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Distribution and Molecular Identification of *Meloidogyne* spp. Parasitising Flue-cured Tobacco in Yunnan, China

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Abstract

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Twenty-one populations of root-knot nematodes (RKNs) were recovered from diseased roots collected from flue-cured tobacco in 21 locations in Yunnan (China) during 2014–2015. Molecular diagnosis on species was performed based on characteristics of sequences of D2-D3 expansion domains of the 28S rDNA and sequence characterised amplified regions (SCAR). SCAR results identified 13 populations as *Meloidogyne arenaria,* six as *M. javanica* and two as *M. incognita*. *Meloidogyne arenaria* is the predominant species with a detection rate of 61.9%, followed by *M. javanica* (28.5%) and *M. incognita* (9.5%). The tobacco RKNs were mainly distributed in western, central, and southwestern Yunnan, particularly in the western areas. *Meloidogyne arenaria* and *M. javanica* mainly occurred in Lincang (southwestern Yunnan) and Yuxi (central Yunnan), and *M. incognita* only in Zhaotong (northeastern Yunnan). Phylogenetic analysis based on inferences using LSU D2-D3 sequence data revealed that all 21 populations of *Meloidogyne* in this study are in a monophyletic clade in relation to other RKNs.

Keywords: tobacco; 28S LSU rDNA; SCAR; root-knot nematode

Tobacco (*Nicotiana tabacum* L.) is an important cash crop around the world. In China, flue-cured tobacco leaf production was up to 2.3 million tons in 2015. Flue-cured tobacco is the most widely planted in Yunnan province, with yearly 1.05 million acres and more than 0.89 million tons which accounted for 39% of total yields in the country (http://www.eastobacco.com/zxbk/tpxw/201604/t20160422_401153.html). However, tobacco production is quite challenging due to infection by plant-parasitic nematodes. Root-knot nematodes (RKNs) have been recognised as the most economically damaging parasites associated with the tobacco (Shew & Lucas 1991; Díaz-Silveira & Herrera 1998). They commonly cause several symptoms such as root galls, leaf chlorosis and yellowing, plant wilting, and eventually dying which in general generate a yield loss of 20–50% (Qin *et al*. 1991).

RKNs belong to the genus *Meloidogyne* Göldi 1887. More than 100 species in this genus have been described so far (Hunt & Handoo 2009; Moens *et al*. 2009). Traditionally, *Meloidogyne* species were identified based on morphological characters, isozyme patterns, and host plant response to infection (Esser *et al*. 1976; Eisenback 1985; Esbenshade & Triantaphyllou 1985). Unfortunately, species discrimination of *Meloidogyne* has always been challenging due to highly conserved morphology across species (Blok & Powers 2009). Recently, molecular tools have been employed to distinguish nematode species (Powers & Harris 1993; Powers 2004; Adam *et al*. 2007; Blok & Powers 2009). Several molecular markers such

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as sequence characterised amplified region (SCAR), restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNA (RAPD), satellite DNA probes, and molecular techniques including real-time PCR assays and DNA sequencing have become available for RKN identification (Zijlstra *et al*. 2000; Adam *et al.* 2005; Powers *et al*. 2005; Berry *et al*. 2008; Blok & Powers 2009; Ye *et al*. 2015; Zeng *et al*. 2015). Five species of RKNs, i.e. *M. arenaria*, *M. javanica*, *M. incognita, M. enterolobii*, and *M. inornata* were detected from tobacco in southern Brazil (de Araújo Filho *et al*. 2016). Four species of RKNs including *M. arenaria*, *M. javanica*, *M. incognita*, and *M. hapla* were considered as pathogens of the tobacco in Yunnan, China (Chen *et al*. 1997). However, there is controversy about the dominant species of tobacco RKNs in Yunnan (Chen *et al*. 1997; Wang *et al*. 1998). Little has been known about the RKN species composition and distribution from flue-cured tobacco in Yunnan Province, China in the last 20 years.

The objective of the present study was to identify species of RKNs collected from the main flue-cured tobacco variety from Yunnan by using sequences of D2-D3 expansion domains of the 28S rDNA and SCAR markers in order to adopt measures to control nematodes.

MATERIAL AND METHODS

Nematode populations. Twenty-one populations of RKNs were obtained from diseased tobacco roots from 21 locations in Yunnan Province (Table 1).

