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Full Length Article

Development of Species-specific Primer Pairs for the Molecular Diagnosis of *Ditylenchus arachis*

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Abstract

Ditylenchus arachis was firstly found in hulls and seeds of peanut (*Arachis hypogaea*) in 2014 and is an economically important nematode pest of peanut in northern China. The rDNA-ITS regions of 7 *D. arachis* populations were PCR amplified. Sequence analysis showed that the interspecific diversity was found between *D. arachis* and other *Ditylenchus* species based on ITS-1 sequences. A set of species-specific primers (named DARF /DARR) for rapid identification of *D. arachis* was designed based on the sequence analysis of rDNA-ITS 1. The primers were sensitive to generate a specific band of the correct size (171 bp) from DNA template of a single different developmental stage of *D. arachis*. The reliability of the primers was tested by screening 18 different geographical populations of *D. arachis* in China, and an expected band size was produced for all populations tested. The closely related *Ditylenchus* species and general nematodes collected from soil of peanut were also tested for the primers, no specific band was amplified. Given its specificity, sensitivity and reliability, the specific primers should be of great benefit to accurate identification of *D. arachis,* which is fundamental for effective management of the nematode. © 2018 Friends Science Publishers

Keywords: Peanut; Stem nematode; rDNA-ITS; Identification; Specificity; Sensitivity

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Introduction

Ditylenchus arachis was originally described in many peanut fields in Hebei Province and Shandong Province and was the first reported stem nematode infecting peanut in China (Zhang *et al.*, 2014). *D. arachis* is one of the important damaging nematodes of peanut in China which can attack underground portion of the peanut, included the roots, pegs, hulls and seeds. Damage consists of root reduction or inhibition associated with root rotting, black discoloured seeds (Zhang *et al.*, 2014). Our recent surveys revealed that *D. arachis* was widely distributed in peanut-producing regions of northern China, resulting in substantial economic losses in peanut (Zhang, 2016; Wang, 2017). Furthermore, *D. arachis* was also confirmed that it could parasitize many common weeds in local peanut fields (Zhang, 2016). Thus, the fast, reliable and sensitive identification of *D. arachis* is absolutely necessary for effective nematode control.

Unfortunately, it is quite often difficult to differentiate *Ditylenchus* species based on their morphology and morphometrics because of only a few diagnostically valid characteristics (Fortuner, 1982; Sturhan and Brzeski, 1991; Zhang *et al.*, 2014). For instance, *D. arachis* has morphological similarity with *D. destructor* and *D. myceliophagus*, which are also widely distributed in northern China (Huang *et al.*, 2010), and it differentiates each other only based upon a combination of few morphological characteristics (Zhang *et al.*, 2014). However, recognition of these characters requires considerable time and technical skills

Within the last few years, species identification of *Ditylenchus* based on morphological characteristics has been supplemented with molecular observations (Marek *et al.*, 2005, 2010; Subbotin *et al.*, 2005; Vovlas *et al.*, 2011; Zhang *et al.*, 2014; Jeszke *et al.*, 2015). And there is a little molecular-based diagnosis for *Ditylenchus* species by using species-specific primers (Subbotin *et al.*, 2005; Kerkoud *et al.*, 2007; Wan *et al.*, 2008; Marek *et al.*, 2010; Jeszke *et al.*, 2015). However, a species-specific molecular protocol for the diagnosis of *D. arachis* is lacking. Relatively highly conserved sequences are found in the internal transcribed spacer region of rDNA, which are commonly used as genetic markers for the identification of nematodes (Zijlatra *et al.*, 1995; Htay *et al*., 2016). The purpose of this study was to develop a set of species-specific primer pairs with sensitivity and reliability based on the sequence analysis for the molecular diagnosis of *D. arachis*.

