root plants in China, including roots of the medical plant milkvetch (*Astragalas membranaceus* Bge.) and rhizomes of the common vegetable yam (*Dioscorea opposite* Thune.) showing symptoms of infestation similar to those caused by *D. destructor* in potato and other plants (Sturhan and Brzeski, 1991). We extracted nematodes from roots and stems and conducted morphological and ITS characterization studies on them.

* Corresponding author e-mail: xiehui@scau.edu.cn

MATERIALS AND METHODS

Nematode populations

The two populations of the nematodes were extracted from the diseased roots of *A. membranaceus* (Fig. 1 A-D) and the diseased tubers of *D. opposite* (white arrows in Fig. 1 E-F), the former from Dingxi city, Gansu province, and the latter from Pingyao county, Shanxi province, in China. Nematodes were extracted by a modified Baermann funnel method (Hooper, 1991). Some of the nematodes were killed and fixed in 4% formalin for long-term preservation and for further morphological study, while the rest were put in 1.5-ml Eppendorf tubes and preserved at 4 °C in a refrigerator for live conservation and for scanning electron microscopy (SEM) and molecular studies.

Morphological study

Fixed nematodes were transferred to anhydrous glycerol by a modification of Seinhorst's glycerol-ethanol method (Seinhorst, 1959). Specimens were mounted in glycerin on permanent slides. Twenty females and twenty males of each population were examined and measured using an Olympus CX40 microscope (Japan).

Live nematodes were washed three times with distilled water, prefixed by glutaraldehyde (2.5% final concentration) and fixed again by 1% osmic acid, then dehydrated by passing them through a gradual ethanol gradient of 10, 20 ····· 100% at room temperature. The dehydrated specimens were then critical point dried with liquid CO_2, mounted on stubs and coated with gold-palladium before observation with a XL-30 (FEI Corp.) SEM at 10 kV.

Table I. Measurements and morphometric indices of *Ditylenchus destructor* from *Astragalas membranaceus* and *Dioscorea opposite* [Measurements in µm and in the form: mean ± standard deviation (range)].

