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Research Note

Characterisation of *Trichodorus similis* (Nematoda: Trichodoridae) associated with potato from the Czech Republic

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Summary

Trichodorus similis associated with potato in the Czech Republic was described and illustrated. This study provides additional information on morphometrical and morphological characters of *T. similis* and integrates morphological and genetic data obtained by species-specific polymerase chain reaction and sequencing (ITS1 and D2-D3 expansion segments of 28S rDNA). The knowledge on morphological variability and genetic diversity is extended, and a rapid and accurate molecular diagnostics was successfully applied.
Keywords: D2-D3 rDNA; ITS1, morphology; species-specific PCR, Triplonchida

Introduction

Trichodorids can cause substantial crop losses directly by feeding on plant roots and indirectly as vector of *Tobraviruses*; both vector and virus are polyphagous and are of considerable economic importance in a number of agricultural crops (Taylor & Brown, 1997; MacFarlane *et al*., 2002). Previous records of trichodorids from the Czech Republic are from apple, birch, elm, grass, maple, oak, peach, pine, strawberry and sweet cherry (Kumari *et al*., 2007; Kumari & Decraemer, 2009, 2011; Kumari, 2010; Kumari & Subbotin 2012). Research has been initiated in the Czech Republic to determine the presence or extent of trichodorids associated with potato. *Trichodorus similis* Seinhorst, 1963, originally described from the Netherlands, a vector of Tobacco rattle virus (TRV) (Taylor & Brown, 1997) was found at Kaliště region from a potato field, whereas *T. viruliferus* and *Paratrichodorus* sp. were recovered from another locality (unpublished).

Trichodorus similis has been reported from many other European countries: Belgium (Coolen *et al*., 1980), Bulgaria (Peneva & Choleva, 1994), Denmark (Sønderhousen *et al*., 1969), France (Scotto la Massese, 1985), Germany (Hallmann *et al*., 2007), Greece (Brown *et al*., 1996), Italy (Roca & Lamberti, 1984), Norway (Stølen & Markussen, 1985), Poland (Brzeski & Szczygiel, 1974), Russia (Ambrosov *et al*., 1979), Slovakia (Lišková & Sturhan, 1999), Spain (López-Pérez *et al*., 2001), Sweden (Andersson *et al*., 1992), the Netherlands (van Hoof, 1967) and UK (Cooke, 1973), occasionally in the USA, in different habitat types, associated with various crops or wild plants (Decraemer, 1995). The species have been reported to occur in Belarus and Slovenia (http://www.faunaeur.org/), however these records should be confirmed. Recently, it was reported from Turkey (Kepenekci, 2014; Kepenekci *et al*., 2014) associated with grapevine. The conventional identification based on morphological and morphometrical characters, which often reveals intraspecific variability, is complex, difficult and time-consuming. Polymerase chain reaction based on species-specific primers allows quick and precise identification of trichodorid species (Boutsika *et al*., 2004b). Further, identification by molecular methods supports the taxonomical determination based on the analysis of morphological characters. Likewise, morphological identification is also needed to support the molecular tests identification therefore the objective of this study was to characterise a *T. similis* population associated with potato using both morphological and molecular methods.

Table 1. Morphometrics of *Trichodorus similis* males from Kaliště region. Measurements in µm.

Character	Mean ± SD (range)	n
L	730 ± 44 (658 – 783)	6
Onchiostyle	38.9 ± 22 (37 – 40)	5
Pharyngostom	44 ± 0.6 (43 – 45)	6
Pharynx	136.7 ± 2.9 (132 – 142)	6
Anterior to guide ring	18.4 ± 0.6 (17.5 – 19)	5
Anterior to nerve ring	59.7 ± 2 (56 – 61)	6
Anterior to SE	77.0 ± 6.9 (71 – 85)	5
Anterior to CP1	32.3 ± 2.9 (29.5 – 37)	5
Distance CP1 to CP2	20.2 ± 2 (17 – 22)	5
Distance CP2 to CP3	14.8 ± 2.2 (11 – 16)	5
Distances CP3 to SE	11 ± 5 (7.5 – 20)	3
Distance CP1 to SE	46 ± 4 (42 – 50.5)	3
Body diam. at cardia	26 ± 1.6 (23 – 28)	6
Mid – body diam.	28.2 ± 2.5 (25 – 31)	6
Anal body diam.	23.5 ± 2.1 (20 – 26.5)	6
Spicule length	36.6 ± 1 (34 – 38)	6
Gubernaculum	15.6 ± 0.5 (15 – 16)	5
Distance SP1 to cloacal opening	26.7 ± 1.2 (25 – 28)	6
Distance SP1 to SP2	29.2 ± 2.5 (25 – 32)	6
Distance SP2 to SP3	40.2 ± 5.7 (35.5 – 52)	6
Tail	15.2 ± 1.2 (13 – 16.5)	6
a	26.0 ± 1.9 (23.6 – 29.0)	6
b	5.3 ± 0.30 (4.8 – 5.7)	6
c	48.2 ± 3.2 (44.1 – 53.2)	6
c'	0.6 ± 0.03 (0.6 – 0.7)	6