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Materials and Methods

Nematode Populations

Ditylenchus species, including 18 *D. arachis* populations, 6 *D. destructor* populations, 3 *D. myceliophagus* populations and one *D. nanus* population, were identified from geographically disparate regions of China. *D. dipsaci* population was obtained from the Netherlands supplied by Xiamen Entry-exit Inspection and Quarantine Bureau (Table 1). Also, *Meloidogyne hapla* and other parasitic-nematode species commonly occurring in peanut soils were sampled. The above nematodes were isolated from the infected plant tissues or rhizosphere soil (or rhizosphere medium) using the modified Baermann funnel method. These nematode specimens were handpicked by pipette and placed into 1 *M* NaCl for further molecular studies.

DNA Extraction

A single nematode was handpicked onto a 10 μL drop of distilled water on a coverslip, cut into two pieces under a dissecting microscope, transferred 8 μL water with the nematode into a PCR tube. Then the sample tube was stored at -70°C for 20 min, and incubated at 99°C for 10 min. After cooling, 1 μL proteinase K (1 mg mL^{-1}) (Takara, Dalian, China) and 1 μL 10×PCR buffer were added and mixed. The sample tube was incubated for 60 min at 65°C, then 10 min at 95°C (Liu *et al*., 2011). Finally, the samples were stored at -20°C until further use.

PCR Amplification

The rDNA-ITS sequences of 7 populations (Table 1, DarA01~ DarA07) were amplified by PCR. Amplification reactions using the universal primers (ITSA: 5′-TTGATTACGTCCCTGCCCTTT-3′ and ITSB: 5′-TTTCACTCGCCGTTAC TAAGG-3′) (Vrain *et al*., 1992) were performed in a 50 μL comprising 10 μL template DNA, 3 μL each primer (10 μM), 5 μL 10× PCR buffer (with Mg^{2+}), 4 μL dNTP (2.5 m*M*), 0.4 μL rTaq DNA (5 U μL^{-1}, TaKaRa) and 24.6 μL sterile ddH_2O. The negative control with no template was designed. The amplification conditions were consisted of an intial denaturation 94°C for 5 min; 35 cycles of 1 min at 94°C, 1 min at 55°C and 1.5 min at 72°C; and a final extension at 72°C for 10 min. After PCR, the amplified products were analyzed by electrophoresis with a 2% agarose gel stained with ethidium bromide in 1×TAE buffer and visualized under UV light. The PCR products were cloned and sequenced by Sangon Biological Technology Co. Ltd (Sango, Shanghai, China).

Primer Design

The ITS sequences of D. arachis amplified in our study were submitted to NCBI database (http://www.ncbi.nlm.nih.gov/). The ITS sequences of *Ditylenchus* species, including *D. destructor* (GQ469491, KF221213, KF221214, DQ471335), *D. myceliophagus* (AM232236), *D. africanus* (KF219617), *D. dipsaci* (GQ469495) and *D. gigas* (HQ219240), which were retrieved from NCBI and were used for the design of specific primers (Table 2). The nucleotide sequences of rDNA-ITS were aligned using the MUSCLE algorithm (Edgar, 2004) in MEGA 5 (Tamura *et al*., 2011) to identify conserved regions. The specific forward and reverse primers were designed from the ITS regions using Primer Premier 5.0 (Lalitha, 2000) to generate an expected fragment of about 170 bp in size and verified using BLAST (http://www.ncbi.nlm.nih.gov/blast) to exclude nonspecific reactions with other closely related species. Additionally, the quality of the primers was assessed by Oligo 6.0 software. The primers were synthesized by Sango Biotech Co., Ltd. To ensure its specificity, optimization of PCR condition was conducted by varying the annealing temperatures.

Primer Test

To test the sensitivity of the species-specific primers, the DNA of 1, 2, 3, 4 and 5 female *D. arachis* were extracted from DarA01 population for PCR amplification. Amplification was carried out in a 25 μL reaction mixture containing the 2 μL template DNA, 0.7 μL each primer (10 m*M*), 12.5 μL PCR Master Mixture and 9.1 μL sterile ddH_2O. The negative control with no template was designed. Amplification conditions were consisted of initial denaturation at 94°C for 4 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 45 s; and final extension at 72°C for 8 min. After PCR, the amplified products were loaded into a 2% agarose gel for electrophoresis, and the DNA bands were stained with ethidium bromide in 1×TAE buffer and visualized by UV light.

