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MOLECULAR DIAGNOSTICS OF SUGAR BEET NEMATODE *HETERODERA SCHACHTII* SCHMIDT

The methods of molecular diagnosis of sugar beet cyst nematode Heterodera schachtii are described including PCR-RFLP with universal primers; PCR with species specific ITS rDNA primers; PCR in real time with SYBR green I dye.
***Keywords**: sugar beet cyst nematode, polymerase - chain reaction, primers*

Introduction. Among the sedentary endoparasitic nematodes, species from family *Heteroderidae* represent the most dangerous pathogens affecting the agricultural production and causing severe plants disease leading to serious yield reduction. The most harmful species are: *Punctodera punctata*, *Globodera rostochiensis* and *G. pallida*, *Heterodera avenae*, *H. glycines*, *H. humuli*, *H. trifolii*, *H. medicaginis*, *H. goettingiana* and *H. schachtii* [1]. It has been demonstrated that these species identification can be made on a base of morphological and morphometrical studies of nematodes of different age and gender, observation of the color of females during their transformation into cysts and implementation of bioassays. However morphological identification requires specialized expertise and is extremely time consuming, whereas other procedures are labor intensive. Therefor over the last 15 years molecular identification techniques based on DNA, cDNA or RNA sequence specificity has been developed for routine nematode diagnostics, including sugar beet nematode (*H. schachtii*) [2]. *H. schachtii* is a major pest in sugar beet production in many countries. PCR diagnostic techniques can be used successfully for species identification, providing high sensitivity and specificity as well as appropriate timeframe for the procedure (4-8 hours in total). These include PCR-RFLPs [3], PCR with species-specific primers [4] and Real-time PCR with SYBR green I dye [5].

PCR-RFLP [3]

The methods can only be used on nematodes morphologically identified as *Heterodera sp.*, as the primers are not specific for *Heterodera spp.* Restriction of PCR amplicon forms RFLP patterns that are used to distinguish *H. schachtii* from more than 20 species of *Heterodera* genus.

One to four cysts (extracted from soil or plant roots) are nucleic acid source. Cysts are transferred into 10 μl of double distilled water in an Eppendorf tube and crushed with a microhomogenisor. 8 μl of worm lysis buffer and 2 μl proteinase K (600 μg/μl) are added to each tube.

Worm lysis buffer:

KCl	125 mM
Tris HCl, pH = 8.3	25 mM
$MgCl_2$	3,75 mM
DTT	2,5 mM
Tween 20	1,125 %
Gelatine	0,025 %

The solution is incubated at 65ºC for 60 min and at 95ºC for 10 min consecutively. No DNA clean-up is required. Aliquots of DNA extracted are stored at –20°C (or at –80°C for longer periods).

The assay is designed to the internal trancribed spacer (ITS) region of the rDNA sequences of *Heterodera spp*. (fig. 1).

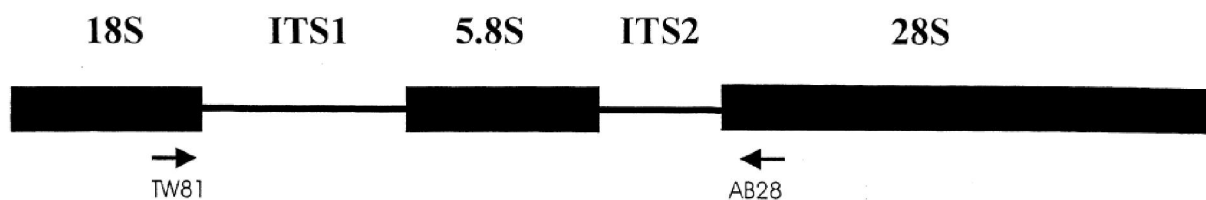

Fig. 1. Structure of the ITS regions and the positions of the primers (W81 and AB28) used for polymerase chain reaction.

TW81 5′-GTTTCCGTAGGTGAACCTGC-3′ [6]
AB28 5′-ATATGCTTAAGTTCAGCGGGT-3′ [6]

PCR master mix:

	Concentration per 100-µl single reaction
10 X PCR Buffer, containing 15 mM $MgCl_2$ (Qiagen)	10 µl (final concentration 1 X: 1.5 mM $MgCl_2$)
5 X Q-solution (Qiagen)	20 µl
dNTPs (Qiagen)	200 µM
Primer AB28	1,5 µM
Primer TW81	1,5 µM
Taq DNA Polymerase, 5 U/µl (Qiagen)	0,8 U
DNA	10 µl
ddH_2O	

PCR cycling parameters:

94°C	4 min	
94°C	1 min	35 cycles
55°C	1,5 min	
72°C	2 min	
72°C	10 min	

DNA amplicons (5 µl product; the remainder is stored at –20°C) are separated by electrophoresis on 1% agarose gel and visualized under UV light according to standard procedures [7]. DNA ladder should be used to identify the approximate size of amplicons obtained. The PCR is estimated as positive if a single amplicon is obtained with an approximate size 1060 bp.