DNA extraction. Five RKN females were hand-picked into 50 µl of worm lysis buffer (WLB) containing Proteinase K for DNA extraction (Williams *et al*. 1992). DNA samples were stored at –20°C until used as a PCR template.

DNA amplification and sequencing. The primers used for PCR and DNA sequencing are presented in Table 2. The primers Mi-F/Mi-R are species-specific for *M. incognita*, Fjav/Rjav for *M. javanica*, and Far/Rar for *M. arenaria*. Primers RK28SF/MR were used for PCR amplification and DNA sequencing for 28S rDNA D2-D3. The 25-µl PCR was performed using TaqMix DNA polymerase (Guangzhou Dongsheng Biotech Ltd., Guangzhou, China) according to the manufacturer's protocol. The thermal cycler programs for PCR were as follows: denaturation at 95°C for 5 min

Table 1. Populations of root-knot nematodes collected from tobacco fields in 21 locations

No.	Population codes	Locations (Villages/Towns, Counties, Cities)	Geographical coordinates
1	LC-YD-1	Haixin, Yongkang, Yongde, Linchang	99.266055, 24.023452
2	LC-YD-2	Daba, Dedang, Yongde, Linchang	99.286349, 23.976857
3	LC-CY-1	Dongmi, Yanshuai, Cangyuan, Lincang	99.566799, 23.295057
4	LC-FQ-1	Tianxin, Luodang, Fengqing, Lincang	100.030098, 24.533861
5	LC-SJ-1	Bangbing, Shuangjiang, Lincang	99.855325, 23.261205
6	LC-SJ-2	Yakou, Shuangjiang, Lincang	99.672758, 23.501379
7	LC-GM-1	Yanliu, Daxing, Gengma, Lincang	99.811342, 23.797391
8	LC-GM-2	Daxing, Gengma, Lincang	99.813336, 23.801312
9	LC-ZK-2	Yingpan, Yingpan, Zhenkang, Lincang	102.541627, 24.362121
10	LC-ZK-1	Chagou, Zhenkang, Lincang	99.566799, 23.295057
11	YX-CJ-1	Longtan, Jiucun, Chengjiang, Yuxi	102.996589, 24.686844
12	YX-CJ-2	Luojia, Haokou, Chengjiang, Yuxi	102.917331, 24.677799
13	YX-HT-1	Dawan, Beicheng, Hongta, Yuxi	102.573944, 24.456685
14	YX-HT-2	Longshu, Gaocang, Hongta, Yuxi	102.524578, 24.318802
15	YX-HT-3	Caiyuan, Longquan, Hongta, Yuxi	102.524776, 24.319325
16	YX-YM-1	Shuitang, Pubei, Yimen, Yuxi	102.190833, 24.616749
17	ZT-SY-1	Shouwang, Zhaoyang, Zhaotong	103.75524, 27.266037
18	ZT-SY-2	Buka, Zhaoyang, Zhaotong	103.722035, 27.213835
19	WS-YS-1	Pingyuan, Yanshan, Wenshan	103.756612, 23.754958
20	WS-YS-2	Hongfu, Pingyuan, Yanshan, Wenshan	103.352464, 23.669483
21	QJ-ZY-1	Dapo, Dapo, Zhanyi, Qujing	103.67085, 25.685974

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Table 2. Primers used for PCR and DNA sequencing

Primers	Genes	Sequences (5'→3')	TM (°C)	References
MR	28S D2/D3	AACCGCTTCGGACTTCCACCAG	60	Hu *et al.* (2011)
RK28SF	28S D2/D3	CGGATAGAGTCGGCGTATC	60	Ye *et al.* (2015)
Far	SCAR	TCGGCGATAGAGGTAAATGAC	61	Zijlstra *et al.* (2000)
Rar	SCAR	TCGGCGATAGACACTACAAACT	61	Zijlstra *et al.* (2000)
Fjav	SCAR	GGTGCGCGATTGAACTGAGC	64	Zijlstra *et al.* (2000)
Rjav	SCAR	CAGGCCCTTCAGTGGAACTATAC	64	Zijlstra *et al.* (2000)
Mi-F	SCAR	GTGAGGATTCAGCTCCCCAG	62	Meng *et al.* (2004)
Mi-R	SCAR	ACGAGGAACATACTTCTCCGTCC	62	Meng *et al.* (2004)

