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Characterisation of *Meloidogyne* species from China using isozyme phenotypes and amplified mitochondrial DNA restriction fragment length polymorphism

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Abstract

Forty-six *Meloidogyne* populations from 14 provinces of China were characterised in terms of malate dehydrogenase, esterase phenotypes and *Hin*fI restriction profiles of amplification products from the mitochondrial DNA (mtDNA) region between the *COII* and *lrRNA* genes. Isozyme phenotyping revealed that 29 of the populations were *M. incognita*, six were *M. javanica*, six were *M. arenaria*, three were *M. hapla* and two were *M. enterolobii*. *Hin*fI restriction patterns of the *COII-lrRNA* region correlated with nematode isozyme phenotypes and enabled reliable differentiation and identification of the five root-knot nematodes occurring in China. The size and sequence of the mtDNA amplification product were determined for the first time for *M. enteroloii*, a potentially economically important crop pathogen. Sequence comparison showed that the sequence of the intergenic region between the *COII* and *lrRNA* genes for *M. enterolobii* was identical to that reported for *M. mayaguensis*. Together with published observations on morphology, host range and esterase phenotype of the two nominal species, the mtDNA sequence evidence suggests that *M. mayaguensis* could be conspecific with *M. enterolobii*.

Introduction

Root-knot nematodes (*Meloidogyne* spp.) are a major group of pathogens of agricultural crops in China. These root endoparasites are widely distributed from the tropical south to the temperate north, infecting many crop species and causing crop losses up to 70% (Xu et al., 1994). According to several surveys, *M. incognita* is the predominate species of root-knot nematodes in China, followed by *M. javanica*, *M. arenaria* and *M. hapla* (Pan, 1984; Li and Yu, 1991; Zhang and Weng, 1991; Wang et al., 2001). Other species of *Meloidogyne* that have been recorded from China include *M. enterolobii*, *M. graminicola*, *M. brevicauda* and *M. mali*, of which *M. enterolobii* is regarded as a potential threat to agriculture as the nematode is able to reproduce on resistant tobacco and tomato cultivars and cotton (Yang and Eisenback, 1983; Xu et al., unpubl. data).

Effective management of root-knot nematodes often requires rapid and accurate species identification of target nematode populations. *Meloidogyne* species are traditionally differentiated from each other by morphological characters and isozyme phenotypes. Morphological identification demands considerable skill and could be unreliable due to significant intraspecific morphological variations in *Meloidogyne* spp. Isozyme phenotyping has been shown to be a valuable tool for precise identification of major *Meloidogyne* species (Esbenshade and Triantaphyllou, 1985a,b; Carneiro et al., 2000). A limitation of this technique is that juveniles, males and eggs cannot be reliably diagnosed, which hinders its use in routine examination of soil samples that often contain only second-stage juveniles.

Recent progress in the development of PCR-based molecular diagnostics has offered a potential replacement for the traditional procedures. Powers and Harris (1993) discriminated five major species of *Meloidogyne* by amplification and restriction of the intergenic region between the *COII* and *lrRNA* genes in the mitochondrial genome (mtDNA-PCR–RFLP). Zijlstra et al. (2000) achieved rapid identification of *M. incognita*, *M. javanica* and *M. arenaria* by developing species-specific sequence characterised amplified region (SCAR) primers. For practical application, the mtDNA method appears more attractive as DNA markers can be readily amplified with two conserved primers from a single juvenile of a number of *Meloidogyne* species. However, the general applicability of this assay still needs to be confirmed since only limited nematode species and populations have been examined and differing results for some species have been reported (Williamson et al., 1994; Orui, 1998; Blok et al., 2000). In the present study, we investigated malate dehydrogenase (Mdh) and esterase (Est) phenotypes and mtDNA-PCR–RFLP patterns of 46 *Meloidogyne* populations collected from 14 provinces of China. Our objectives were to determine the species identity of the populations and assess the utility of mtDNA polymorphism for reliable diagnosis of major root-knot nematodes occurring in China.