The sequence variability of different developmental stages of the nematode for the species-specific primers were also investigated, the DNA of a single egg, 2nd-stage juvenile (J_2), J_3, J_4, male and female of *D. arachis* were extracted from DarA01 population. The PCR reaction process and products detection were the same as described above, and the test was repeated for 3 times.

To validate the specificity and reliability of the species-specific primers on different populations of *D. arachis* or non-target nematodes, 18 populations of *D. arachis* from different peanut plantations in the several provinces of northern China were used as the target nematodes. And six populations of *D. destructor*, three populations of *D. myceliophagus*, one population of *D. nanus*, *D. dipsaci*, *Meloidogyne hapla*, *Pratylenchus brachyurus*, *Rotylenchulus reniformis* and *Aphlenchoides composticola* were used as the control (Table 1). The DNA extraction of single female or juvenile for each species was extracted and applied to PCR amplification, which was conducted according to the method described above and repeated in triplicate.

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Table 1: Isolates and sources of nematode species used in this study

Nematodes species	Code	Host	Origin
Ditylenchus arachis	DarA01	*Arachis hypogaea*	Julu County, Xingtai City, Hebei Province, China
D. arachis	DarA02	*A. hypogaea*	Dacui County, Qianan City, Hebei Province, China
D. arachis	DarA03	*A. hypogaea*	Shangzhuang County, Qianan City, Hebei Province, China
D. arachis	DarA04	*A. hypogaea*	Handan City, Hebei Province, China
D. arachis	DarA05	*A. hypogaea*	Laiwu City, Shangdong Province, China
D. arachis	DarA06	*A. hypogaea*	Zaozhuang City, Shangdong Province, China
D. arachis	DarA07	*A. hypogaea*	Feixian County, Linyi City, Shangdong Province, China
D. arachis	DarA08	*A. hypogaea*	Mengyin County, Shangdong Province, China
D. arachis	DarA09	*A. hypogaea*	Taian City, Shangdong Province, China
D. arachis	DarA10	*A. hypogaea*	Liaocheng City, Shangdong Province, China
D. arachis	DarA11	*A. hypogaea*	Shahe County, Xingtai City, Hebei Province, China
D. arachis	DarA12	*A. hypogaea*	Zhuozhou City, Hebei Province, China
D. arachis	DarA13	*A. hypogaea*	Gaoyi Country, Shijiazhuang City, Hebei Province, China
D. arachis	DarA14	*A. hypogaea*	Shangqiu City, Henan Province, China
D. arachis	DarA15	*A. hypogaea*	Suqian City, Jiangsu Province, China
D. arachis	DarE16	*Eleusine indica*	Julu County, Xingtai City, Hebei Province, China
D. arachis	DarD17	*Digitaria sanguinalis*	Julu County, Xingtai City, Hebei Province, China
D. arachis	DarS18	*Setaria viridis*	Julu County, Xingtai City, Hebei Province, China
D. destructor	DdeI01	*Ipomoea batatas*	Julu County, Xingtai City, Hebei Province, China
D. destructor	DdeI02	*I. batatas*	Lulong County, Qinhuangdao City, Hebei Province, China
D. destructor	DdeI03	*I. batatas*	Laiwu City, Shangdong Province, China
D. destructor	DdeI04	*I. batatas*	Feixian County, Linyi City, Shangdong Province, China
D. destructor	DdeI05	*I. batatas*	Laiyang City, Shangdong Province, China
D. destructor	DdeI06	*I. batatas*	Yishui County, Linyi City, Shangdong Province, China
D. myceliophagus	DmyA01	*A. hypogaea*	Longyan City, Fujian Province, China
D. myceliophagus	DmyP02	*Pleurotus ostreatus*	Laiwu City, Shangdong Province, China
D. myceliophagus	DmyA03	*Anthurium andraeanum*	Xiamen City, Fujian Province, China
D. nanus	DnaC	*Chamaedorea elegans*	Zhangzhou City, Fujian Province, China
D. dipsaci	DdiH	*Hyacinthus orientalis*	The Netherlands
Meloidogyne hapla	MhaA	*A. hypogaea*	Laiyang City, Shangdong Province, China
Pratylenchus brachyurus	PbrA	*A. hypogaea*	Xiamen City, Fujian Province, China
Rotylenchulus reniformis	RreA	*A. hypogaea*	Xiamen City, Fujian Province, China
Aphlenchoides composticola	AcoA	*A. hypogaea*	Julu County, Xingtai City, Hebei Province, China