Character	Population from *Dioscorea opposite*		Population from *Astragalas membranaceus*	
	Female (20)	Male (20)	Female (20)	Male (20)
L	917.8±85.19(810-1062.5)	833.9±51.42(765-981.3)	858.9±54.6(725-962.5)	872.4±56.9(763.8-995)
W	27.1±3.39(22.5-35)	20.9±2.13(18.3-28.3)	24.2±1.8(21.3-27.5)	22.7±1.2(20-25)
Stylet	10.1±0.28(9.5-11)	10.1±0.24(10-11)	11.9±0.9(11-13)	11.3±0.7(10-12)
DGO	0.9±0.12(0.6-1)	0.8±0.19(0.5-1)	1.3±0.3(1-2)	1.2±0.3(0.7-2)
VMB	50.7±2.35(45-50)	52.1±3.83(45-62.5)	52.3±3.8(43.8-60)	51.2±2.5(45-55)
AEGL	141.7±8.66(127.5-162.5)	142.3±8.9(127.5-167.5)	141.8±8.8(127.5-162.5)	142.6±7.6(127.5-155)
AEPL	102.6±6.02(93-112)	100.4±6.15(88-113)	103±5.4(93-115)	101.7±6.2(89-111)
Metacorpus	9.3±1.12(7-11)	8.9±0.67(7-10)	8.4±0.9(7.5-10)	8.3±0.7(7-9.3)
Metacorpus	15.7±1.21(13-18)	14.9±1.14(12-17)	16.4±1.4(15-20)	16±1.3(13-18)
OES	130.6±8.57(116.5-150.5)	131.2±8.83(116.5-156)	128.8±8.7(114.5-148.5)	130.3±7.6(114.5-143)
Overlap	13.6±7.79(4-31)	9.8±6.07(0-21)	10.1±5.8(3-30)	10±5.5(2-20)
a	34.2±3.18(28.3-42.9)	40.3±4.65(27.1-49.1)	35.7±3.3(29.7-44.3)	38.6±2.2(32.8-41.7)
b	7.2±0.71(6.1-8.8)	6.3±0.44(5.7-7.3)	6.5±0.5(5.4-7.9)	6.6±0.5(5.7-7.5)
b'	6.5±0.78(5.3-7.9)	5.9±0.28(5.4-6.4)	6.1±0.5(5.1-7.5)	6.1±0.4(5.3-6.9)
c	14±1.7(11.8-18.3)	13.2±0.86(11.7-14.6)	13.2±1.1(10.7-15.9)	13.8±0.9(12.5-15.1)
c'	4.7±0.48(3.7-6)	5.1±0.43(4.3-6)	4±0.3(3.4-4.7)	4.6±0.3(4-5.2)
V	80.2±1.5(77.2-84.1)		79.9±3.2(75.2-92.7)	
V'	86.5±1.22(83.6-89.4)		85.8±1.5(82.6-88.3)	
T		59.4±5.85(44.5-67.1)		43.7±4.3(35-51.2)
O	0.12±0.05(0.1-0.3)	0.11±0.02(0.1-0.2)	0.11±0.03(0.1-0.2)	0.1±0.03(0.1-0.2)
PUS/VBW	3.2±0.35(2.4-4)		3.8±0.5(2.9-4.8)	
Overlap/w	0.5±0.32(0.1-1.3)	0.5±0.3(0-1.1)	0.4±0.2(0.1-1.2)	0.4±0.2(0.1-0.8)
Procorpus/OES	0.2±0.02(0.2-0.3)	0.3±0.02(0.2-0.3)	0.2±0.03(0.2-0.3)	0.2±0.02(0.2-0.3)
Isthmus/OES	0.3±0.03(0.3-0.4)	0.3±0.04(0.2-0.4)	0.3±0.03(0.3-0.4)	0.3±0.05(0.2-0.4)
Gland/OES	0.3±0.03(0.3-0.4)	0.3±0.04(0.2-0.4)	0.3±0.03(0.3-0.4)	0.3±0.05(0.2-0.4)
Hemizonid	3.6±0.62(3-5)	3.7±0.54(3-5)	4±0.53(3.5-6)	3.9±0.58(3-5)
MB	0.4±0.03(0.3-0.4)	0.39±0.02(0.35-0.43)	0.41±0.04(0.3-0.5)	0.4±0.03(0.3-0.4)
VBW	24±2.66(20-27.5)		22±2.1(17.5-25)	
PUS	76.1±7.59(62.5-90)		83.5±10.9(60-107.5)	
VA	114.9±13.51(95-145)		113.3±12.9(95-142.5)	
PUS/VA	0.7±0.07(0.6-0.9)		0.74±0.1(0.5-0.9)	
Tail	65.8±5.53(57.5-76.3)	63.3±4.89(53.8-72.5)	65.4±5.4(55-77.5)	63.3±4.6(55-72.5)
ABW	14.2±1.31(12.5-16.3)	12.4±0.59(10-13)	16.3±1.5(13.8-20)	13.7±0.8(12.5-15)
Guber length		6.9±0.45(6-8)		7.9±0.7(7-9.3)
Spicule length		19.3±1.03(18-22)		22.8±1.5(20-25)

The abbreviations used in the tables are partly according to de Man, while other abbreviations are as follows: W = width of body; DGO = distance from the base of the stylet to the dorsal esophageal gland orifice; MB = distance from the cephalic region to the middle of the metacorpus; AEGL = distance from cephalic region to the end of the oesophagus; AEPL = distance from the cephalic region to the excretory pore; Guber Length = gubernarculum length; OES = Oesophagus length; Overlap = length of gland overlapping intestine; PUS = length of post-vulva uterine sac; VBW = body width at vulva; ABW = body width at anus; VA = distance from vulva to anus.

Molecular study

DNA extraction. Single nematode DNA extractions were achieved as described by Ma *et al.* (2006) and Subbotin *et al.* (2005). A single adult live nematode was picked, washed three times with sterile water, and cut into 2-3 fragments in 8 µl of triple-distilled water on a glass slide with a sterile scalpel. The sample was then transferred into 200 µl PCR tubes with 1 µl 10x PCR buffer (Takara Biotech, Dalian, China) and 1 µl 20 mg/ml proteinase K (Takara Biotech). The tubes containing the mixture were put into a -80 °C refrigerator for 30 min. or more for better cell lysis, incubated for 1 h at 65 °C and for 15 min. at 95 °C consecutively to make the proteinase K inactive. After centrifugation, the supernatant DNA suspension was used for PCR reaction with an appropriate reaction mixture (below).