Materials and methods

Nematode populations

Forty-six *Meloidogyne* populations used in this study are listed in Table 1. All the populations were derived from single egg-masses from field populations. The original field populations were randomly collected from infected economic plants in 14 provinces of China. Nematodes were routinely maintained on tomato cv. Sukang 8 at 20–28 °C in a greenhouse.

Isozyme analysis

Malate dehydrogenase and Est activities were analysed according to the procedure of Esbenshade and Triantaphyllou (1985a) with minor modifications. Protein extracts from one to four females were loaded into individual lanes of 7% polyacrylamide gels. In each case, a reference population of *M. javanica* was included. After electrophoresis, the gels were stained first for Mdh for 10 min, and then for Est for an additional 20 min. Designation of enzyme phenotypes followed Esbenshade and Triantaphyllou (1985b) and Carneiro et al. (2000).

DNA preparation

Nematode egg-masses were hand-picked from infected tomato roots and second-stage juveniles (J2) were hatched in water at 25 °C. Total genomic DNA was then extracted from ca. 10 µl of packed juveniles using the method of Cenis (1993), except that an incubation of the nematode solution for 1 h at 65 °C was added following the grinding of nematodes. The quality and quantity of extracted DNA samples were checked by subjecting them to electrophoresis and ethidium bromide staining together with uncut λ DNA standards. For PCR amplification from single J2, individual J2 were hand-picked and ruptured with a steel needle in a 5-µl drop of sterile water on a glass slide. The crude nematode lysates were then transferred to PCR reaction tubes and used directly as templates for amplification.

PCR–RFLP procedure

Primers C2F3 (5′-GGTCAATGTTCAGAAATTTGT-GG-3′; Powers and Harris, 1993) and MRH106 (5′-AATTTCTAAAGACTTTTCTTAGT-3′; Stanton et al., 1997) were used to amplify the intergenic region between the cytochrome oxidase subunit II (*CO II*) and 16S rRNA (*lrRNA*) genes in the mitochondrial genome of *Meloidogyne*. Primer MRH106 is located ~130 bp downstream from primer 1108 which was originally used by Powers and Harris (1993). PCR was conducted in a 25-µl reaction volume containing 10 ng of purified DNA or 5 µl of crude J2 lysate, 0.2 µM of each primer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 1 × *Ex Taq* PCR buffer and one unit of *Ex Taq* DNA polymerase (TaKaRa Biotech, Dalian, China). For amplification using purified DNA, reaction mixtures were subjected to a preheating at 94 °C for 4 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 50 °C and 2 min at 70 °C, and a final incubation at 72 °C for 10 min using a Mastercycler Personal thermal cycler (Epperndorf, Hamburg, Germany). For amplification using crude J2 lysates, the number of cycles was raised to 40. Following amplification, 5 µl of PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualised under UV illumination. *Hin*fI-digestion of amplified products was conducted in a 10-µl volume

