Identification of Cereal Cyst Nematodes in Central Saudi Arabia using rDNA Internal Transcribed Spacer Region and RAPD Markers

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ABSTRACT

The ribosomal DNA internal transcribed spacer (ITS) region and RAPD markers were used to identify five populations of *Heterodera avenae* collected from wheat fields of Buraida, Unizah, Badaei, Sheri, and Zolfy, from the central region of Saudi Arabia. PCR amplification of the ITS regions of rDNA from all populations yielded one fragment of 1.2 kb. RAPD assays were performed to determine the genetic diversity among *H. avenae* populations. Based on the pair-wise comparisons of amplification products, the genetic similarity was calculated and ranged from 0.51 to 0.74. The smallest genetic similarity was between the populations of *H. avenae* from Unizah and Sheri (0.51). The other population of *H. avenae* from Unizah was the closest relative to Buraida population (0.71). A dendrogram was constructed using UPGMA analysis that allowed the distinction of two groups of populations: (1) the populations of *H. avenae* from Buraida, Unizah and Badaei and (2) the populations of *H. avenae* from Sheri and Zolfy. This study gives accurate species identification of *H. avenae* using DNA markers for the first time from these locations.

Key words: Cereal cyst nematode, *Heterodera avenae*, ITS-rDNA, RAPD.

INTRODUCTION

The cereal cyst nematode, *Heterodera avenae* limits wheat production in Saudi Arabia, especially in Al-Karj, Hail and Al-Qassim, the three major wheat-producing regions (Al-Hazmi *et al.*, 1994 and 2001; Al-Rehiayani, 2002). *Heterodera avenae* has a wide distribution and a large genetic intraspecific variability, including many pathotypes (Andersen and Andersen, 1982). The cyst nematodes of *Heterodera avenae* group, represent a complex of species and populations that may reflect convolution within a large range of wild and cultivated species throughout the world (Stone and Hill, 1982; Sturhan and Rumpenhorst, 1996).

Several morphological studies have been identified for the characterization of these species (Cook 1982; Mulvey and Golden 1983). Unfortunately, the conservative morphology of nematodes diminishes the

discriminatory power of the identification by using the morphological characters (Curran and Robinson, 1993). Confusion could also arise from mixed species being present as many species have host plants in common.

Molecular approaches are particularly useful in the detection and identification of specific species, especially those that are morphologically identical at the species level (Dyble *et al.*, 2002). The analysis of nuclear ribosomal DNA has been investigated in systematic studies of numerous organisms and is suitable for separation of populations at below the species level (Vrain *et al.*, 1992; Powers *et al.*, 1997). Internal transcribed spacer (ITS) regions were found to be useful to differentiate species within the *H. avenae* group (Ferris *et al.*, 1994; Bekal *et al.*, 1997; Subbotin *et al.*, 1999). The randomly amplified polymorphism DNA (RAPD) marker (Williams *et al.*,1990) was applied to differentiate between different species and races of plant parasitic nematodes such as *Meloidogyne spp., Globodera spp.,* and *Heterodera spp.* based on their different DNA sequences (Chacon *et al.*, 1994; Lopez-Brana *et al.*, 1996).

Heterodera avenae from Saudi Arabia has been identified using only morphological and pathotype studies (Al-Hazmi *et al.*, 2001). In this study, the rDNA internal transcriber region and RAPD markers have been used to identify the five populations of the cereal cyst nematodes collected from the five wheat-producing regions of Central Saudi Arabia (Buraida, Unizah, Badaei, Sheri, and Zolfy).

MATERIALS AND METHODS

Nematode preparation

Five populations of cereal cyst nematodes collected from five wheat-producing regions of Central Saudi Arabia (Buraida, Unizah, Badaei, Sheri, and Zolfy) were used in this study. The nematode populations were analyzed directly from white females produced on wheat (cv. Yecora rojo). White females were washed from roots under tap water and recovered on a 250 µm aperture sieve. They were then picked under a stereomicroscope and stored at –20°C. On average, 30 white females were individually analyzed for each population.