TM – annealing temperature

followed by 35 cycles of denaturation at 94°C with 30 s; annealing at different temperatures (62°C for the Mi-F/Mi-R, 64°C for the Fjav/Rjav, 61°C for the Far/Rar, and 55°C for the RK28SF/MR) for 45 s, and extension at 72°C for 2 minutes. The final extension was performed at 72°C for 10 min (Zijlstra *et al.* 2000; Meng *et al.* 2004; Ye *et al.* 2007). Sequence characterised amplified region (SCAR) PCR products were checked using 1% agarose gel under electrophoretic running conditions (120 V, 45 min). PCR products were cleaned using an EZ Spin Column DNA Gel Extraction Kit (Bio Basic Inc., Markham, Canada) according to the manufacturer's protocol before being sequenced by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd. (Shanghai, China) using an ABI PRISM 3730 sequencing system.

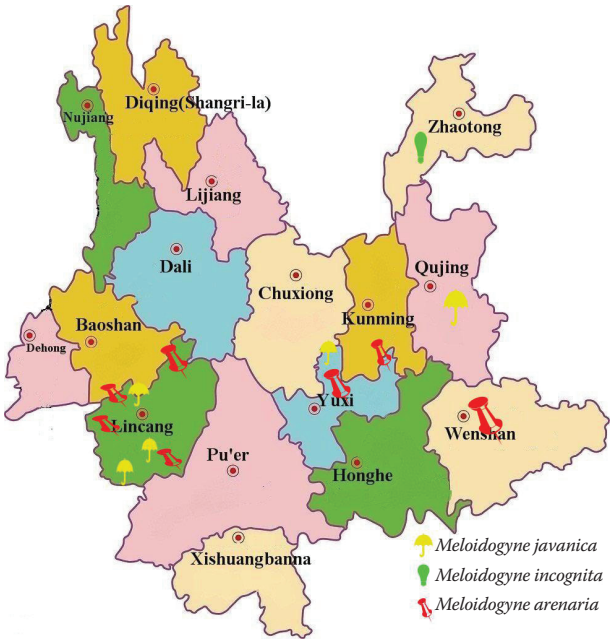

Figure 1. Distribution of *Meloidogyne* spp. identified in tobacco fields in Yunnan, China (symbols represent the species)

Phylogenetic analyses. DNA sequences with the highest matches against our isolates based on BLASTN result in GenBank were used for phylogenetic analysis. DNA sequences were edited with ChromasPro1.5 (Technelysium Pty Ltd, Helensvale, Australia) and

Table 3. Species of *Meloidogyne* spp. from flue-cured tobacco in Yunnan, China

Population code	*M. arenaria*	*M. incognita*	*M. javanica*
LC-YD-1	+		
LC-YD-2			+
LC-CY-1			+
LC-FQ-1	+		
LC-SJ-1	+		
LC-SJ-2	+		
LC-GM-1			+
LC-GM-2			+
LC-ZK-1	+		
LC-ZK-2	+		
YX-CJ-1	+		
YX-CJ-2	+		
YX-HT-1	+		
YX-HT-2	+		
YX-HT-3	+		
YX-YM-1			+
ZT-SY-1		+	
ZT-SY-2		+	
WS-YS-1	+		
WS-YS-2	+		
QJ-ZY-1			+

(+) annealing temperature

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aligned using ClustalW in MEGA5.05 (Tamura *et al.* 2011). Writing of likelihood scores and estimating of parameters including base frequencies, the proportion of invariable sites, and the gamma distribution shape were conducted by using PAUP v4.0b10 (Sinauer Associates, Inc. Publishers, Sunderland, USA) and used in phylogenetic analyses. DNA sequence data was evaluated using MODELTEST v3.7 (Posada & Buckley 2004). Bayesian analysis was performed to confirm the tree topology using MrBayes v3.1.0 (Huelsenbeck & Ronquist 2001), running the chain for 4 000 000 generations and setting the 'burnin' at 1000. Markov Chain Monte Carlo methods were used within a Bayesian framework to estimate the posterior probabilities of the phylogenetic trees (Larget & Simon 1999) using the 50% majority rule.