Materials and Methods

Morphological study

Specimens from the Kaliště field population were extracted by sieving on 1 mm, 150 µm and 75 µm and placing the residual on 99 µm sieves on a Baermann funnel for 24 – 48 hours (Brown & Boag, 1988). Nematodes were heat killed, fixed in TAF, processed in slow glycerin process and mounted in anhydrous glycerin on slides. Photographs were taken using an Axio Imager.M2–Carl Zeiss compound microscope with a digital camera (ProgRes C7) and specialised software (CapturePro Software 2.8). Measurements were made using an Olympus BX41 light microscope, a digitising tablet (CalComp Drawing Board III, GTCO CalCom Peripherals, Scottsdale, AZ, USA), and computer Digitrak 1.0f programme, (Philip Smith, Scottish Crop Research Institute, Dundee, UK).

Polymerase Chain Reaction

Total genomic DNA was prepared by a rapid technique of Klimyuk *et al*., (1993) and subsequent modification of the technique for nematodes by Stanton *et al*., (1998). The DNA template was amplified by polymerase chain reaction (PCR) using the species-specific primer SIMIREV2 (5'-CAC TCG TCG GAC TCA AAC C-3') and an antisense primer UNIVERSAL (5'-CCC GTC GCT ACT ACC GAT T-3') (Boutsika *et al*., 2004b). Amplification was done using one cycle of 95 °C for 3 min, followed by 35 cycles of 94 °C of 30 s, 55 °C for 30 s and 72 °C for 30 s, and finally one cycle of 72 °C for 10 min for annealing, using a DNA Engine PTC-1148 thermal cycler (Bio-Rad, USA) with heated lid. An aliquot (4 µl) of each amplification product was mixed with 1 µl of 6x loading dye (Fermentas, MBI) and electrophoresed in high resolution 1.5 % agarose gel in TAE buffer. The bands were visualized and photographed under UV (312 nm) after syber safe (1 µg/ml) binding to the DNA fragments. A 100 base pair marker (Fermentas, MBI) was included on the gel.

Sequencing

Two individuals of the Kaliště population (out of 10 with positive species-specific PCR products) were used for sequencing D2–D3 expansion segments of the 28S and ITS1 regions of ribosomal DNA. D2–D3 expansion segment of the 28S gene was amplified and sequenced using the following D2A primers: 5'-ACA AGT ACC GTG AGG GAA AGT TG-3' and D3B: 5'-TCG GAA GGA ACC AGC TAC TA-3' (Nunn, 1992). Internal transcribed spacer 1 (ITS1) was amplified and sequenced using the primers BL18:

Table 2. Morphometrics of *Trichodorus similis* females from Kaliště region. Measurements in µm.

Character	Mean ± SD (range)	n
L	758 ± 63 (630 – 863)	25
Onchiostyle	39.3 ± 0.84 (38 – 42)	25
Pharyngostom	45.3 ± 1.84 (42 – 49)	25
Pharynx	138.7 ± 7.81 121 – 152)	25
Anterior to guide ring	19.0 ± 0.70 (18 – 20)	21
Anterior to nerve ring	63.4 ± 3.1 (59 – 66)	22
Anterior to SE	86.9 ± 5.07 (81 – 98)	8
Body diam. at cardia	27.4 ± 1.52 (24 – 30)	25
Mid-body diam.	30.8 ± 2.06 (26 – 34)	25
Length anterior genital branch	140.6 ± 9.23 (129 – 156)	6
Length posterior genital branch	134.9 ± 8.29 (125 – 152)	6
a	24.7 ± 1.7 (21.3 – 28.1)	25
b	5.5 ± 0.4 (4.7 – 6.3)	25
V	55.8 ± 2.5 (50.2 – 62.3)	25
G1	17.8 ± 1.6 (15.4 – 19.9)	6
G2	17.2 ± 2.0 (15.6 – 21.4)	6