Table 2: Sequence information from GenBank for designing species-specific primers

Nematodes species	Host	Origin	GenBank accession no.
Ditylenchus arachis	*Arachis hypogaea*	Julu County, Xingtai City, Hebei Province, China	JX040545
	A. hypogaea	Shangzhuang County, Qianan City, Hebei Province, China	JN594665
	A. hypogaea	Dacui County, Qianan City, Hebei Province, China	JN605348
	A. hypogaea	Laiwu City, Shangdong Province, China	JN635037
	A. hypogaea	Handan City, Hebei Province, China	KX426049
	A. hypogaea	Zaozhuang City, Shangdong Province, China	KX426050
	A. hypogaea	Feixian County, Linyi City, Shangdong Province, China	KX426051
D．destructor	*Solanum tuberosum*	the Czech Republic in Montreal	GQ469491
	Ipomoea batatas	Feixian County, Linyi City, China Shangdong Province, China	KF221213
	I. batatas	Julu County, Xingtai City, China Hebei Province, China	KF221214
	Astragalas mongholicus	China	DQ471335
D. africanus	*A. hypogaea*	South Africa	KF219617
D. myceliophagus	Mushroom	Wenzhou City, Zhejiang Province, China	AM232236
D. dipsaci	*Beta vulgaris*	Mueter, Germany	GQ469495
D. gigas	*Vicia faba*	Cordoba, Spain	HQ219240

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Results

Sequence Amplification of rDNA-ITS

PCR amplification of rDNA-ITS generated a single band with a length of approximately 1030 bp in 7 populations tested by using the universal primers ITSA and ITSB (Fig. 1). And these 7 sequences were submitted to GenBank under accession No. JX040545, JN594665, JN605348, JN635037 and KX426049-KX426051, respectively (Table 2).

Design of Species-specific Primers

Sequence analyses showed that the rDNA-ITS-1 sequences were divergent between *D. arachis* and its similar species. Species-specific primer pairs DARF (forward: 5´- GGGAAGGCGAAGCTAAGCTA -3´ and

DARR (reverse: 5´- AACTTAGAGGCCAACGAAGCC -3´) were designed from the rDNA-ITS1 sequences of *D. arachis*. The length of the expected PCR amplified fragment using the set of specific primers was 171 bp (Fig. 2).

Test of Species-specific Primers

To evaluate the sensitivity of the species-specific primers, the PCR amplified products for different numbers of female *D. arachis* from DarA01 population amplified using the primers (DARF and DARR) were shown in Fig. 3. A single band with a length of 171 bp was obtained from DNA templates extracted from 1, 2, 3, 4 and 5 female *D. arachis*. And the sensitivity of the species-specific primers was further demonstrated using DNA extracted from the different development stages of *D. arachis.* The single band of 171 bp in length was produced from the DNA templates of a single of egg, J_2, J_3, J_4, male and female of *D. arachis* by the species-specific primers (Fig. 4). The method proved suitable for *D. arachis* sensitive identification of DNA samples from a single adult, a single juvenile, even a single egg of *D. arachis*.

The specificity and reliability of the specific primers were confirmed by yielding the expected fragment sizes (171 bp) for all the populations of *D. arachis* (Fig. 5) and no products were detected for the tested non-target species (Fig. 6).