Table 1. *Meloidogyne* populations used in this study

Species	Population	Origin	Primary host	Isozyme phenotypes		mtDNA-PCR–RFLP	
				Mdh	Est	PCR product (bp)	*Hin*fI digestion (bp)
M. incognita	MIAH1	Dangsan, Anhui	Eggplant	N1	I2	1800	1300, 400, 100
	MIFJ1	Nanan, Fujian	*Myrica rubra*	N1	I2	1800	1300, 400, 100
	MIGD2	Guangdong	*Wisteria sinensis*	N1	I2	1800	1300, 400, 100
	MIGD3	Guangzhou, Guangdong	Cabbage	N1	I2	1800	1300, 400, 100
	MIGX1	Nanning, Guangxi	Banana	N1	I2	1800	1300, 400, 100
	MIGX2	Nanning, Guangxi	Banana	N1	I2	1800	1300, 400, 100
	MIHB1	Wuhan, Hubei	Cowpea	N1	I2	1800	1300, 400, 100
	MIHN1	Henan	Yam	N1	I2	1800	1300, 400, 100
	MIHN2	Zhengzhou, Henan	Kiwi fruit	N1	I2	1800	1300, 400, 100
	MIHuN1	Hunan	Tobacco	N1	I2	1800	1300, 400, 100
	MIHuN2	Hunan	Lettuce	N1	I2	1800	1300, 400, 100
	MIJS1	Nanjing, Jiangsu	Fig	N1	I2	1800	1300, 400, 100
	MIJS2	Huaiyin, Jiangsu	Cucumber	N1	I2	1800	1300, 400, 100
	MIJS3	Nanjing, Jiangsu	Tomato	N1	I2	1800	1300, 400, 100
	MIJS4	Ganyu, Jiangsu	Tomato	N1	I2	1800	1300, 400, 100
	MIJS5	Liangyungang, Jiangsu	Cucumber	N1	I2	1800	1300, 400, 100
	MIJS6	Rugao, Jiangsu	*Buxus sinica*	N1	I2	1800	1300, 400, 100
	MIJS7	Nanjing, Jiangsu	*Salvia plebeia*	N1	I2	1800	1300, 400, 100
	MIJS8	Nanjing, Jiangsu	Cowpea	N1	I2	1800	1300, 400, 100
	MISD1	Pingyi, Shandong	*Cyclamen persicum*	N1	I2	1800	850, 450, 400, 100
	MISD2	Juye, Shandong	Eggplant	N1	I2	1800	1300, 400, 100
	MISD3	Juye, Shandong	Cowpea	N1	I2	1800	1300, 400, 100
	MISH1	Shanghai	*Hosta plantaginea*	N1	I2	1800	1300, 400, 100
	MITW1	Taiwan	*Rhododendron simsii*	N1	I2	1800	1300, 400, 100
	MITW2	Taiwan	*Gerbera jamesonii*	N1	I2	1800	1300, 400, 100
	MITW3	Taiwan	*Aloe vera*	N1	I2	1800	1300, 400, 100
	MIYN1	Lunan, Yunnan	Tobacco	N1	I2	1800	1300, 400, 100
	MIZJ1	Hangzhou, Zhejiang	Tomato	N1	I2	1800	1300, 400, 100
	MIZJ2	Hangzhou, Zhejiang	Celery	N1	I2	1800	1300, 400, 100
M. javanica	MJFJ1	Fuzhou, Fujian	Tobacco	N1	J3	1800	1800
	MJGD1	Guangzhou, Guangdong	*Serissa serissoides*	N1	J3	1800	1800
	MJGX1	Nanning, Guangxi	Banana	N1	J3	1800	1800
	MJGX2	Nanning, Guangxi	Tomato	N1	J3	1800	1800
	MJHN1	Zhengzhou, Henan	Tomato	N1	J3	1800	1800
	MJYN1	Kunming, Yunnan	Tobacco	N1	J3	1800	1800
M. arenaria	MAJS1	Xuzhou, Jiangsu	Tomato	N1	A2	1300	1200, 100
	MAJS2	Nanjing, Jiangsu	*Pinus massoniana*	N1	A2	1300	1200, 100
	MAJS3	Ganyu, Jiangsu	Tomato	N1	A2	1300	1200, 100
	MASD1	Anqiou, Shandong	Ginger	N1	A2	1300	1200, 100
	MATW1	Taiwan	*Lycium chinense*	N1	A2	1300	1200, 100
	MAYN1	Kunming, Yunnan	Tobacco	N1	A1	1800	1700, 100
M. hapla	MHSD1	Heze, Shandong	*Paeonia suffruticosa*	H1	H1	670	570, 100
	MHSD2	Shandong	Peanut	H1	H1	670	570, 100
	MHYN1	Kunming, Yunnan	*Salvia splendens*	H1	H1	670	570, 100
M. enterolobii	MEHN1	Hainan	*Psidium guajava*	N1a	VS1–S1	850	850
	MEHN2	Hainan	*Psidium guajava*	N1a	VS1–S1	850	850

containing 5 µl of PCR product, 1 µl of *Hin*fI restriction buffer and 15 units of *Hin*fI (TaKaRa Biotech). Digestion was allowed to proceed for 4 h at 37 °C. Restricted products were assayed on a 2% agarose gel.