Nematode DNA extraction

Total genomic DNA was extracted using the method described by Caswell-Chen *et al.*, (1992) and Bekal *et al.*, (1997). Each population of nematode was washed in distilled water, squashed in 100 µl of extraction buffer (100mM EDTA, 100mM NaCl, 100mM Tris pH 7.5, 0.5% SDS, 200 µg

proteinase K), and incubated at 55°C for 3 hours to overnight. Following incubation, an equal volume of phenol-chloroform-isoamylalcohol (25-24-1) was added to the tubes, mixed, and centrifuged at 2600g for 10min. The aqueous phase was removed to a fresh tube and an equal volume of ice-cold isopropanol was added followed by centrifugation as above to precipitate the DNA. The pellet was washed in 70% ethanol and dissolved in TE buffer (10mM Tris-HCl, pH8.0, 0.1mM EDTA).

ITS-rDNA PCR amplification

A pair of 21-mer primers, 5'-TTGATTACGTCCCTGCCCTTT-3' and 5'-TTTCATTCGCCGTTACTAAGG-3', were used to amplify the two internal transcribed regions, ITS1 and ITS2 of *H. avenae*. These primers were synthesized by Dr. S. Bekal, IDT (Integrated DNA Technologies, INC.), University of Illinois-Urbana, USA. Amplifications were carried out in 25 µL reaction volumes, containing 1X Taq polymerase buffer (50 mM KCl, 10mM Tris, pH 7.5, 1.5 mM MgCl2) and 1 unit of Taq polymerase (Pharmacia Biotech, Germany) supplemented with 0.01% gelatin, 0.2 mM of each dNTPs (Pharmacia Biotech, Germany), 25 pmol of each ITS1 and ITS2 primers, and 50 ng of total genomic DNA. Amplification was performed in a thermal cycler (Thermolyne Amplitron) programmed for 1 cycle of 30s at 94°C; and 40 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C; followed by 5 min at 72°C. An aliquot of 10 µl from each reaction product was resolved by electrophoresis on 1.5% agarose gel in 1X TAE buffer, stained with ethidium bromide, and visualized with UV light.

RAPD-PCR amplification

Twenty random primers obtained from Operon Technologies, Alameda, USA were used for RAPD analysis conducted in a thermal cycler (Thermolyne Amplitron). The reaction mixture (25 µl) contained 1x PCR buffer with Mg Cl2 (50 mM K Cl, 10 mM Tris- HCl (pH 9.0), 2 mM Mg Cl2 and Triton X-100), 200 µM each of dATP, dCTP, dGTP, and dTTP, 50 ng template DNA, 50 pM of oligonucleotide primer and 1.5 unit of Taq polymerase. The mixtures were subjected to the following conditions: hold at 94° C for 2 min for starting, followed by 40 cycler of 94°C for 1 min, 36°C for 1 min and 72° C for 2 min, and a final hold at 72°C for 5 min. PCR products were visualized along with a DNA marker on 2% agarose gel with 1X TAE buffer and detected by staining with ethidium bromide. Gels were photographed using Polaroid films under UV light.

Data Handling and Cluster Analysis:

Data were scored for computer analysis on the basis of the presence or absence of the amplified products for each random primer. If a product was present in a population, it was designated "1", if absent it was designated "0" after excluding unreproducible bands. Pair-wise comparisons of poulation, based on the presence or absence of unique and shared polymorphic products, were used to generate similarity coefficients based on simple matching. The similarity coefficients were then used to construct a dendogram by UPGMA (Unweighted Pair-Group Method with Arithmetical Averages) using NTSYS-PC (Rohlf, 1993).

RESULTS

PCR-rDNA

The amplification of the rDNA ITS regions of each population yielded one fragment of approximately 1.2 kb long. No variation in this fragment was observed over all populations of *H. avenae* (fig.1).

