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Ribosomal DNA sequence analysis of *Heterodera filipjevi* and *H. latipons* isolates from Russia and comparisons with other nematode isolates

Virginia R. Ferris*, Sergei A. Subbotin, Anita Ireholm***, Yitzhak Spiegel****, Jamal Faghihi* and John M. Ferris***

*Department of Entomology, Purdue University, West Lafayette, IN, 47907-1158, U.S.A., e-mail: vferris@entm.purdue.edu
**Institute of Parasitology of Russian Academy of Sciences, Leninskii Prospect, 33, Moscow, 117071, Russia,
***Swedish University of Agricultural Science, S-230 53 Alnarp, Sweden,
****Division of Nematology, Agricultural Research Organization, The Volcani Center, P. O. Box 6 Bet Dagan, Israel 50250.

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Summary. Ribosomal DNA (ITS rDNA) sequence data for two geographically separated isolates of *Heterodera filipjevi* from Russia were identical, and were 99.7% similar to rDNA sequence from Swedish East and West Gotland strains of *H. avenae*. These data are consistent with the view that the 'Gotland strain' of *H. avenae* and *H. filipjevi* are conspecific. The ITS rDNA sequence from an isolate of *H. latipons* from the Rostov region of Russia was 98.1% similar to the ITS rDNA sequence of an isolate from Gilat, Israel, the type locality of *H. latipons*, which suggests the possibility that the two morphologically similar, but geographically separated, isolates might be sibling species.
Key words: *Heterodera avenae*, *H. filipjevi*, *H. latipons*, ribosomal DNA, rDNA sequence, phylogenetic analysis, Russia, Israel, Sweden.

Heterodera filipjevi (Madzhidov, 1981) Stelter, 1984 is an important nematode pest of cereals in European and Middle Asian countries (Subbotin, *et al.*, 1999). It was believed that this species, described from Tadzhikistan, had a limited geographic distribution, but recent morphological, biochemical and molecular studies indicate that populations from widely dispersed geographic regions, originally identified as *H. avenae* Wollenweber, 1924, or strains of *H. avenae* (Gotland and pathotype 3), or even as *H. mani* (Mathews, 1971), are probably conspecific with *H. filipjevi*. Ferris *et al.* (1989, 1994) compared isolates of *H. avenae sensu stricto* from Sweden, Australia and America with isolates of the Swedish 'Gotland strain' of *H. avenae* using 2-D PAGE protein patterns and sequence data for the two internal transcribed spacers of the ribosomal RNA gene (ITS rDNA). These researchers found significant differences between the 'Gotland strain' isolates of *H. avenae* and *H. avenae sensu stricto*. Subbotin *et al.* (1999) reported that, based on canonical discriminant analysis of morphometric and restriction fragment length polymorphism (RFLP) data, the East Gotland strain from Etelhem Sweden was similar/identical to *H. filipjevi* populations from Russia. An objective of the present research was to compare sequence data for ITS rDNA from two populations of *H. filipjevi* from Russia with previously obtained ITS rDNA sequence data from 'Gotland strain' isolates of *H. avenae*, including an Etelhem isolate (Ferris *et al.*, 1994).

Heterodera latipons Franklin, 1969 is an important cereal cyst nematode in many Mediterranean countries (Franklin, 1969). Widespread geographic populations have many morphological similarities, but the extent of genetic diversity among these populations is presently unknown. It is possible that some of these populations might actually comprise sibling species. A second objective of this study was to compare ITS rDNA sequence from a Russian isolate of *H. latipons* with ITS rDNA sequence for *H. latipons* from the type locality in Israel.

MATERIALS AND METHODS

Nematode isolates. Populations used for this study included two isolates of *H. filipjevi* and one of *H. latipons* from Russia. The *H. filipjevi* isolates were from Pushkin, Leningrad region (host: false wheat - *Elytrigia repens* L.) and from Vad, Nizhnii Novgorod

region (host: oats - *Avena sativa* L.). The *H. latipons* isolate (host: false wheat - *Elytrigia repens* L.) was from the Rostov region. Other isolates from earlier work (Ferris *et al.*, 1989, 1994) used for comparisons in this study, included the following isolates from Sweden: *H. avenae sensu stricto* of pathotype Ha12 from Nässja, three isolates of the East Gotland pathotype (from Etelhem, Stacketorp and Alnarp), and two isolates of the 'West Gotland' pathotype (from Hjelmsäter and N. Härene). All cereal cyst nematodes used in the earlier studies were from growth chamber cultures of barley cv. Varde (*Hordeum vulgare* L.). Details can be found in the original publications (Ferris *et al.*, 1989, 1994). The isolate of *H. latipons* from oats growing in Israel was from Gilat, Israel, the type locality (Franklin, 1969).

