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A new primer set for amplification of ITS-rDNA in *Ditylenchus destructor*

Niloufar Mahmoudi[1, 2]*, Davoud K. Nejad[3], Fatemeh Shayanmehr[4]

[1]Peoples' Friendship University of Russia, *Moscow, Russian Federation*
[2]All-Russian Plant Quarantine Centre, *Moscow*, *Russian Federation*
[3]Semnan University, *Semnan, Iran*
[4]Tarbiat Modares University, *Tehran, Iran*
*Corresponding author: niloofarmahmoodi@ymail.com

Abstract. A technique was developed for the identification of *Ditylenchus destructor* nematode belonging to the *Ditylenchus* genus, based on the use of different primers for polymerase chain reaction (PCR). Two universal ribosomal primers were amplified to the internal transcribed spacer region ITS-rDNA. The sequencing of PCR products confirmed the polymorphism between species. The primers were sensitive to generate a particular band of the correct size (300bp) from the DNA template of a single, separate *D. destructor* stage of development. Screening populations of *D. destructor* from Iran and the Russian Federation have tested the reliability of the primers, and the expected size of the band was produced for all test populations. *Ditylenchus destructor* closely related species have also been tested and no specific band was amplified. Such results showed that the primers currently developed are useful for quantifying the *D. destructo*r density in potato tuber.

Keywords: Potato nematode, ITS-rDNA, *Ditylenchus destructor*, Primer design

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Introduction

Since the advent of polymerase chain reaction (PCR) and a large amount of genetic data produced with DNA sequencing, molecular-based detection tools have been widely developed and successfully used for plant parasite nematodes diagnosis. Molecular detection

tools have the following advantages compared to other approaches, (i) can be used in a high throughput manner, (ii) DNA information can be easily acquired with a large number of databases and sequencing information, (iii) inexpensive, fast and accurate, (iv) DNA markers are independent of phenotypic variation and nematode developmental stage [1]. DNA-based detection tools make excellent nematode diagnostic methods because they are simple, accurate and quick [2, 3] and can be used with a wide variety of sample types, including host tissue, eggs, egg masses, soil extracts and fixed samples [4]. The sequences contain readily detectable genetic markers in the form of tandem repeats used to create phylogenetic trees [5] for the evaluation and diagnosis of genetically related populations [6]. Because nematodes species descriptions have historically been focused on the idea of morphological or typological organisms, molecular techniques have recently shown that many assumed monospecific species are siblings or cryptic species, genetically distinct but shared common morphological diagnostic characteristics [7—9]. [10, 11] and [12] used and recommended the specific primers to identify *D. destructor* rDNA ITS regions have also been reported to be used successfully for phylogenetic analysis [11—14]. Nonetheless, the definition of nematode species has been debated recently, indicating that species delimitation should be based primarily on an amalgamation of polyphasic taxonomy concepts, which assembles and assimilates all available data and information (phenotypic, genotypic and phylogenetic) used to delimit taxa at all levels [8, 9, 15]. The main accessible strategy that possibly can separate among the haplotypes is those of [12, 16, 17] *D. gigas* have been developed [12, 13, 18, 19]. This method is suitable for the identification of species in monospecific samples but cannot be used if the sample contains more than one nematode species using species-specific SCAR or ITS-rRNA primers [11, 12, 20—22]. This study aimed to develop a PCR species-specific primers with sensitivity and reliability based on the sequence analysis for the molecular identification of *D. destructor* from other *Ditylenchus* species.

Materials and methods

DNA extraction. Nematodes were extracted from potatoes (*Solanum tuberosum* L.) collected from different regions in the Russian Federation and Iran. Several nematode specimens from the population were put into a drop of water and cut by a scalpel under a camera-equipped ZEISS Axioskop50® microscope. DNA extraction from the material under this research was laid out by treating the specimens with Proteinase K that was followed by removing proteins with no extraction with organic solvents. For this purpose, a DNA-Ekstran-2 set No EX-511—100 (Synthol, Moscow) was used.

PCR with Species-specific primers. The first PCR amplification mixture (final volume 25 µl) was prepared as follows in a PCR tube (Table 1).

Table 1

PCR reaction mixture composition

Reagents	Volume µl
Master Mix	5 µl
Primer DITdesR	0.6 µl
Primer DITuniF	0.6 µl
H20	13.8 µl
DNA	5 µl
Total	25 µl

The primer DITdesR and DITuniF were used for PCR Species-specific amplification. The contents were mixed gently by overtaxing. The reaction was performed in a thermal cycler, which involved the following stepwise procedure: denaturation of the template at 95 °C for 3 minutes, annealing at 95 °C for 35 seconds followed by extension at 63.5 °C for 30 seconds, 72 °C, 30 seconds and 5 minutes at 72 °C for 35 cycles. Negative control was included with each set of amplification. Then, The PCR products were subsequently partitioned according to their size on 1 % agarose gel electrophoresis and visualized by Gel documentation or purified for sequencing.