RESULTS

Species of RKNs. Three RKN species from 21 RKN populations from diseased tobacco roots were identified based on molecular characterisation, including *Meloidogyne arenaria* from 13, *M. javanica* from six, and *M. incognita* from two populations. *Meloidogyne arenaria* is the predominant species with a detection rate of 61.9% (Table 3).

Distribution of RKNs. The distribution of three RKNs is presented in Figure 1. It shows that the tobacco RKNs were mainly distributed in central, western, and southwestern Yunnan, especially in the western areas. *Meloidogyne arenaria* and *M. javanica* mainly occurred in Lincang (southwestern Yunnan) and Yuxi (central Yunnan) with prevalence of 61.9 and 28.5%, respectively, and *M. incognita* only in Zhaotong (northeastern Yunnan) with 9.5%.

Molecular phylogenetic relationships* of *RKNs. A phylogenetic tree based on sequences of D2-D3 expansion domains of the 28S rRNA gene is presented in Figure 2. The dendrogram inferred from LSU D2-D3 using *Pratylenchus parazeae* and *Helicotylenchus leiocephalus* species as outgroup taxa suggested that: (*i*) all the selected 42 *Meloidogyne* populations are in a monophyletic clade in relation to *P. parazeae*

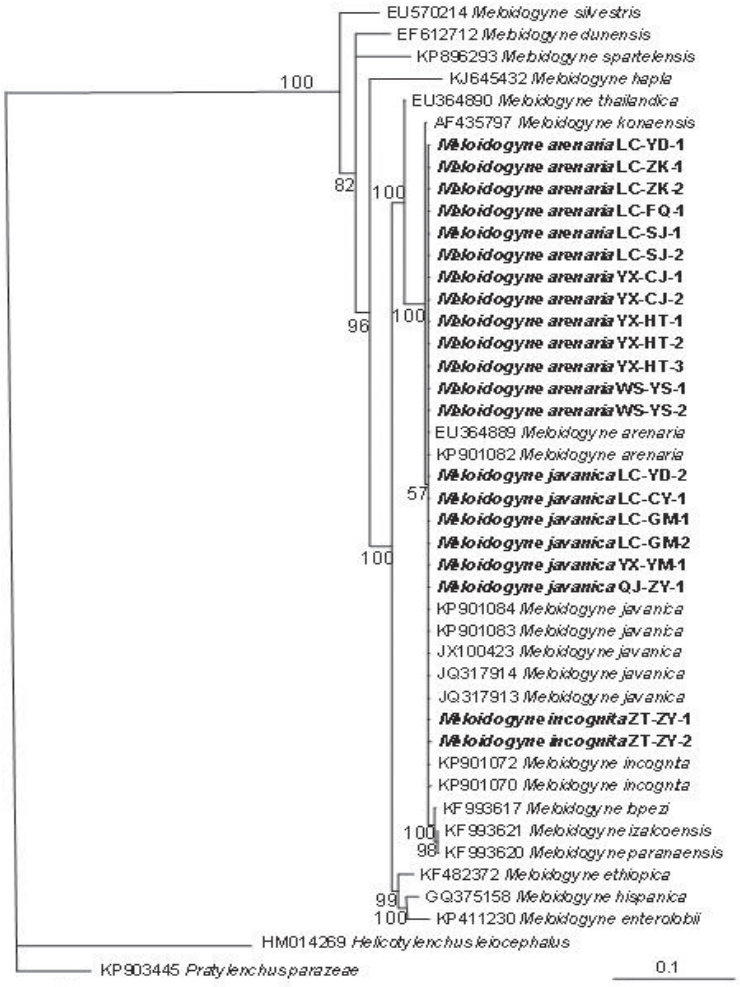

Figure 2. The 10001st Bayesian consensus tree inferred from 28S D2-D3 under GTR+G model –lnL = 2812.7192; AIC = 5643.4385; freqA = 0.2050; freqC = 0.2134; freqG = 0.3170; freqT = 0.2646; R(a) = 0.3968; R(b) = 2.1167; R(c) = 1.1724; R(d) = 0.2998; R(e) = 3.0376; R(f) = 1.0000; Pinvar = 0.0000; Shape = 0.9882; posterior probability values exceeding 50% are given on appropriate clades