Enzyme *MvaI* (Gibco Co., BRL) is used for amplicon restriction following the manufacturer's instructions.

The digested DNA is separated by electrophoresis on 1,5% agarose gel and visualized under UV light according to standard procedures [7]. DNA ladder should be used to identify the RFLP profiles (table 1).

Table 1

Approximate sizes of restriction fragments (*Mva*I) of rDNA ITS regions for cyst forming nematodes *Heterodera spp.* [3, 8]

Heterodera spp.	Approximate sizes of restriction fragments of rDNA ITS regions (bp)
H. schachtii	1010, 840, 760, 630, 220, 150, 80
H. avenae (type A)	400, 330, 290
H. avenae (type B)	400, 330, 290
H. arenaria	400, 330, 290
H. filipjevi	400, 330, 290
H. aucklandica	400, 330, 290
H. iri	420, 330, 290
H. latipons	420, 330, 290
H. hordecalis	440, 330, 290

H. trifolii	760, 220, 80
H. medicaginis	760, 220, 80
H. ciceri	760, 220, 80
H. salixoplila	400, 330, 290
H. oryzicola	470, 300, 210
H. glycines	760, 220, 80
H. cajani	760, 300
H. humuli	760, 300
H. ripae	760, 300
H. fici	690, 200, 80
H. litoralis	560, 310, 290, 240
H. carotae	760, 300
H. cruciferae	760, 300
Heterodera sp.	800, 260
H. cyperi	780, 320
H. goettingiana	760, 300
H. urticae	760, 300

PCR with species specific primers [4]

Either several cysts, one cyst, or single juveniles alone (extracted from soil or plant roots) are nucleic acid source.

Nematodes are transferred into 8 µl distilled water in an Eppendorf tube and crushed with a microhomogenisor. 10 µl worm lysis buffer and 2 µl proteinase K (600 µg/ml) are added and the tubes.

Worm lysis buffer:

KCl	500 mM
Tris HCl, pH = 8.0	100 mM
$MgCl_2$	15 mM
DTT	1,0 mM
Tween 20	4,5 %

The solution is incubated at 65ºC for 60 min and at 95ºC for 10 min consecutively. No DNA clean-up is required. Aliquots of DNA extracted are stored at –20°C (or at –80°C for longer periods).

The assay is designed to the internal trancribed spacer (ITS) region of the rDNA sequences of *Heterodera spp.* [4] (fig. 2).

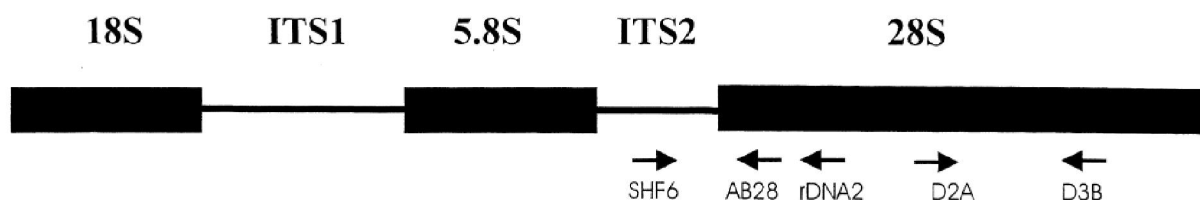

Fig. 2. Structure of the ITS regions and the positions of the primers (SHF6, AB28, rDNA2, D2A, D3B) used for polymerase chain reaction.

SHF6	5′-GTTCTTACGTTACTTCCA-3′	[4]
AB28	5′-ATATGCTTAAGTTCAGCGGGT-3′	[6]
rDNA2	5′-TTTCACTCGCCGTTACTAAGG-3′	[9]
D2A	5′-ACAAGTACCGTGAGGGAAAGTTG-3′	[10]
D3B	5′-TCGGAAGGAACCAGCTACTA-3′	[10]

PCR master mix:

	Concentration per 100-μl single reaction
10 X Qiagen PCR buffer	2,5 μl
5X Q-solution (Qiagen)	5 μl
10 mM dNTPs (Qiagen)	0,5 μl
Primer 1	1,5 μM
Primer 2	1,5 μM
Taq DNA Polymerase (5 U/μl) (Qiagen)	1,5 U
DNA	2 μl
ddH_2O	12 μl

The assay for each DNA sample includes 2 individual reactions or run as duplex PCR with:

1) species-specific primers for identification purpose (SHF6 and AB28 or SHF6 and rDNA2);
2) universal primers (D2A and D3B) for internal control.