Cloning and sequencing

The PCR product amplified from population MEHN1 of *M. enterolobii* was excised from an agarose gel and purified using an UNIQ-10 DNA purification kit (Sangon Biotechnology, Shanghai, China). The gel-purified fragment was then cloned into the pMD 18-T vector (TaKaRa Biotech). Sequencing was done on an ABI PRISM 377-96 sequencer using BigDye terminator chemistry (Perkin Elmer Applied Biosystems, Foster City, USA). Two clones from population MEHN1 were sequenced. Sequences were compiled using BioEdit Version 5 (T.A. Hall, North Carolina State University, USA). Sequence alignment was performed with CLUSTAL W (http://www.genome.ad.jp).

Results

Isozyme analysis

Malate dehydrogenase and Est activities were investigated for the 46 Chinese populations of *Meloidogyne*. Three Mdh phenotypes (i.e. N1, H1 and N1a) and six Est phenotypes (i.e. I2, J3, A1, A2, H1 and VS1–S1) were detected (Figure 1). Based on the established relationship of Mdh–Est profiles and species of *Meloidogyne*, five species were identified from the populations, of which 29 were *M. incognita* (Mdh–Est phenotype N1–I2), six were *M. javanica* (N1–J3), six were *M. arenaria* (N1–A2 and N1–A1), three were *M. hapla* (H1–H1) and two were *M. enterolobii* (N1a–VS1–S1) (Table 1).

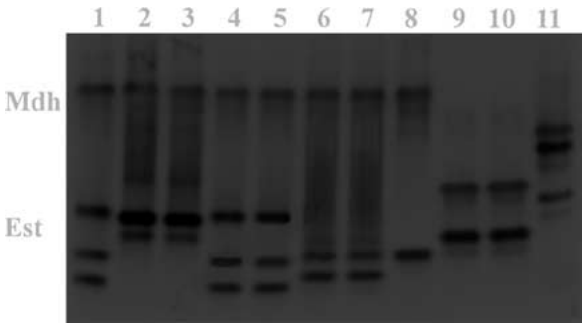

Figure 1. Malate dehydrogenase (Mdh) and esterase (Est) phenotypes of representative populations of *M. incognita*, *M. javanica*, *M. arenaria*, *M. hapla* and *M. enterolobii* from China. 1: MJYN1 (Mdh–Est phenotype N1–J3); 2: MIJS1 (N1–I2); 3: MIHN1 (N1–I2); 4: MJFJ1 (N1–J3); 5: MJGD1 (N1–J3); 6: MAJS1 (N1–A2); 7: MATW1 (N1–A2); 8: MAYN1 (N1–A1); 9: MHSD1 (H1–H1); 10: MHSD2 (H1–H1); 11: MEHN1 (N1a–VS1–S1).

mtDNA-PCR–RFLP analysis

Using mtDNA primers C2F3 and MRH106, an amplification product was obtained from either purified DNA or crude J2 lysate from each of the populations listed in Table 1. Fragments of four different sizes were amplified (Table 1, Figure 2). Specifically, the populations of *M. incognita*, *M. javanica* and *M. arenaria* of Mdh–Est phenotype N1–A1 had a PCR product of ca. 1800 bp; the *M. arenaria* populations of N1–A2 produced a smaller product of ca. 1300 bp; the *M. hapla* populations produced a product of ca. 670 bp; and the *M. enterolobii* populations gave a fragment of ca. 850 bp (Figure 2). Taking into account the fact that C2F3/MRH106 products are ~130 bp larger than those amplified with primers C2F3 and 1108 (Powers and Harris, 1993; Stanton et al., 1997), the amplification product sizes revealed in this study for *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla* were generally in agreement with previous reports (Powers and Harris, 1993; Williamson et al., 1994; Stanton et al., 1997; Orui, 1998; Blok et al., 2002). Interestingly,