Amplification, cloning and sequencing. The methods for handling the nematodes and obtaining the rDNA were essentially as previously described by Ferris *et al.* (1993, 1994, 1995), and are briefly summarized here. Female nematodes (usually one or two) taken from a vial of 70% alcohol were rinsed in sterile water and crushed in 20 µl cold TE buffer, using a Radnoti (Thomas Scientific, Swedesboro, NJ, USA) 25 µl-size glass homogenizer. The homogenate was either used immediately or stored at -20° C. Five such preparations were usually made for each isolate. Prior to amplification by polymerase chain reaction (PCR), the homogenate was thawed briefly, and spun in a microfuge for 3 minutes at 16,000 g. About 15 µl of the supernatant was discarded, 60 µl Instagene (BioRad, Hercules, CA, USA) was added to the pellet, and the procedure completed according to manufacturer's directions for use of Instagene. Usually, 10 µl of a 1:10 dilution of the preparation was used for each 25 µl PCR reaction, but occasionally this was varied to improve amplification. Standard PCR (Saiki, 1990), as described in Ferris *et al.* (1993) was used with reagents from Perkin Elmer (Norwalk, CT, USA) and Promega (Madison, WI, USA), and a COY Tempcycler model 50 (COY, Inc., Ann Arbor, MI, USA). The PCR was for 30 cycles with denaturation at 94 °C for 1 min, annealing at 43 °C for 1 min, and extension at 72 °C for 1 min. The amplified DNA band was monitored by electrophoresis in SeaPlaque GTG agarose (FMC, Rockland, ME, USA), excised, and diluted in 1 ml sterile water. Thereafter, approximately 1 ng of the diluted DNA was amplified for 30 cycles as described previously, except that annealing was at 60 °C. Primers for PCR amplification were those described by Ferris *et al.* (1993). The amplified region of about 1 kb in length spanned the two ITS regions and included the 5.8S gene that lies between them. Multiple amplifications were carried out for each isolate in order to be able to detect any sequence variation, but none occurred with these isolates. Amplified rDNA was cloned into the pGEM-T vector (Promega) and transformed into the *E. coli* strain JM109 according to the manufacturer's protocols. Clones were checked, using PCR, for the correct insert of the predicted size (approximately 1kb), and plasmid preparations made using the Wizard Plus Miniprep kit (Promega). Manual sequencing, using Sequenase version 2.0 (U.S. Biochemical, Cleveland, OH, USA), was used for all earlier isolates previously described (Ferris *et al.*, 1994); but sequences were obtained for the isolates from Russia with an automatic sequencing system (ALFexpress, Pharmacia Biotech, Piscataway, NJ, USA) used in the Purdue Sequencing Laboratory. In each instance, the double-stranded sequencing was from multiple clones, usually 2 to 4 clones were sequenced per isolate, to assure accuracy. Internal primers used for manual sequencing were those reported previously by Ferris *et al.* (1994). Sequence obtained from the automatic system was long enough, and of sufficient quality, that internal primers were not necessary.

Sequence comparisons. Sequence data for each isolate, including ITS1, 5.8S, and ITS2 rDNA, were compared with the data obtained for the other isolates, using the computer program GAP in the Sequence Analysis Software Package of the Genetics Computer Group, followed by PILEUP (Devereaux *et al.*, 1984).

RESULTS

Sequence data for the Pushkin isolate of *H. filipjevi*, the Etelhem isolate of the East Gotland strain, and the Russia and Israel isolates of *H. latipons*, are shown in Fig. 1. The rDNA sequences of the two isolates of *H. filipjevi* were identical. The Swedish East and West Gotland sequences also were identical, and differed from the sequence of the *H. filipjevi* isolates by only three base pairs (bp 952-954 in ITS2, Fig. 1). Pairwise percentage sequence similarities for these isolates, and for *H. avenae*, are shown in Table 1. The *H. filipjevi* sequence was 99.7% identical to the sequence from the Swedish East and West Gotland strains. The rDNA sequence of the isolate of *H. latipons* from Russia was 98.1% similar to that from the type locality, Gilat, Israel.