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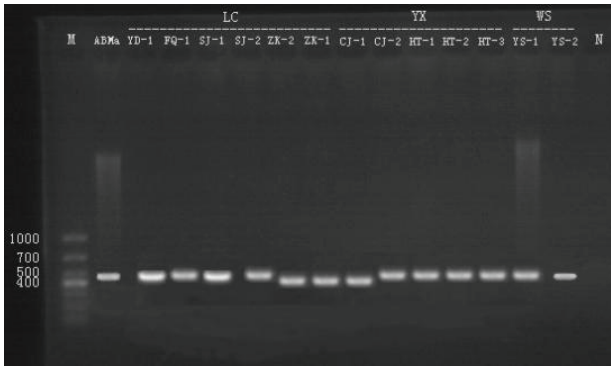

Figure 3. PCR product with the *Meloidogyne arenaria* species-specific Far/Rar primers

M – 1KB DNA Marker; ABMa – positive control (*M. arenaria* from *Anubias barteri* var. *caladiitolia*); LC – Lincang; YX – Yuxi; WS –Wenshan; N – negative control (water); other codes – sample population codes in this study

and *H. leiocephalus* with 100% posterior probability (pp); (*ii*) 21 studied populations are distinctly in different clades with *M. silvestris, M. dunensis, M. spartelensis, M. hapla, M. thailandica, M. konaensis, M. ethiopica, M. hispanica, M. enterolobii;* (*iii*) 21 populations of *Meloidogyne* in the present study are clustered in a monophyletic clade with other selected populations of *M. arenaria* (EU364889, KP901082), *M. incognita* (KP901070, KP901072), and *M. javanica* (KP901084, KP901083, JQ317913, JQ317914, JX100423), and these three species are not in separate clades; (*iv*) 21 populations in this study are grouped in a poorly supported (57% pp) clade with other three populations of *Meloidogyne* from GenBank (*M. lopezi, M. izalcoensis, M. paranaensis*).

SCAR marker analysis. A single specific band of 420, 720, and 999 bp in size was produced through PCR by *M. arenaria*-specific primers Far/Rar from 13 populations, *M. javanica*-specific primers Fjav/Rjav from six, and *M. incognita*-specific primers Mi-F/Mi-R from two populations, respectively. The same result was observed in corresponding positive controls. However, no band was produced in any negative control (water) (Figures 3 and 4). In addition, PCR amplification was negative by other species-specific primers (gel photo not shown).

DISCUSSION

Chen *et al.* (1997) showed that *M. arenaria* was the dominant species of tobacco RKNs in Yunnan, China. However, Wang *et al.* (1998) reported that *M. javanica* was dominant in Yunnan. Hu *et al.* (1998) showed that