Sequencing and Phylogenetic analysis. The amplified PCR product obtained after purification by Thermo Scientific Gene JET Gel Extraction Kit was sequenced by Sanger's dideoxy cycle by Genetic Analyzer AB-3500 (Applied Biosystems, USA). Primitive comparison of sequencing results with the GeneBank genetic sequence database was done by the NCBI BLAST web site (http://www.ncbi.nlm.nih.gov/BLAST). The results are presented in percentage values, the DNA sequence available in the GeneBank homologous to those examined were analyzed along with the newly sequenced one.

Design of Species-specific Primers. The ITS sequences of *D. destructor* including MN122076, MN307126, MN307128, MN493767, MN658597, MN658599, MN658637, MN658638 and *D. dipsaci*: MG676655, MG676656, MG676656, *D. gigas*: KJ653270, KJ653267 which were retrieved from NCBI and were used for the design of specific primers (Table 2). The specific forward and reverse primers were designed from the ITS regions using Primer Premier DITdesR and DITuniF to generate an expected fragment of about 126 bp in length and verified using BLAST (http://www.ncbi.nlm.nih.gov/blast) to exclude nonspecific reactions with other closely related species.

Table 2

Sequence information from GenBank for designing species-specific primers

Accession Number	Species	Country
GQ469492	*Ditylenchus destructor*	China
GQ469491	*Ditylenchus destructor*	Czech Republic
KJ653270	*Ditylenchus gigas*	Iran
KJ653267	*Ditylenchus gigas*	Iran
MG676655	*Ditylenchus dipsaci*	Japan
MG676656	*Ditylenchus dipsaci*	Japan
MG676657	*Ditylenchus dipsaci*	Japan

Results and discussion

Ditylenchus destructor DNA sequence analysis. The sequenced ITS-rRNA gene, deposited in NCBI GenBank under accession numbers; MN122076, MN307126, MN307128, MN493767, MN658597, MN658599, MN658637, MN658638 (Table 3) are 1013 bp, 1160 bp, and 1108 bp, 637bp, 553bp, 501bp, 624bp long, respectively. BLAST search at NCBI revealed that all molecular markers of *D. destruc*tor from Russia Federation

and Iran, newly obtained in this study, matched with the corresponding sequences of *D. destructor* present in the database (Table 4). The sequence analysis revealed sequence variability between different geographical populations of *D. destructor* isolated from different host plants.

Table 3

Submitted Sequences in NCBI GenBank

Region	Accession Number
Moscow region	MN122076
Hamedan region	MN307126
Hamedan region	MN307128
Hamedan region	MN493767
Briansk region	MN658597
Briansk region	MN658599
Nizhny Novgorod region	MN658637
Nizhny Novgorod region	MN658638

Table 4

Reference sequences of *D. destructor* used in the phylogenetic analysis (http://www.ncbi.nlm.nih.gov)

Accession Number of D. destructor	Country	Host Plant
MH992393	China	Potato
EU400636	China	Sweet potato
EU400627	South Korea	Sweet potato
EF208213	China	Potato
HQ235698	Iran	Potato
FJ707365	Czech Republic	Potato
MG673926	China	Carrot
EU400638	China	Sweet potato
MG675235	China	Carrot
EU400643	China	Sweet potato
KY435979	China	Carrot
EU400639	China	Sweet potato
GQ469490	USA	Potato
JX162205	Canada	Garlic
DQ328727	Russia	Potato
JN166693	Iran	Potato
MK979365	China	Potato
MG673926	China	Carrot
KX766417	China	Potato
LC030371	Japan	Potato
GQ469491	Czech Republic	Potato
DQ471335	China	Potato

Design of Species-specific Primers. A first primer named dsn.1 (Table 5) was designed to have some nucleotides mismatches observed when comparing *D. destructor*, *D. dipsaci*, and *D. gigas* (Fig 1, 2). At present, a set of species-specific primers for *D. destructor* were developed based on the sequence differences in the rDNA-ITS region of *D. destructor*. The PCR amplification by the species-specific primers demonstrated that it could amplify

a single, stable and clear band for a single adult and different geographical populations of *D. destructor*. The specificity and reliability of the primers were also demonstrated in vitro conditions. Given the specificity, sensitivity, and reliability of the primers for *D. destructor*, the diagnostic primers could provide a rapid and reliable molecular marker for identification or detection of *D. destructor*. Typically, testing diagnostic primers usually need more samples with similar morphological characteristics and more geographically disparate locations [23]. 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 site to produce the same specific band.