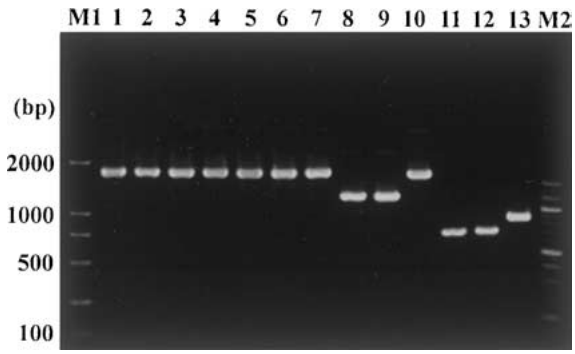

Figure 2. PCR products amplified from representative populations of *M. incognita*, *M. javanica*, *M. arenaria*, *M. hapla* and *M. enterolobii* from China using primers C2F3 and MRH106. 1: MIJS1; 2: MIHN1; 3: MIYN1; 4: MISD1; 5: MJFJ1; 6: MJGD1; 7: MJYN1; 8: MAJS1; 9: MATW1; 10: MAYN1; 11: MHSD1; 12: MHSD2; 13: MEHN1; M1: DL2000 DNA marker (TaKaRa Biotech, Dalian, China); M2: 100-bp DNA ladder (New England Biolabs, Beverly, USA).

the amplification product from *M. enterolobii* was comparable in size to that reported for *M. mayaguensis* (Blok et al., 2002).

When the amplification products were digested with the restriction enzyme *Hin*fI, distinctive enzyme phenotype-correlated RFLP profiles were generated that allowed the differentiation of the five species studied (Figure 3). Differing restriction sites on the 1800-bp product separated *M. incognita*, *M. javanica* and *M. arenaria* of N1–A1 from each other. Three fragments of ca. 1300, 400 and 100 bp were produced in all the *M. incognita* populations except for population MISD1 which had an additional restriction site on the 1300-bp fragment, resulting in four fragments of 850, 450, 400 and 100 bp; two fragments of ca. 1700 and 100 bp were generated in the *M. arenaria* population MAYN1 of N1–A1; and no restriction of the 1800-bp fragment occurred in the *M. javanica* populations. The 1300- and 670-bp products, species-specific for *M. arenaria* of N1–A2 and *M. hapla*, respectively, were cleaved into two fragments of ca. 1200 and 100 bp, and 570 and 100 bp, respectively. The 850-bp fragment characteristic of *M. enterolobii* was not restricted by *Hin*fI.

Since the size of the amplification product from *M. enterolobii* resembled that of *M. mayaguensis*, the C2F3/MRH106 product from population MEHN1 was cloned, and two clones were sequenced. No intrapopulation sequence variations were detected.