DISCUSSION

Molecular and biochemical data have contributed to improvements in the identification and placement of nematode populations of species of the *H. avenae* group. In addition to the research described above, Bossis and Rivoal (1996), using 2-D PAGE,

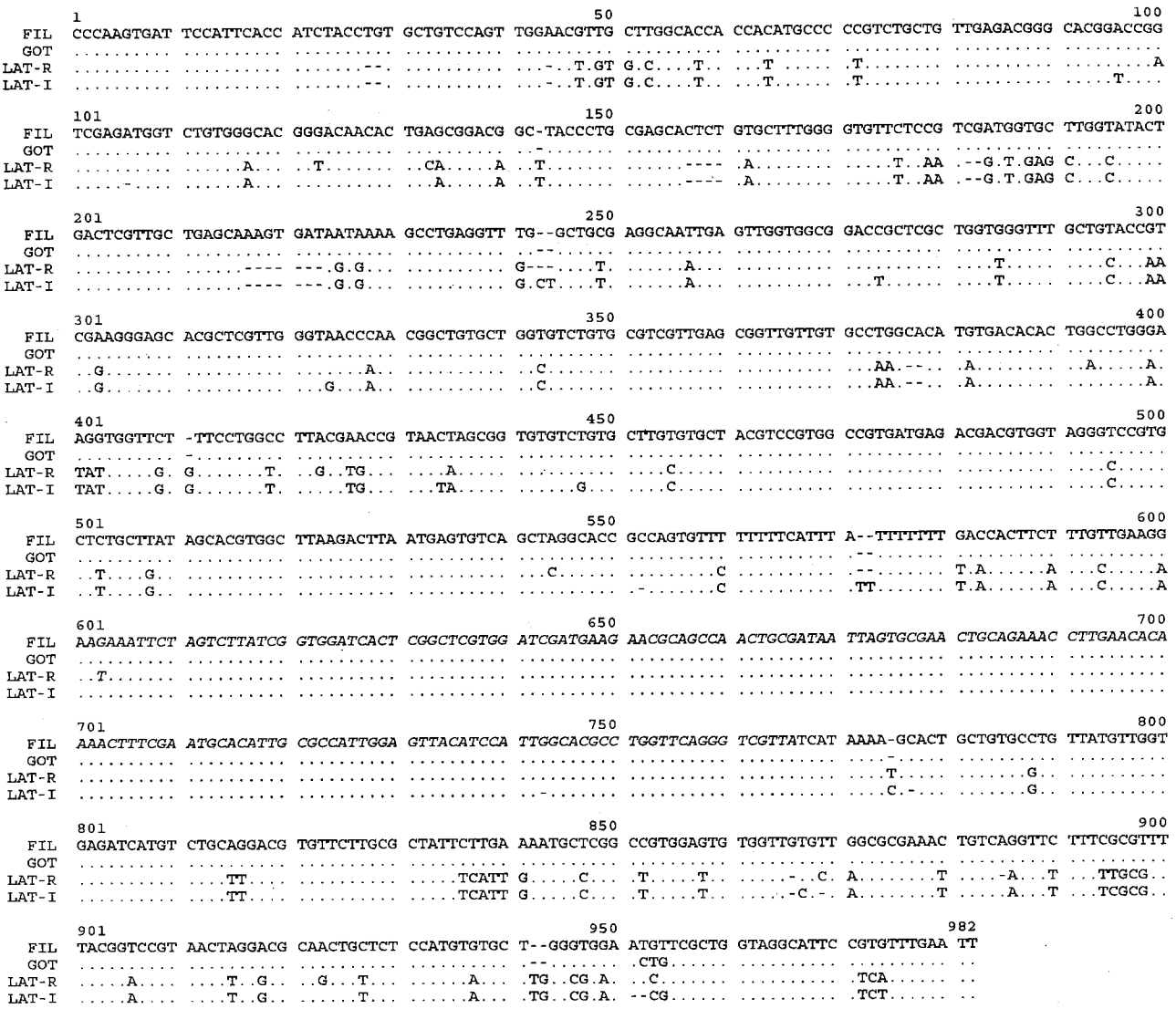

Fig. 1. Nucleotide bases of rDNA sequence of ITS1, 5.8S (italics), and ITS2 for *Heterodera filipjevi* from Russia (FIL); *H. avenae*, 'East Gotland' strain from Etelhem, Sweden (GOT); *H. latipons* from Russia (LAT-R); and *H. latipons* from Israel (LAT-I). All base notations are for the non-transcribed strand. Periods indicate sequence identity and hyphens indicate gaps. Sequence differences are uncorrected for multiple changes at a site.

also showed clear genetic dissimilarities between *H. avenae sensu stricto* and the 'Gotland strain' isolates. López-Braña *et al.* (1996) used random amplified polymorphic DNA (RAPD) patterns to separate *H. avenae sensu stricto* from Gotland strain populations from Spain (Torralba de Calatrava) and Sweden (Etelhem).