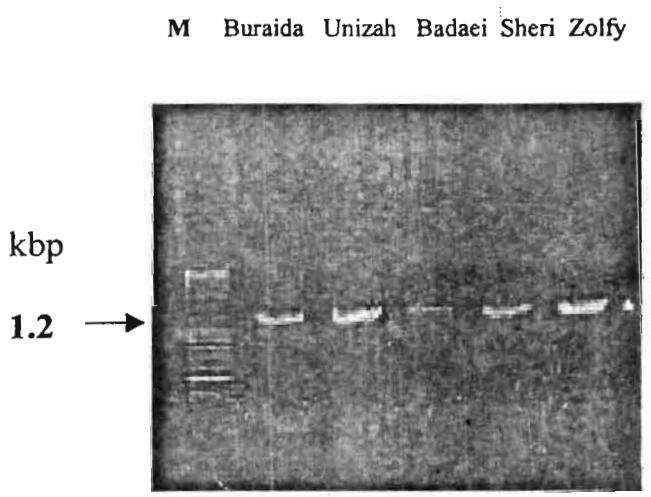

Fig. 1. Agarose gel of amplified ITS regions from five population of *H. avenae* (*left to right*) Buraida, Unizah, Badaei, Sheri, and Zolfy. M lane is 1 kbp ladder DNA marker.

RAPD analysis

A total of twenty 10-base primers were tested in each of five *H. avenae* populations to detect and characterize RAPD polymorphism. Of the primers tested, 12 produced amplification products that were monomorphic across all populations or were polymorphic but not reliable. The remaining eight primers revealed a total of 26 polmorphic fragments that could be scored reliably in all five *H. avenae* populations (Table 1). The number of polymorphic amplification products revealed by each of these primers ranged from 1 to 7(e.g., Fig.1).

Table 1. Selected operon primers with the number of amplified products and polymorphic fragments.

Primers	Amplified products (a)	Polymorphic fragments (b)	% polymorphism (b/a*100)
OP-A2	7	6	86
OP-A3	3	2	66
OP-A4	3	3	100
OP-F5	2	1	50
OP-F6	4	3	75
OP-F10	4	2	50
OP-F14	9	7	78
OP-F15	4	2	50

M Buraida Unizah Badaei Sheri Zolfy

Fig. 2. Polymorphism revealed using primer OP-F6 to amplify genomic DNA purified from five populations of *H. avenae* (*left to right*) Buraida, Unizah, Badaei, Sheri, and Zolfy. M lane is 1 kbp ladder DNA marker.

Cluster analysis

The RAPD markers produced by eight primers were used to construct a similarity matrix (Table 2). The genetic similarity among all populations of *H. avenae* ranged from 0.51 to 0.74. The smallest genetic similarity resulted from the population of *H. avenae* from Unizah and Sheri (0.51). While, the population of *H. avenae* from Unizah was the closet relative to Buraida population (0.71). The two populations of *H. avenae* from Zolfy and Sheri were most closely related (about 0.74 similarity) than other populations. The dendrogram constructed from simple matching with UPGMA analysis revealed two main clusters (Fig. 3). Cluster I contained the populations of *H. avenae* from Buraida, Unizah and Badaei. Cluster II contained the populations of *H. avenae* from Sheri and Zolfy.

Table 2. Simple matching coefficients of similarity determined from analysis using 26 RAPD loci.

Population	Buraida	Unizah	Badaei	Sheri	Zolfy
Buraida	1.000				
Unizah	0.714	1.000			
Badaei	0.571	0.686	1.000		
Sheri	0.686	0.514	0.600	1.000	
Zolfy	0.657	0.600	0.629	0.743	1.000