PCR cycling parameters:

94°C	4 min	
94°C	30 s	10 cycles
45°C	40 s	
72°C	1 min	
94°C	30 s	20 cycles
55°C	40 s	
72°C	1 min	
72°C	10 min	

DNA amplicons are separated by electrophoresis on 1% agarose gel and visualized under UV light according to standard procedures [7]. DNA ladder should be used to identify the approximate size of amplicons obtained.

The PCR is estimated as positive if a single 200 bp (for the set of primers SHF6 and AB28) or 255 bp (for the set of primers SHF6 and rDNA2) amplicon is obtained.

The internal control primers D2A and D3B amplify the D2–D3 expansion region of the 28S gene (800 bp) and indicate the presence of template nematode DNA in the sample, and as a consequence prove the success of the PCR reaction.

Real-Time PCR with SYBR green I dye [5]

Either cysts or at least 5 juveniles (extracted from soil or plant roots) are nucleic acid source.

Nematodes are transferred to an Eppendorf tube containing 20 μl double distilled water and crushed with a microhomogenizor. 12 μl of proteinase K (600 mg/ml) in 1 X PCR buffer (Qiagen, Germany) are added to the tubes to make a final volume of 132 μl. The tubes are incubated at 65ºC for 60 min and at 95ºC for 15 min consecutively. Aliquots of DNA extracted are stored at –20°C (or at –80°C for longer periods).

The assay is designed to the internal trancribed spacer (ITS) region of the rDNA sequences of *Heterodera spp.* using the following primers combination SH6Mod 5’-CGTGTTCTTACGTTACTTCCA-3’ (modified from Amiri et al., 2002) [4] and SH4 5’-AGCATGCGAAGGATTGG-3’.

Amplification reaction is performed in real-time with 12 μl 1 X SYBR Green I PCR Master Mix (Applied Biosystems), 0,1 μM of each primer, and 4 μl DNA sample in a final volume of 25 μl.

RT-PCR cycling parameters:

50°C	2 min	
95°C	10 min	
95°C	15 s	30 cycles
65°C	1 min	

At the end of amplification DNA melting curve analysis of the resulted amplicon should be performed: the meting temperature for amplicons of 84.6°C+0.1 indicates the presence of *H. schachtii* DNA in the sample.

Conclusions. PCR provides efficient tool for a precise, reliable and rapid identification of sugar beet nematodes from 40 (out of 60) species of the genus *Heterodera*. Real-time PCR is even faster since it eliminates the time-consuming post-PCR agarose gel electrophoresis. These methods of identification are highly sensitive: positive detection can be obtained even when a single second-stage juvenile or a cyst is mixed with other nematode species. Precise identification of nematodes in field soil helps effective integrated pest control development.

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Анотація

Пилипенко Л.А., Калатур К.А.

Методи молекулярно-генетичної діагностики бурякової нематоди Heterodera schachtii Schmidt

У статті детально подано методи молекулярно-генетичної діагностики бурякової цистоутворюючої нематоди Heterodera schachtii: полімеразно – ланцюгова реакція (ПЛР) з універсальними для роду Heterodera праймерами та подальшим вивченням поліморфізму довжин рестрикційних фрагментів; ПЛР із видоспецифічними ITS rDNA праймерами; ПЛР в режимі реального часу з SYBR green I барвником.

Ключові слова: *бурякова цистоутворююча нематода, полімеразно – ланцюгова реакція, праймери*

Аннотация

Пилипенко Л.А., Калатур Е.А.

Методы молекулярно-генетической диагностики свекловичной нематоды Heterodera schachtii Schmidt

В статье подробно представлены методы молекулярно-генетической диагностики свекловичной цистообразующей нематоды Heterodera schachtii: полимеразно - цепная реакция (ПЦР) с универсальными для рода Heterodera праймерами с дальнейшим изучением полиморфизма длины рестрикционных фрагментов; ПЦР с видоспецифичными ITS rDNA праймерами; ПЦР в режиме реального времени с SYBR green I красителем.

Ключевые слова: *свекловичная цистообразующая нематода, полимеразно - цепная реакция, праймеры*