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RESEARCH ARTICLE

Morphological and molecular characterizations of cereal cyst nematode *Heterodera avenae* Wollenweber, 1924 from the Czech Republic

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Abstract

The cereal cyst nematode, *Heterodera avenae* Wollenweber, 1924, is a major pest of cereal crops throughout the world and causes serious yield losses, especially of wheat. Previous studies have shown that this species is widely distributed in the Czech Republic. In this study, seven populations of *H. avenae* were molecularly studied, and one population was morphologically described. Three regions (18S, 28S, and internal transcribed spacer 1) of ribosomal DNA were sequenced and the analysis of the 18S gene of six populations did not reveal any variation, whereas the internal transcribed spacer 1 and 28S sequences of six populations differed by only two nucleotides from a population in Žilina. Precise and quick identification of cereal cyst nematodes is important for effective control measures and ribosomal sequence analyses of seven populations in this study will be useful in future phylogenetic studies of *Heterodera* spp. occurring in the Czech Republic.

Keywords: *Heterodera avenae*, PCR, ribosomal DNA, sequencing, nematode

1. Introduction

Cereal crops, such as barley, wheat and oats, are among the major staple crops that have economic importance worldwide. These crops are parasitized by many pathogens and pests, including plant parasitic nematodes. Among plant parasitic nematodes, cyst-forming nematodes (*Heterodera* spp.) are considered very damaging because of the resulting crop losses. Currently, the genus *Heterodera* contains more than 90 species (Subbotin *et al.* 2010), and the most economically important cereal cyst nematode (CCN) is a composite group of 12 species (*H. arenaria* Coooper, 1955; *H. aucklandica* Wouts & Sturhan, 1995; *H. avenae* Wollenweber, 1924; *H. bifenestra* Coooper, 1955; *H. filipjevi* (Madzhidov, 1981) Stelter, 1984; *H. hordecalis* Andersson, 1975; *H. iri* Mathews, 1971; *H. latipons* Franklin, 1969; *H. mani* Mathews, 1971; *H. pratensis* Gäbler, Sturhan, Subbotin & Rumpenhorst, 2000; *H. spinicauda* Wouts, Schoemaker, Sturhan & Burrows, 1995; *H. turcomanica* Kirjanova & Shangalina, 1965) that infects graminaceous cereals and grasses, resulting in high crop losses worldwide (Yan and Smiley 2010). Among them, the most economically important species are *H. avenae*, *H. filipjevi* and *H. latipons* (Akar *et al.* 2009). Damage to crops by these nematodes is the second in importance to damage caused by root-knot nematodes (Jones *et al.* 2013). *H. avenae* has been reported as the most prevalent and damaging nematode for wheat and barley in different parts of the world (Ibrahim *et al.* 1999), and these nematodes are documented to be

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of economic importance for wheat in locations where both drought occurs and cereal monoculture is predominant (Nicol and Rivoal 2008). In Europe, more than 50% of the fields in major cereal growing areas are infected by *H. avenae* (Rivoal and Cook 1993). In the Czech Republic, 62% of fields were infected with *H. avenae* (Sabová *et al.* 1989a; Valocká *et al.* 1993), and 8.3% of fields were infected above the economic damage threshold (Sabová *et al.* 1989b). Yield losses in the former Czechoslovakia were 35–88% (Sabová *et al.* 1990).

The species of the *H. avenae* complex are differentiated from each other by small morphological and morphometric characteristics. Morphological identification keys (Mulvey and Golden 1983; Handoo 2002) are published for the *H. avenae* group species, but with the increasing number of species in this group, reliable identification based on morphology is becoming increasingly difficult. Moreover, identification based on the morphology and morphometry of cysts and second-stage juveniles is time-consuming and requires substantial skill. Polymerase chain reactions based on species-specific primers are a reliable tool for quickly and precisely identifying cyst nematode species (Toumi *et al.* 2013; Yan *et al.* 2013). Identification by molecular methods strengthens taxonomical identification based on the analysis of the differences in the morphological characteristics. Likewise, morphological identification is needed to support the identification performed according to molecular tests. The objectives of this study were to: 1) characterise a population from Žilina morphologically and morphometrically; 2) confirm the identity of seven populations of *H. avenae* using polymerase chain reaction; and 3) sequence three regions (18S, 28S and internal transcribed spacer 1 (ITS1)) of the ribosomal DNA from all populations studied.