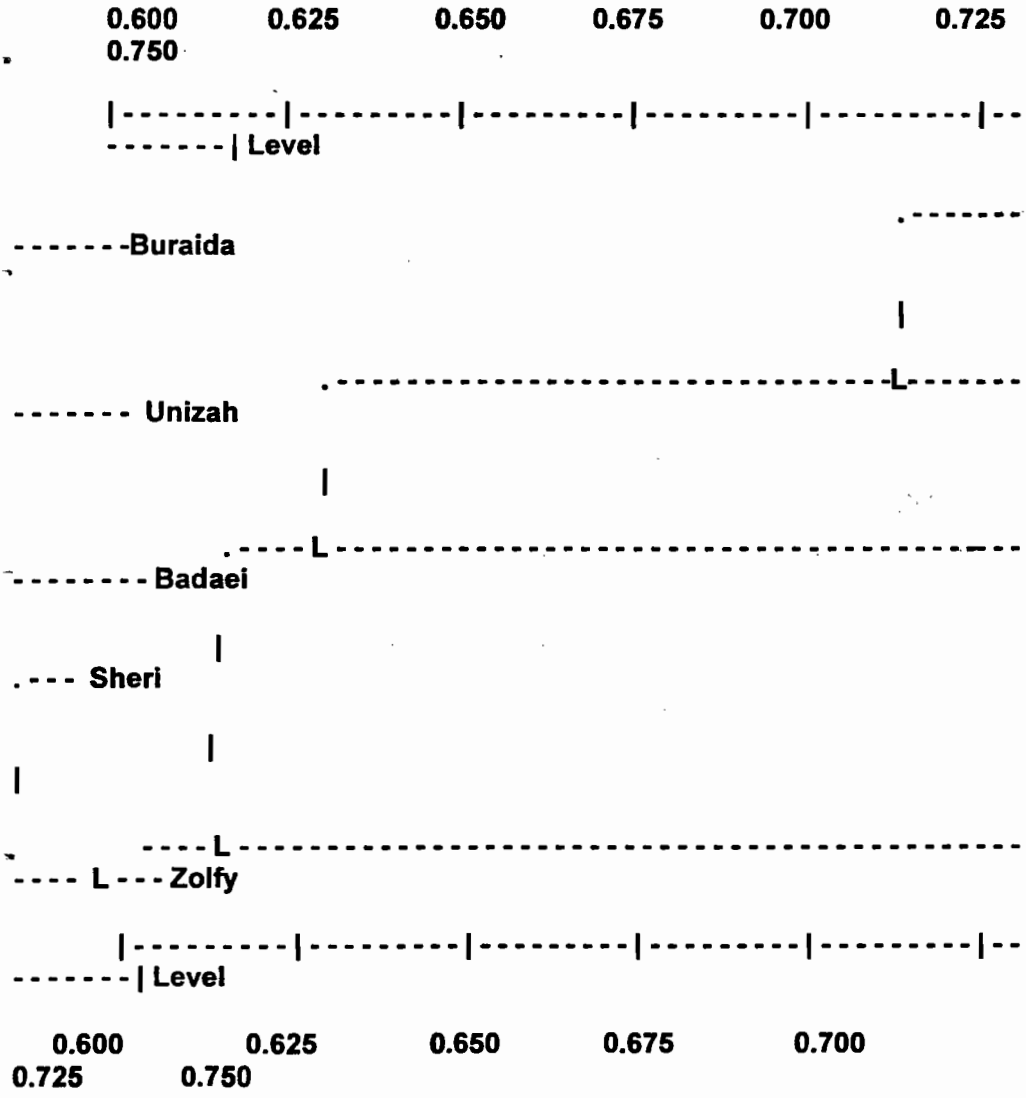

Fig. 3. Dendrogram constructed from similarity coefficients and showing the clustering of five populations of *H. avenae*.

DISCUSSION

Rapid and accurate identification of nematode species is essential for effective nematode management programs. The internal transcribed regions of

ribosomal DNA proved to be a useful method for accurate identification of the cereal nematode, *H. avenae* populations from the central Saudi Arabia. The PCR-rDNA-RFLP method has already been successfully used for the differentiation of other species of the *H. avenae* complex (Bekal *et al.*, 1997; Subbotin *et al.*, 1999) and identification of many other nematode species (Gabler *et al.*, 2000).