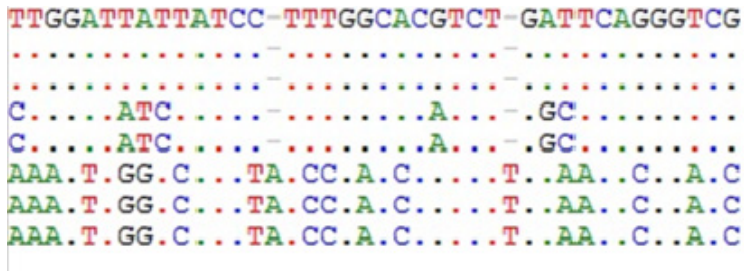

Fig 1. Multi-alignment generated of the rDNA-ITS sequences of *D. destructor* and other *Ditylenchus* species from GenBank used to develop the species-specific forward primer

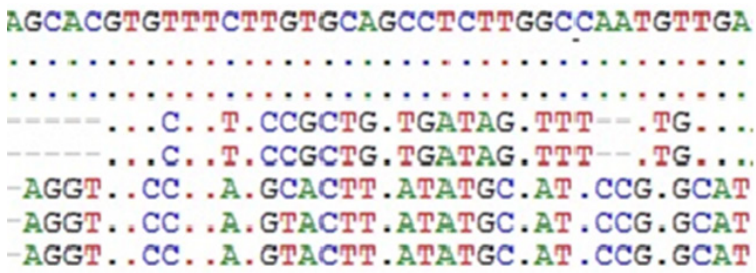

Fig 2. Multi-alignment generated of the rDNA-ITS sequences of *D. destructor* and other *Ditylenchus* species from GenBank used to develop the species-specific reverse primer

Table 5

Primer design for *D. destructor*

Primer.dsn.1	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
Forward primer	TTGGCACGTCTGATTCAGGG	Plus	20	193	212	60.32	55.00
Reverse primer	GTCAACATTGGCCAAGAGGC	Minus	20	318	299	59.76	55.00
Product length	126						

Table 6

Primer design for *D. destructor* by NCBI Primer-Blast

Primer.dsn.2	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
Forward primer	TTTCGAATGCACATTGCGCC	Plus	20	157	176	60.73	50.00
Reverse primer	CTAGGCCAAAGAGACAGCGG	Minus	20	281	262	60.46	60.00
Product length	125						

Test of Species-specific Primers. To evaluate the sensitivity of the species-specific primers, the PCR amplified products for different numbers of *D. destructor* amplified using the primers (dsn.1 and dsn.2) were shown in (Fig 3). A single band with a length of 300 bp was obtained from DNA templates extracted *D. destructor*. The method proved suitable for *D. destructor* sensitive identification of DNA samples. The specificity and reliability of the specific primers were confirmed by yielding the expected fragment sizes (300 bp) for all the populations of *D. destructor* and no products were detected for the tested of the species *D. dipsaci* and *D. gigas* (Fig 4).

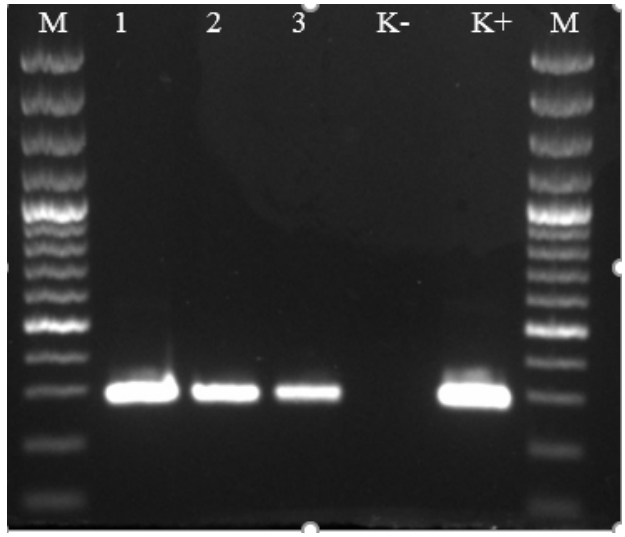

Fig 3. Gel with amplification products obtained in PCR with species-specific primer from the ***D. destructor*** population. Lanes: M = 100 bp DNA ladder; K+=Positive control with ***D. destructor*** DNA; K– = control without DNA