Clarification of the taxonomy of strains and populations of cereal cyst nematodes has been progressed through comparison of populations from the type locality of *H. filipjevi* with other nominal *H. avenae* and 'Gotland strain' populations. Subbotin *et al.* (1996, 1999) showed that cereal cyst nematode populations from Russia and the Ukraine, previously assigned to *H. avenae*, were morphologically and morphometrically conspecific with the Tadzhikistan population of *H. filipjevi*. The authors confirmed these findings with biochemical data from isoelectric focusing patterns of protein extracts. They further showed that only *H. filipjevi* is widely distributed in the European part of Russia. On the basis of electrophoretic and morphological studies, Rumpenhorst *et al.* (1996), Sturhan (1996) and Sturhan and Rumpenhorst (1996) reported that most of the cereal cyst nematode populations from Turkey, Iran and Germany, previously identified as *H. avenae* or *H. mani*, were identical to *H. filipjevi*. Sturhan and Rumpenhorst (1996) published identical protein patterns from cysts of the 'Gotland strain', the strain known as pathotype 3, and *H. filipjevi* populations.

Table 1. Pairwise percentage similarity values for the nucleotide bases shown on Figure 1 plus comparable rDNA data for *H. avenae* (Ferris *et al.*, 1994)*.

	FIL	GOT	LAT-R	LAT-I	HAV
FIL	-	99.7	90.2	89.4	96.5
GOT	-	-	90.0	89.5	96.8
LAT-R	-	-	-	98.1	87.0
LAT-I	-	-	-	-	87.1

*Abbreviations for species/isolates are as in Figure 1. HAV = *H. avenae.*

Bekal *et al.* (1997), in a study in which internal transcribed spacer (ITS) rDNA was cut with restriction enzymes, found no differences in restriction fragment length polymorphisms (RFLPs) between *H. filipjevi* populations and 'Gotland strain' populations from Sweden (Etelhem), Spain (Torralba de Calatrava) and Bulgaria. Subbotin *et al.* (1999) found, using morphometric and rDNA - RFLP data, that the populations used by Bekel *et al.* (1997) from Etelhem, Sweden and from Spain were conspecific with further populations of *H. filipjevi* from Russia.

Data from our research are consistent with suggestions of Sturhan & Rumpenhorst (1996), Bekal *et al.* (1997), and Subbotin *et al.* (1999) that the Swedish Gotland strain isolates are conspecific with *H. filipjevi*; and on the basis of presently available data we believe them to be so. Similarity in ITS rDNA between the *H. latipons* isolate from Russia and that from Israel is high (98.1%), suggesting a close phylogenetic relationship. However, similarities in ITS rDNA sequence between *Globodera rostochiensis* and *G. pallida*, the two potato cyst nematode species, are only slightly less (96.7%) than for the two *H. latipons* populations (Ferris *et al.*, 1995). Our original diagnosis that the Russian isolate used here was *H. latipons* was based on data discussed in Subbotin *et al.* (1999). More recent observations by one of us (SAS) of further populations from Asia, and comparions with type slides from the Rothamsted collection, suggest the possibility that our isolate from Russia, could, in fact, be a sibling species of *H. latipons*. In addition to further morphometric and other research on similar populations from diverse parts of the world, molecular sequence data based on other genes will likely be needed to resolve the question of the extent of variability and species integrity of these geographic isolates that appear to be similar to *H. latipons*.

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Ferris V.R., Subbotin S.A., Ireholm A., Spiegel Y., Faghihi J., Ferris J.M. Анализ последовательностей рибосомальной ДНК изолятов *Heterodera filipjevi* и *H. latipons* из России и сравнение их с другими изолятами нематод.

Резюме. Последовательности рибосомальной ДНК (ITS rDNA) двух географически удаленных изолятов *Heterodera filipjevi* из России оказались идентичными и на 99.7% соответствовали рДНК последовательностям Восточной и Западной Готландской расы *H. avenae* из Щвеции. Полученные данные подтверждают, что Готландская раса *H. avenae* и *H. filipjevi* конспецифичны. Последовательности рибосомальной ДНК изолята *H. latipons* из Ростовской области России были на 98.1% сходны с последовательностями ITS рДНК изолята этого вида из Гилата (Израиль) - типового места обнаружения этого вида. Эти данные показывают, что два этих морфологически сходных и географически удаленных друг от друга изолята возможно представляют собой виды-близнецы.