RAPD analysis showed that polymorphism among populations of *H. avenae* can arise through: nucleotide changes that prevent amplification by introducing a mismatch at one priming site; deletion of a priming site; insertions that render priming sites too distant to support amplification; and insertions or deletions that change the size of the amplified product (Williams *et al.*, 1990). RAPD procedure used in this study was relatively simple, and the polymorphisms detected were repeatable. Compared with RFLP analysis, a major advantage of the RAPD method may be the reduced effort required for screening. Blok *et al.*, (1997) showed that the RAPD-PCR technique could be used to study the genetic variation within and between the tropical *Meloidogyne* species. Studies showing the comparison of RAPD-PCR and RFLP data showed similar levels of interspecific variation but greater intraspecific variation in case of RAPD-PCR.

The dendogram constructed with molecular data in this study supported the observations of Sturthan and Wouts (1995) and Subbotin *et al.*, (1999) that there is an extensive variation within the *H. avenae* group. Therefore, this study proved that RAPD marker can distinctly separate populations within the *H. avenae* group from central Saudi Arabia.

This is the first report indicating the occurance and identification of species of *H. avenae* in these locations using DNA markers. Further DNA observations and biological studies are needed to identify at which taxonomical level populations of cyst nematodes can be separated.

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REFERENCES

Al-Hazmi, A.S., R. Cook, and A.A.M. Ibrahim. 2001. Pathotype characterization of the cereal cyst nematode, *Heterodera avenae*, in Saudi Arabia. Nematology 3: 379-382.

Al-Hazmi, A.S., A.A.M. Ibrahim, and A.T. Abdul-Razig. 1994. Occurrence, morphology and reproduction of *Heterodera avenae* on wheat and barley in Saudi Arabia. Pakistan Journal of Nematology 12: 117-129.

Al-Rehiayani, S. 2002. The cereal nematode, *Heterodera avenae*, in Al-Qassim area, Saudi Arabia. Nematology 4:260.

Andersen, S., and K. Andersen. 1982. Suggestions for determination and terminology of pathotypes and genes for resistance in cyst-forming nematodes, especially *Heterodera avenae*. *EPPO Bulletin* 12: 379-386.

Bekal, S., J.P. Gauthier, and R. Rivoal. 1997. Genetic diversity among a complex of cereal cyst nematodes inferred from RFLP analysis of the ribosomal internal transcribed spacer region. Genome 40: 479-486.

Blok, C.B., M.S. Phillips, J.W. McNicol, and M. Fargette. 1997. Genetic variation in tropical *Meloidogyne spp.* as shown by RAPDs. Fundam. Appl. Nematol. 20: 127-133.

Caswell-Chen, E.P., V.M. Williamson, and F.F. Wu. 1992. Random amplified polymorphic DNA analysis *Heterodera cruciferae* and *H. schachtii* populations. J. Nematol. 24: 343-351.

Chacon, M.R., E. Rodriguez, R.M.E Parkhouse, P.R. Burrows, and T. Garate. 1994. The differentiation of parasitic nematodes using random amplified polymorphic DNA. J. Helminthol. 68: 109-113.

Cook, R. 1982. Cereal and grass hosts of some graminaceous cyst nematodes. EPPO (Eur. Mediterr. Plant Prot. Organ.) Bull. 12: 399-411.

Curran, J. and Robinson. 1993. Molecular aids to nematode diagnosis. *In* Kevans, D.L., Trudgill, D.L. & Webster, J.M (Eds). Plant parasitic nematodes in temperate agriculture. CABI, pp.545-564.

Dyble, J., H.W. Paerl, and B.A. Neilan. 2002. Genetic characterization of *Cylindrospermopsis raciborskii* (Cyanobacteria) isolates from diverse geographic origins based on *nif*H *and cpc*BA-IGS nucleotide sequence analysis. Applied and Environmental Microbiology, pp. 2567-2571.

Ferris, V.R., J.M. Ferris, J. Faghihi, and A. Ireholm. 1994. Comparisons of isolates of *Heterodera avenae* using 2-D PAGE protein patterns and ribosomal DNA. Journal of Nematology 26: 144-141.