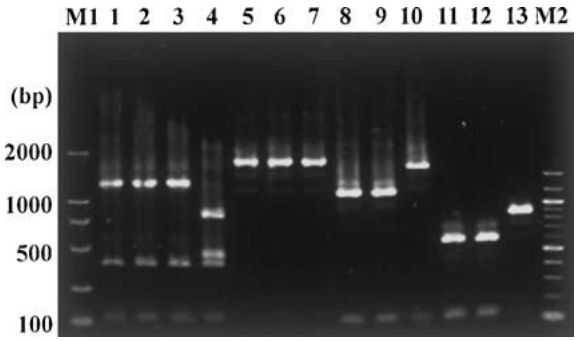

Figure 3. *Hin*fI-restriction patterns of PCR products amplified from representative populations of *M. incognita*, *M. javanica*, *M. arenaria*, *M. hapla* and *M. enterolobii* from China using primers C2F3 and MRH106. 1: MIJS1; 2: MIHN1; 3: MIYN1; 4: MISD1; 5: MJFJ1; 6: MJGD1; 7: MJYN1; 8: MAJS1; 9: MATW1; 10: MAYN1; 11: MHSD1; 12: MHSD2; 13: MEHN1; M1: DL2000 DNA marker (TaKaRa Biotech, Dalian, China); M2: 100-bp DNA ladder (New England Biolabs, Beverly, USA).

The sequence was determined to be 841 bp in size (data not shown). Sequence comparison showed that, to our surprise, the 662-bp region between primer C2F3 and primer 1108 (excluding primer sequences) of *M. enterolobii* was identical to the orthologous region reported for *M. mayaguensis* (data not shown; Blok et al., 2002). This raises questions over the validity of the species status of *M. mayaguensis*.

Discussion

The species identity of 46 *Meloidogyne* populations collected from 14 provinces in China was revealed by isozyme phenotyping and confirmed by mtDNA-PCR–RFLP analysis in the present study (Table 1). *M. incognita* was identified from 63% of the samples, *M. javanica* and *M. arenaria* from 13% of the samples, respectively, *M. hapla* from 6.5% of the samples and *M. enterolobii* from 4.5% of the samples. The detection rates for the former four species are in agreement with those of previous investigations carried out in different regions of China (Pan, 1984; Li and Yu, 1991; Zhang and Weng, 1991; Wang et al., 2001), and confirm that *M. incognita* is the predominant species of root-knot nematodes in China. The detection of *M. enterolobii* on Guava (*Psidium guajava*) constitutes the third report of the nematode from the Hainan Province (Yang and Eisenback, 1983; Zhao, 2000).

Characterisation of 46 populations of *M. incognita*, *M. javanica*, *M. arenaria*, *M. hapla* and *M. enterolobii* demonstrated that *Hin*fI restriction patterns of the mtDNA region between the *COII* and *lrRNA* genes strictly correlated with nematode Mdh–Est phenotypes except for the variant *M. incognita* population MISD1 (Table 1). Since isozyme phenotypes have been shown to be reliable criteria for identification of major *Meloidogyne* species (Esbenshade and Triantaphyllou, 1985a,b), the reliability of the mtDNA-PCR–RFLP assay for diagnosis of the five root-knot nematodes occurring in China was generally validated. Compared with the traditional isozyme phenotyping method, the mtDNA diagnostic used in the present study is easier to perform and has the power to diagnose a single juvenile. Therefore, this technique will be a valuable tool for routine species identification of root-knot nematodes in soil samples. Availability of such a tool will undoubtedly enhance the effectiveness of control measures for crop root-knot diseases in China.

Powers and Harris (1993) designed primers C2F3 and 1108 to amplify the intergenic region between the

mitochondrial *COII* and *lrRNA* genes to discriminate five major *Meloidogyne* species. In their original description, populations of *M. arenaria* were differentiated from *M. incognita* and *M. javanica* by a size polymorphism in the amplification products. However, it was evident from later studies that some *M. arenaria* populations also produce the same size of amplification product as that of *M. incognita* and *M. javanica* (Stanton et al., 1997; Orui, 1998; Blok et al., 2002). Sequence information from Australian populations indicated that this form of *M. arenaria* (i.e. mtDNA haplotypes C and H or Est phenotype A1) could be separated from *M. incognita*, but not from *M. javanica* by *Hin*fI restriction of the products amplified with C2F3 and 1108 (Hugall et al., 1994; Stanton et al., 1997). To overcome this problem, we used the downstream primer MRH106 to couple with C2F3 to incorporate an additional 130-bp *lrRNA* sequence into the amplification products. According to the restriction map by Stanton et al. (1997), this 130-bp sequence from primer 1108 to primer MRH106 contained an *Hin*fI restriction site polymorphism between *M. javanica* and populations of *M. arenaria* showing Est phenotype A1. Consistent with the result of the Australian study, the Chinese *M. arenaria* population MAYN1 of N1–A1 was successfully distinguished from *M. javanica* by *Hin*fI digestion of the C2F3/MRH106 products (Figure 3).