ITS-rDNA PCR amplification. The primers set (Ma *et al.*, 2006) was forward primer PF1: 5'-CGTAACAAGGTAGCTGTAG-3' and reverse primer PR1: 5'-

TTTCACTCGCCGTTACTAAGG-3', synthesized by Invitrogen Biotech (Shanghai, China). The reaction volume was 50 µl, consisting of DNA suspension 8 µl, 10x PCR buffer (including Mg^{2+}) 5 µl, dNTP (2.5 mM) 2 µl, each primer (10 µM) 1.5 µl, r*Taq* DNA polymerase 0.3 µl (Takara Biotech), and made up to 50 µl with triple-distilled water; the procedure was pre-heating at 94 °C 5 min., followed by 40 cycles of 1 min at 94 °C, 30 s at 55 °C, 1 min. at 72 °C, and a final incubation for 10 min. at 72 °C. After DNA amplification, 5 µl of the product was run on a 1% 1x TAE buffer agarose gel, and stained with ethidium bromide. After checking, the gels were photographed with a UVP Gel Image System (Alpha Innotech, USA). The remainder of the product was stored

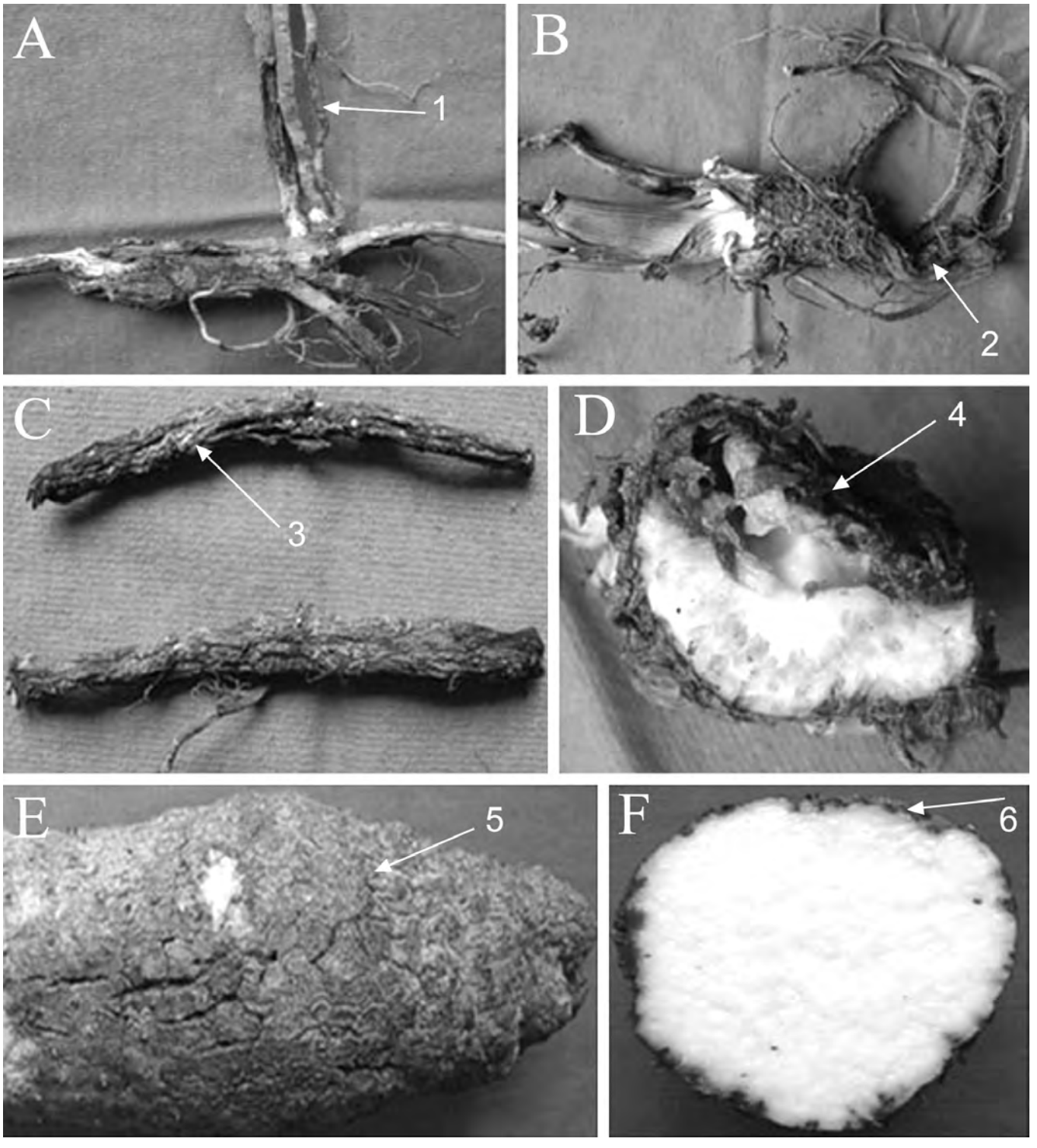

Fig. 1. Symptoms caused by *Ditylenchus destructor* on the roots. A-D *Astragalas membranaceus*: rot root on the whole roots (arrows 1-3) and on a transverse section (arrow 4). E-F *Dioscorea opposite*: ramous surface of an entire tuber (arrow 5) and on a transverse section (arrow 6). (The nematodes were extracted from these diseased tissues).

at -20 °C and then used for sequencing. The ITS amplification products were purified and sequenced by Invitrogen Biotech using an ABI 3730 genetic Analyzer. The two sequences obtained are deposited in the GenBank database under accession numbers EF088931 and DQ471335.