Discussion

D. arachis has a short life cycle and can survive as a state of anhydrobiosis in hulls or seeds left in the field or stored seeds (Zhang *et al*., 2014; Zhang, 2016; Wang, 2017), the surviving low population densities may be enough to form large populations and lead to severe damage. To select effective control schemes, an accurate and rapid detection of *D. arachis* at the species level is essential. But due to the high degree of morphological similarity, morphometric characters are not always reliable tools for the identification of *Ditylenchus* species (Wendt *et al*., 1993). At present, a set of species-specific primers for *D. arachis* were developed for the first time by in silico analysis based on the sequence differences in the rDNA-ITS1 region of *D. arachis* and other sequences of *Ditylenchus* species deposited in GenBank. When testing the primer reliability, the primer pairs produced a unique specific band of 171 bp with genomic DNA from 18 populations of *D. arachis* which were collected in four provinces of China. The PCR amplification by the species-specific primers demonstrated that it could amplify a single, stable and clear band for a single adult, even an egg or one J_2, J_3, J_4 and different geographical populations of *D. arachis,* which were collected from peanut fields in four provinces.

The specificity and reliability of the primers were also demonstrated *in vitro* with *D. destructor*, *D. myceliophagus* and a range of non-target parasitic-nematode species respectively which were typically found in peanut fields.

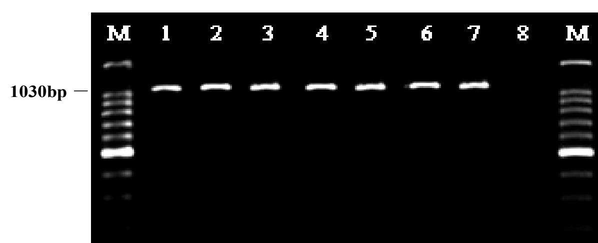

Fig. 1: Amplification products of rDNA-ITS region from 7 *D. arachis* populations using the universal primers. Lanes 1-7: DarA01, DarA02, DarA03, DarA04, DarA05, DarA06, DarA07; lane 8, negative control without DNA; M, D2000 DNA Ladder (TaKaRa, China)

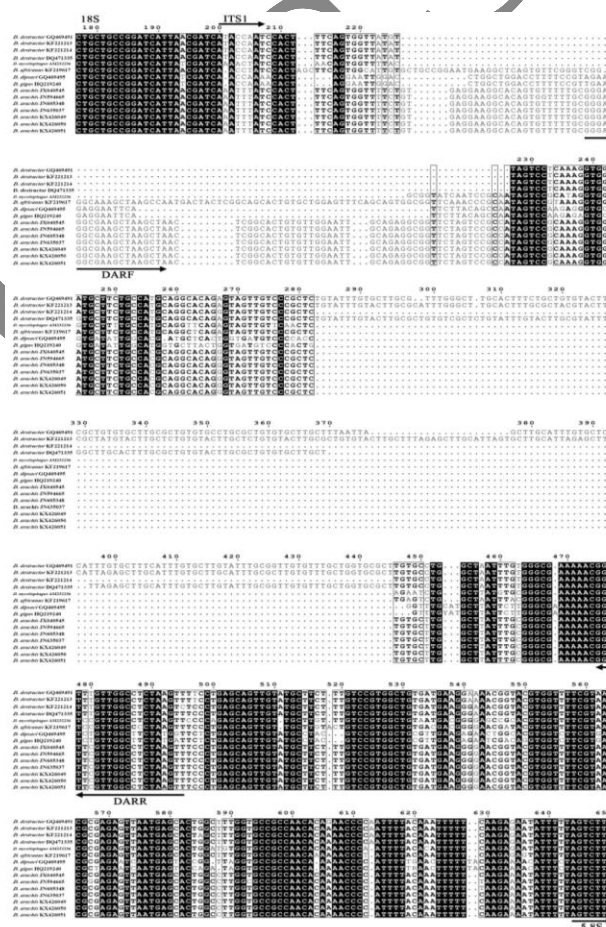

Fig. 2: Multi-alignment generated of the rDNA-ITS 1 sequences of *D. arachis* and other *Ditylenchus* species from GenBank used to develop the species-specific primers

D. arachis remains to be less noticed in northern China probably because the symptoms caused by the nematode are very similar to the black hull rot disease, and the nematodes has a weak stylet and easy to be misdiagnosed as harmless fungivorous nematodes, such as *D. myceliophagus*.