*Hin*fI restriction patterns of the C2F3/1108 amplification products from *M. incognita* and *M. javanica* have been reported by several studies with differing results (Powers and Harris, 1993; Williamson et al., 1994; Orui, 1998; Blok et al., 2002). While Powers and Harris (1993) found three restriction fragments of ca. 1000, 400 and 300 bp for *M. incognita*, and two fragments of ca. 1000 and 700 bp for *M. javanica*, the other investigators reported two products of ca. 1300 and 400 bp for *M. incognita*, and no restriction of the 1700-bp product for *M. javanica*. Considering the fact that the 130-bp sequence from primer 1108 to primer MRH106 contains a *Hin*fI restriction site in *M. incognita* (see restriction map in Stanton et al., 1997), the restriction phenotypes of the Chinese populations agreed with those from the latter studies except for the variant *M. incognita* population MISD1 which showed an additional *Hin*fI restriction site on the amplification product (Figure 3). The differing results concerning *Hin*fI restriction patterns suggest that intraspecific variations exist in the *COII–lrRNA* region among *M. incognita* and *M. javanica* populations of different origins. Therefore, in practice some caution should be used as to the interpretation of *Hin*fI restriction profiles with respect to species identification of *M. incognita* and *M. javanica*.

The sequence of the C2F3/MRH106 amplification product for *M. enterolobii* was determined in the present study (data not shown). Sequence comparison revealed that the 662-bp sequence between primers C2F3 and 1108 for *M. enterolobii* was identical to that reported for *M. mayaguensis*. This raises intriguing questions over the taxonomic relationship of the two nominal species. *M. enterolobii* was originally found infecting pacara earpod trees (*Enterolobium contortisiliquum*) on Hainan Island of China (Yang and Eisenback, 1983). It has so far been recorded only from the Hainan Province (Zhao, 2000; Xu et al., this study). On the other hand, *M. mayaguensis* has been recorded from a number of countries in Africa, Central America and the Caribbean region (Rammah and Hirschmann, 1988; Decker and Rodriguez Fuentes, 1989; Fargette et al., 1994; Willers, 1997; Trudgill et al., 2000). This nematode is regarded as a considerable threat to agriculture because it is able to reproduce on tomato, soybean and sweet potato cultivars resistant to other major tropical root-knot nematodes (Prot, 1984; Fargette, 1987). Previous studies have shown that *M. mayaguensis* had female perinean pattern, host range and cytogenetic features similar to those of *M. enterolobii* (Yang and Eisenback, 1983; Esbenshade and Triantaphyllou, 1985; Rammah and Hirschmann, 1988). In addition, populations of *M. mayaguensis* showed an Est phenotype (VS1–S1) identical to that of *M. enterolobii* (Esbenshade and Triantaphyllou, 1985; Fargette and Braaksma, 1990; Xu et al., this study). *M. mayaguensis* was differentiated from *M. enterolobii* only by some minor morphological differences and by a different Mdh phenotype (N3c in *M. mayaguensis* vs. N1a in *M. enterolobii*). However, it can argued that the minor differences might actually reflect intraspecific variations as similar intraspecific variations in morphological characters and Mdh phenotype have been recorded for *M. incognita*, *M. javanica* and *M. arenaria* (Esbenshade and Triantaphyllou, 1985b). Together with these observations, the mtDNA sequence evidence presented here suggests that *M. mayaguensis* could be conspecific with *M. enterolobii*. If this proves true in future studies, *M. enterolobii*, being a senior nomen, should be considered a species of wide distribution and a threat to agriculture worldwide.

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