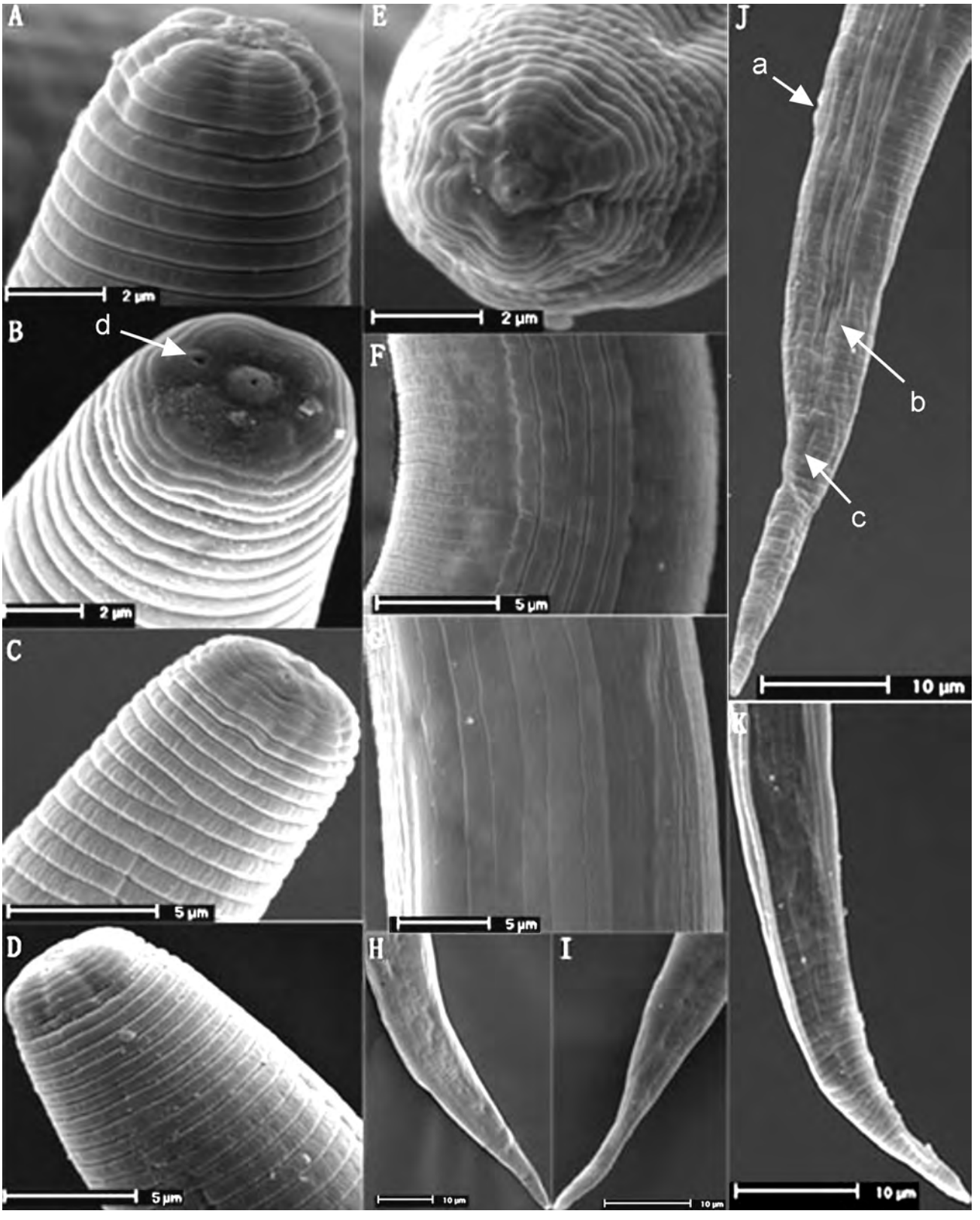

Fig. 2. SEM photographs of *D. destructor* from *A. membranaceus* and *D. opposite*. *Astragalas membranaceus* population: A, D, E, lip region of females, six lips; F, six incisures in lateral side; I, J, tails of females showing anus (arrow a), the position of four incisures in lateral field (arrow b), the position of two incisures in lateral field (arrows c). *Dioscorea opposite* population: B, C, lip region of females, with arrow pointing to the amphid aperture; G, incisures in lateral side; H, K, tails of females.

18S rDNA gene amplification. The primers were designed according to the GenBank database sequences of the 18S conserved region of *Ditylenchus* spp. with the software of DNAstar. Forward primer DF1 was 5'-TAACGAGTATCTATGAGAGGGCAAG-3' and reverse primer DR2 was 5'- TGATCGTTAATGATCCG-GCAGC-3'. The reaction conditions were similar to those for ITS amplification, but with the annealing temperature at 52 °C and extension of 2 min at 72 °C.

PCR products were purified by NaAC (3M) and two times the volume of ethanol, washed twice with 70% ethanol, then dissolved in 20 µl 1x TE buffer. DNA fragments were ligated to pMD18 T- Vector (Takara Biotech) as per the manufacturer's instruction. The ligated DNA was transformed into *Escherichia coli* DH 5x, plated onto LB-Amp+ plates, and cultured for 6~12 h at 37 °C. The positive clones were verified by PCR. Two positive PCR clones of each population were purified and sequenced by Invitrogen Biotech. The two sequences are deposited in the GenBank database under accession numbers EU188727 and EU188747.

RESULTS

Morphological identification (Fig. 2).
Measurements and morphometrics. See Table I.