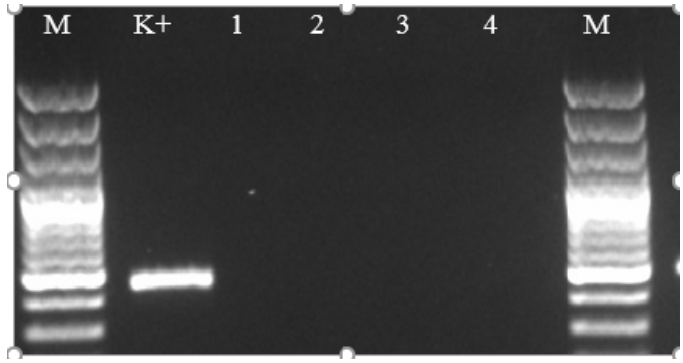

Fig 4. Gel with amplification products obtained in PCR with species-specific primer. Lanes: M = 100 bp DNA ladder; K+=Positive control with ***D. destructor*** DNA; 1,2 = with ***D. dipsaci*** DNA; 3, 4 = with ***D. gigas*** DNA

Conclusions

In this study, a set of species-specific primers (named dsn.1 /dsn.2) was designed firstly for molecular identification of *D. destructor* based on the sequence analysis of rDNA-ITS. The specificity, sensitivity, and reliability of the primers were repeatedly

demonstrated. Therefore, the developed specific primers should be a rapid and accurate molecular protocol for the diagnosis of *D. destructor* and also be fundamental for effective management of the nematode.

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About authors:

Mahmoudi Niloufar — PhD candidate, Department of Agro-Biotechnology, Agrarian and Technological Institute, Peoples' Friendship University of Russia, 8, Miklukho-Maklaya st., Moscow, 117198, Russian Federation; Researcher, Russian Plant Quarantine Center (VNIIKR), 32, Pogranichnaya st., vill. Bykovo, Ramensky district, Moscow region, 140150, Russian Federation; e-mail: niloofarmahmoodi@ymail.com

Nejad Davoud Kartooli — Assistant Professor of Forestry, Faculty of Desert Studies, Semnan University, Mowlawi Boulevard, Motahari Square, 35196–45399, Semnan, Iran, e-mail: Kartooli58@gmail.com; ORCID 0000-0002-0852-6635

Shayanmehr Fatemeh — PhD in Forestry, Natural Resources Faculty, Tarbiat Modares University, 14155-4838, Tehran, Iran

Новый набор праймеров для специфичной амплификации ITS-локусов рДНК *Ditylenchus destructor*

Н. Махмуди[1, 2]*, Д.К. Неджад[3], Ф. Шаянмер[4]

[1]Российский университет дружбы народов, *г. Москва, Российская Федерация*
[2]Всероссийский центр карантина растений, *Московская область, Российская Федерация*
[3]Семнанский университет, *г. Семнан, Иран*
[4]Университет Тарбиат Модарес, *г. Тегеран, Иран*
*niloofarmahmoodi@ymail.com

Аннотация. Разработана методика идентификации нематоды *Ditylenchus destructor*, основанная на использовании различных праймеров для проведения полимеразной цепной реакции (ПЦР). Два универсальных рибосомных праймера были амплифицированы во внутренней транскрибируемой области спейсера ITS-рДНК. Секвенирование продуктов ПЦР подтвердило полиморфизм между видами. Праймеры достаточно чувствительны, чтобы генерировать определенную полосу правильного размера (300 п.н.) из ДНК-матрицы определенной стадии развития *D. destructor*. Скринингом популяций *D. destructor* из Ирана и Российской Федерации проверена надежность праймеров, и ожидаемый размер полосы был получен для всех тестируемых популяций. Близкородственные виды *Ditylenchus destructor* также были протестированы, и специфической имплифицированной полосы не было выявлено. Результаты исследований показали, что данные разработанные праймеры могут использоваться для количественной оценки плотности *D. destructor* в клубнях картофеля.

Ключевые слова: картофельная нематода, ITS-рДНК, *Ditylenchus destructor*, праймеры

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Об авторах:
Махмуди Нилоуфар — аспирант агробиотехнологического департамента, Аграрно-технологический институт, Российский университет дружбы народов, Российская Федерация, г. Москва, 117198, ул. Миклухо-Маклая, д. 8; научный сотрудник, Всероссийский центр карантина растений (ВНИИКР), Российская Федерация, Московская область, 140150, Раменский район, п. Быково, ул. Пограничная, д. 32; e-mail: niloofarmahmoodi@ymail.com
Неджад Давуд Картули — доцент кафедры лесоводства, факультет исследований пустынь, Университет Семнан, Иран, 35196–45399, Семнан, площадь Мотахари, бульвар Молави; e-mail: Kartooli58@gmail.com; ORCID 0000–0002–0852–6635
Шаянмер Фатиме — кандидат наук, факультет природных ресурсов, Университет Тарбиат Модарес, 14155–4838, Иран, Тегеран