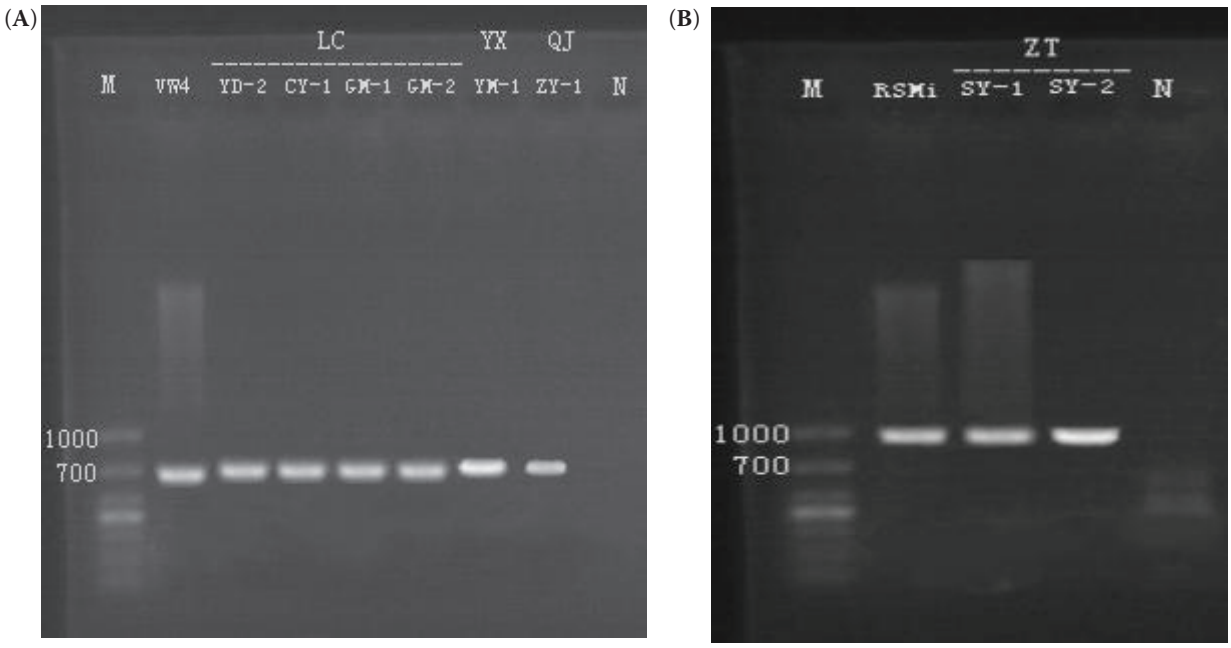

Figure 4. PCR product with the *Meloidogyne incognita* species-specific Fjav/Rjav primers (**A**) and species-specific Mi-F/Mi-R primers (**B**)

M – 1KB DNA marker; RSMi – positive control (*M. incognita* from *Radermachera sinica*); VW4 – positive control (*M. arenaria* from *Anubias barteri* var. *caladiitolia*); LC – Lincang; YX – Yuxi; QJ – Qujin; ZT – Zhaotong; SY – Yanshan; N – negative control (water); YD-2, CY-1, GM-1, GM-2, YM-1, and ZY-1 – sample population codes in this study

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all 40 tested tobacco varieties including main variety K326 were resistant to *M. hapla*, 27.5% (11 varieties) of them were highly resistant to *M. incognita*, while 97.5% (39 varieties) of them were susceptible to *M. arenaria*. These supported *M. arenaria* being a dominant species in the Yunnan tobacco area. The present result was also supported by Chen *et al*. (1997). *Meloidogyne arenaria* was mainly distributed in central, eastern and southeastern Yunnan (Chen *et al*. 1997), but it was detected mainly in the central and southwestern tobacco areas in the present study. *Meloidogyne javanica* was considered as the dominant species of tobacco RKNs in central Yunnan including Yuxi (Qin *et al*. 1991). In this study, *M. javanica* was detected mainly in southwestern tobacco areas in Linchang although it occurred in Yuxi and eastern part in Qujing. Similar results were presented by Chen *et al*. (1997). *Meloidogyne incognita* was considered as the dominant species in western and southwestern Yunnan tobacco regions (Chen *et al*. 1997). From this study, it was also found in the northeastern region (Zhaotong). In addition to these three species, *M. hapla* was considered as a pathogen of tobacco and distributed in the northeastern tobacco area in Zhaotong in Yunnan Province (Qin *et al*. 1991). But this species was not observed in this study. This could be related to limited samples. Thus, extensive sampling is needed in the future.

DNA sequencing has provided useful data for nematode species identification and phylogenetic analysis (Zijlstra *et al*. 1997, 2000; Hugall *et al*. 1999; Powers 2004). DNA markers have played an important role in related aspects. However, sequences of some genes such as SSU 18S, LSU 28S, and ITS of RKN species are highly conserved, and it is difficult to distinguish RKNs to the species level based on these sequences, especially for three common tropical species including *M. incognita*, *M. javanica*, and *M. arenaria* (De Ley *et al*. 1999; Blok & Powers 2009; Kiewnick *et al*. 2014; Janssen *et al*. 2016). Similar results were presented in this study where sequencing the LSU D2-D3 region of the populations we isolated can distinguish these three species from other species of *Meloidogyne*, but could not discriminate among *M. arenaria, M. javanica,* or *M. incognita.* This implies that these three tropical species are derived from a recent event or might be a species complex. SCAR marker has been indicated as a valued molecular characteristic in nematode species identification (Zijlstra *et al*. 2000). In the present study, three species of *Meloidogyne*, i.e. *M. incognita*, *M. javanica*, and *M. arenaria*, among 21 tobacco populations were successfully identified by SCAR analysis. Therefore, the combined analysis of DNA sequencing and SCAR can be employed in accurate species identification in RKNs.

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