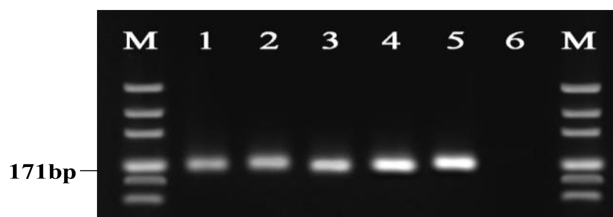

Fig. 3: Amplification products of DNA from different numbers of female *D. arachis* from DarA01 population using the species-specific primers. Lanes 1-5: 1, 2, 3, 4 and 5 females; lane 6, negative control without DNA; M, D600 DNA Ladder (TaKaRa, China)

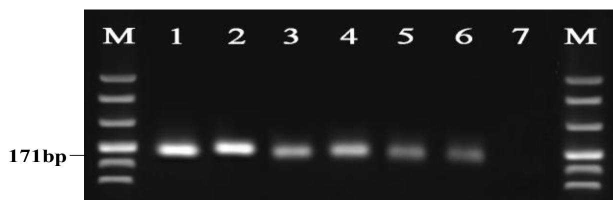

Fig. 4: Amplification products of DNA from different development stages of *D. arachis* from DarA01 population using the species-specific primers. Lanes 1-6: female, male, J_4, J_3, J_2 and egg; lane 7: negative control without DNA; M, D600 DNA Ladder (TaKaRa, China)

Fig. 5: Amplification products of DNA from 18 populations of *D. arachi* using species-specific primers. Lanes1-18 represent 18 populations of *D. arachis* as coded in Table 1 respectively; lane 19: negative control without DNA; M, D600 DNA Ladder (TaKaRa, China)

D. destructor, which is a morphologically close species to *D. arachis*, was also isolated in the same region or peanut field in northern China (Zhang *et al*., 2014). Given the specificity, sensitivity and reliability of the primers for *D. arachis*, the diagnostic primers could provide a rapid and reliable molecular marker for identification or detection of *D. arachis* isolated from hulls, seeds or soil in China. Typically, testing diagnostic primers usually need more samples with similar morphological characteristics and more geographically disparate locations (Oliveria *et al*., 2005). At present, there are nearly 70 species recognized in the genus of *Ditylenchus* (Siddiqi, 2000; Chizhov *et al*., 2010; Giblin-Davis *et al*., 2010; Vovlas *et al*., 2011; Zhang *et al*., 2014). Possibly, the species-specific primers designed in the present work may cross-react with other species in the genus of *Ditylenchus* and mismatches at the primer-binding

Fig. 6: Amplification products of DNA from various species belong to different genera using species-specific primers. Lanes1-6 represent 6 populations of *D. destructor*; Lanes7-9 represent 3 populations of *D. myceliophagus*; Lane 10, *D. nanus*; Lane 11, *D. dipsaci*; Lane 12, *Meloidogyne hapla*; Lane 13, *Pratylenchus brachyurus*; Lane 14, *Rotylenchus reniformis*; Lane 15, *Aphelenchoides composticola*; CK1, DarA01; CK2, negative control without DNA; M, D600 DNA Ladder (TaKaRa, China)

site to produce the same specific band. To avoid the risk of misdiagnosis, the more populations of related species of *Ditylenchus* should be investigated and tested in future.

Conclusion

In this study, a set of species-specific primers (named DARF /DARR) was designed firstly for molecular identification of *D. arachis* based on the sequence analysis of rDNA-ITS 1. The specificity, sensitivity and reliability of the primers were repeatedly demonstrated. Therefore, the developed specific primers should be rapid and accurate molecular protocol for the diagnosis of *D. arachis* and also be fundamental for effective management of the nematode.

Acknowledgements

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