Female. Body with slightly ventral curvature in posterior part when killed by heat; cephalic region low and flattened, continuous or slightly set off from the body, 2~3 µm high and 6-8 µm width, with four annuli and six lips. Lateral fields with six incisures (occasionally more than six incisures found in the population from *A. menbranaceus*), beginning with two incisures at the level of the stylet knobs (about 20 annuli from the lip region), and gradually increasing to three at the level of the back of procorpus of oesophagus and to six at the level of the back of median bulb of oesophagus, before gradually reducing to four at about 1/3 distance along the tail and to two at about 2/3 distance along the tail.

Labial framework moderately developed, stylet not strong but with distinct small basal knobs, stylet conus about half length of stylet. Procorpus of oesophagus slender and long, about 0.2~0.3 times the length of the oesophagus, median bulb fusiform with bulb membrane, isthmus narrow, about 0.2~0.4 times the length of the oesophagus, gland mostly overlapping intestine from dorsal or ventral side, occasionally lateral side or without overlapping. Excretory pore between the middle of the isthmus and anterior part of gland, hemizonid just anterior to the excretory pore.

Epiptygma flattened or projecting; single genital gland stretching forward, ovary usually not reflexed but reflexed in a few females, oocytes in a single or double row in the anterior part and single row nearer the uterus; spermatheca with sperms, post-vulval branch wide and sac-like without sperms; tail with slight ventral curvature, elongated conoid (*A. membranaceus* population) and the posterior cylindrical in the *D. opposite* population, tail tip rounded.