Gabler, C., D. Sturhan, S.A Subbotin, and H.J. Rumpenhorst. 2000. Heterodera pratensis sp. n., a new cyst nematode of the H. avenae complex (Nematoda: Heteroderidae). Russian Journal of Nematology 8: 115-126.

Lopez-Brana, I., M.D. Romero, and A. Delibes. 1996. Analysis of *Heterodera avenae* populations by the random amplified polymorphic DNA technique. Genome 39: 118-122.

Mulvey, R.H., and A.M. Golden 1983. An illustrated key to the cyst-forming genera and species of Heteroderidae in the Western Hemisphere with species morphometrics and distribution. Journal of Nematology 15: 1-59.

Power, T.O., T.C. Todd, A.M. Burnell, P.C.B. Murray, C.C. Fleming, A.L. Szalanskii, B.A. Adams, and T.S. Harris. 1997. The rDNA internal transcribed spacer region as a taxonomic marker for nematodes. Journal of Nematology 29: 441-450.

Rohlf, F.J. 1993. NTSYS-PC numerical taxonomy and multivariate system, version 1.8. Applied Biostatistics Inc., New York.

Stone, A.R. and A.J Hill. 1982. Some problems posed by the *Heterodera avenae* complex. EPPO (Eur. Mediterr. Plant Prot. Organ.) Bull. 12: 317-320.

Sturhan, D. and H.J. Rumpenhorst. 1996. Untersuchungen uber den *Heterodera avenae*-Komplex. *Mitteilungen aus der Biologischen Bundesanstalt fur Land und Forstwirtschaft Berlin Dahlem* 317:75-91.

Sturhan, D. and W.M. Wouts. 1995. On the identify of *Heterodera turcomanica* Kirjanova & Shagalina, 1965 and the synonymy of the genus *Ephippiodera* with *Heterodera* (Nematoda: Heteroderidae). Nematologica 41: 566-574.

Subbotin, S.A., L. Waeyenberge, I.A. Molokanova, and M. Moens. 1999. Identification of *Heterodera avenae* group species by morphological and rDNA-RFLPs. Nematology 1: 195-207.

Vrain, T.C., D.A. Wakarchuk, A.C. Levesque, and R.I. Hamilton. 1992. Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. Fundam. Appl. Nematol. 15: 563-573.

Williams, G.K., A.R. Kubelik, K.L. Livak, J.A. Rafalski, and S.V. Tingey. 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research. 18: 6531-6535.

الملخص العربي

التعرف علي نيماتودا حويصلات الحبوب في المنطقة الوسطى للمملكة العربية السعودية باستخدام الدلائل الجزيئية ال RAPDوال ITS-rDNA

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استخدمت الدلائل الجزيئية ال RAPDوال ITS-rDNA للتعرف علي خمس عشائر من نيماتودا حويصلات الحبوب *Heterodera avenae* جمعت من حقول القمح في بريدة و عنيزة و البدائع و شيري و الزلفي من المنطقة الوسطى للمملكة العربية السعودية. و أعطى تفاعل البلمرة المتسلسل باستخدام ITS-I rDNA لعشائر النيماتودا حزمة واحدة عند ١٢٠٠زوج من النيوكليوتيدات.و باستخدام تحليل ال RAPD لتقدير الاختلافات الوراثية بين عشائر النيماتودا Heterodera avenae . تراوحت درجة التشابة الوراثي بين ٠,٥١ الي ٠,٧٤ وكانت أقل درجة تشابة وراثي بين عشائر النيماتودا من عنيزة و شيري بينما كانت عشائر النيماتودا من بريدة و عنيزة علي درجة تشابة عالية.و على أساس التحليل التجميعي قسمت عشائر النيماتودا إلي مجموعتين: المجموعة الأولي تشتمل علي عشائر النيماتودا من بريدة و عنيزة و البدائع و المجموعة الثانية تشتمل علي عشائر النيماتودا من شيري و الزلفي. و أعطت هذة الدراسة تعرف دقيق لعشائر نيماتودا حويصلات الحبوب *Heterodera avenae* من المناطق تحت الدراسة باستخدام الدلائل الجزيئية.