2. Materials and methods

2.1. Nematode populations and extraction

Soil samples taken at a depth of 0–30 cm were collected from cereals growing areas from the Czech Republic, and soil samples from three unknown localities in Moravia were also included. The sieving and decanting method was used to directly extract male nematodes and second-stage juveniles from soil (Brown and Boag 1998). Nematodes were extracted from the soil by sieving at 1 mm, 150 μm and 75 μm and then placing the residual on 99- and 56-μm sieves on a Baermann funnel from 24–48 h. Male and second-stage juveniles for morphological study were killed with heat, fixed in triethanolamine formalin (TAF), processed in slow glycerin processing and mounted in anhydrous glycerin on slides. Photomicrographs were recorded using a digital camera that was linked to a computer, and measurements were made using imaging software (DP-soft, Olympus, Japan).

Cysts were extracted from a 250-g subsample. Brown cysts were extracted using sieving and flotation methods (Shepherd 1986). The nematode suspension was passed through sieves that had different sizes (150 and 250 mm). The soil-water suspension was placed on a white tissue paper supported by gauze, and the CCN cysts were detected using a stereomicroscope. Eggs were obtained by squashing cysts. Vulval cones (the posterior ends of brown cysts) mounted in glycerol-gelatine on permanent slides were examined, measured and photographed. Cone tops were prepared as described by Mulvey (1972).

2.2. Polymerase chain reaction

The specific amplification primer set, F: HaITS-F6 (5′-ATG CCC CCG TCT GCT GA-3′)+R: HaITS-R4 (5′-GAG CGT GCT CGT CCA AC-3′), was used (Yan *et al.* 2013). Total genomic DNA was extracted from single individuals using a rapid technique (Stanton *et al.* 1998). Individual second-stage juveniles were digested in 0.25 mol L^{-1} NaOH overnight and then heated to 99°C for 3 min. Afterwards, 10 μL of 0.25 mol L^{-1} HCl and 5 μL each of 0.5 mol L^{-1} Tris-HCl (pH 8) and 2% Triton X-100 were added. Then, the mixture was incubated for another 3 min at 99°C. Finally, the DNA suspension was cooled, and the DNA was either used directly for PCR or was stored at –20°C until the template was needed for PCR reactions. PCR was performed in a total volume of 25 μL containing 1 PCR bead (GE Healthcare, Buckinghamshire, UK), 20.5 μL of double distilled sterile water, and 2.0 μL of each primer (10 pmol μL^{-1}) (synthesized by Generi Biotech, Hradec Králové, Czech Republic). To this, 0.5 μL of DNA was added as a template for PCR. Second-stage juveniles of *H. schachtii* from three localities were used as negative controls, and a sterilized water control was included in all PCR experiments. All PCR reactions were performed using a DNA Engine PTC-1148 thermal cycler (Bio-Rad, USA). The DNA was subjected to PCR with the following specifications: first denaturation for 3 min at 95°C, 34 cycles for 30 s at 95°C, 30 s at 64°C, and 30 s at 72°C and final extension at 72°C for 7 min (Yan *et al.* 2013). An aliquot (6 μL) of each amplification reaction was mixed with 1.5 μL of 6× loading dye (Fermentas, MBI) and electrophoresed in a high-resolution 1.5% agarose gel with TAE (Tris-acetate-EDTA) buffer. The bands were visualized and photographed under UV (312 nm) after syber safe (1 μg mL^{-1}) binding to the DNA fragments. A 100-base pair marker (Fermentas) was included on the gel.

2.3. Sequencing

Three regions (18S, 28S and ITS1) of ribosomal DNA were sequenced, and two specimens per population sequenced.