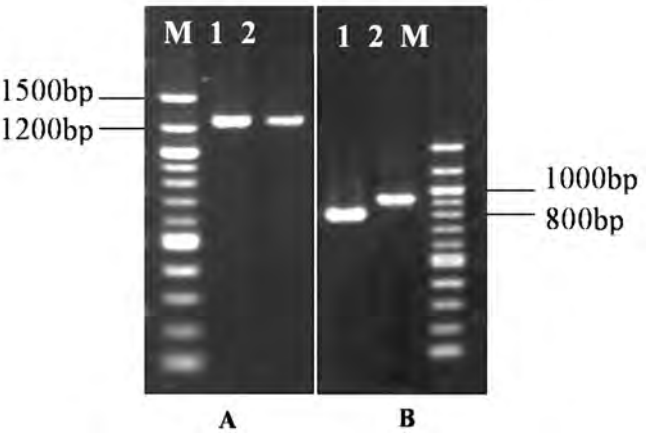

Fig. 3. Electrophoresis photograph of 18S (A) and ITS (B) products of *D. destructor* from *D. opposite* and *A. menbranaceus*. Product of 18S: 1, *D. opposite*; 2, *A. membranaceus*; M, 100 bp ladder Marker. Product of ITS: 1, *D. opposite*; 2, *A. membranaceus*; M, 100 bp ladder Marker.

Male. Similar to female in its general morphology, but with some obvious sexual characteristics. Body more slightly ventrally curved than females, bursa extending to about 3/4 of tail length; strengthened spicules curved ventrally, usually not out of the anus, gubernaculum short and simple.

Molecular identification.

PCR amplification of the ITS region of the two populations yielded two different products, with lengths of 751 bp for the *D. opposite* population and 891 bp for *A. membranaceus* (including 18S, 28S blanking partial sequence, ITS1, 5.8S, ITS2) (Fig. 3B). From a blast in NCBI, the *A. membranaceus* population shared 98% identity with the sequence of GenBank accession number AY987007 from the USA and 40 other sequences (such as GenBank accession number EF208213 and so on); the *D. opposite* population gave a similar result. According to the blast result, the two sequences had different nucleotides in the ITS1 region (*D. opposite* population misses 140 bp nucleotides compared with *A. membranaceus* population), and the other parts were completely identical with max identity of 98%.

PCR amplification of part of the 18S gene yielded a single product with a length of 1225 bp (Fig. 3A), with 1.2% variance between the two populations. From a blast in NCBI, they had more than 98% identity with the deposited GenBank 18S gene sequences of *D. destructor*.

DISCUSSION

The most important morphological and morphometric characteristics were compared between the *A. mem-*

branaceus and *D. opposite* populations, and also with others for *D. destructor* from the literature. The results showed little variation within the two populations and from these and those reported for *D. destructor* infesting potatoes (Thorne, 1945; Brzeski, 1991), iris bulbs and potatoes (Wu, 1960 a, b). Also, the morphological and morphometric characteristics were almost identical to those of *D. destructor* infesting sweet potatoes, potatoes and mints in China (Ding and Lin, 1982; Yin and Zhang, 1983; Zhang *et al.*, 2006).

Morphological identification is difficult with some species of nematodes. Molecular diagnostics are becoming more and more important, especially the study of the ITS-rDNA region, which is a very conserved taxonomic marker for many genera of nematodes. However, occasionally, some misdiagnoses, as with species of *Meloidogyne*, have been made (Powers *et al.*, 1997; Powers, 2004). The 18S ribosomal genes are more conserved than the ITS region, and is very informative at the species level in Nematoda (Powers, 2004). Therefore, in this study, both the ITS and 18S gene were used to identify the nematodes. According to ITS amplification and sequences from blast results of the two populations in the Genbank database, both nematode populations had more than 98% identity with the already deposited sequences of *D. destructor* in the GenBank database. Though their nucleotides numbers were different in ITS1, their ITS2 and 5.8S gene sequences share high homology, with a 98% overall identity. The sequence from the 18S amplification also showed that the two populations may actually be the same species, because few base pairs diverge between them, and there is high identity for both with the already deposited sequences of *D. destructor* in the GenBank database. Thus, the two nematode populations were both identified as *D. destructor*, although they may warrant different infra-species classification status, which will be reported on elsewhere.

In China, *A. membranaceus* is an important medicinal plant with many uses in medical therapy and *D. opposite* is a kind of vegetable. This is the first report of *D. destructor* parasitizing *A. membranaceus* and *D. opposite*, which are, therefore, new hosts for the nematode.

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