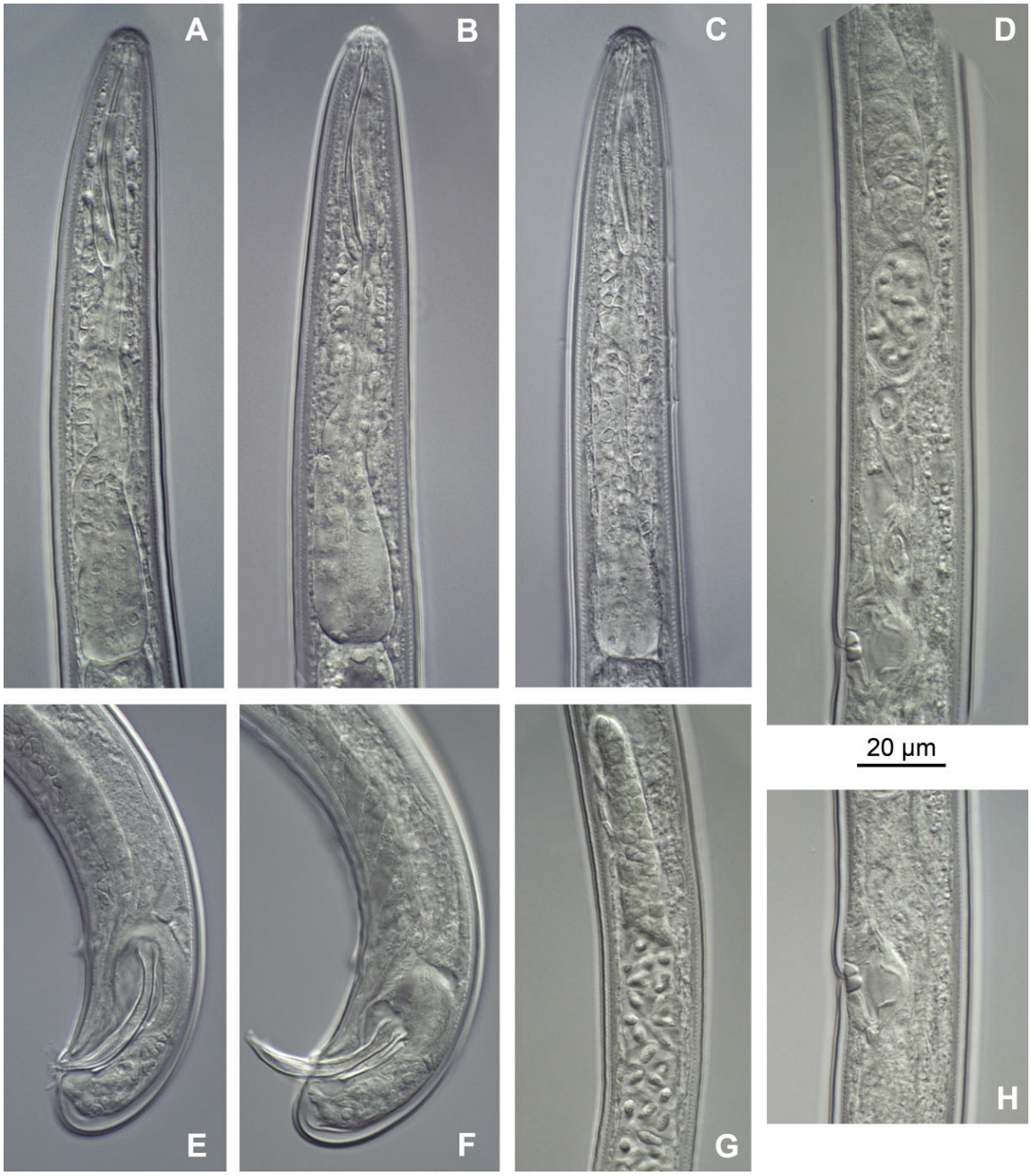

Fig. 1. Photomicrographs of *Trichodorus similis* Seinhorst, 1963, female and male.
A – C: Anterior region of female (A, B) and male (C); D: Female reproductive system (anterior genital branch);
E, F: Posterior body region (male tail and spicules); G: Male gonad; H: Vagina.

5'-CCC GTG GMT ACT ACC GAT T-3' + 5818: 5'-ACG ARC CGA GTG ATC CAC-3' (Boutsika *et al.*, 2004a). PCR mix and cycling profile were same as described above for specific PCR. Aliquots of PCR products were analysed by gel electrophoresis and the remaining products were purified using High Pure Product Purification kit (Roche Diagnostics GmbH, Mannheim, Germany) and sequenced (Macrogen, Netherlands). SequencherTM 4.8 (Genes codes. Corp., Ann Arbor, MI, USA) was used to assemble and view each sequence and check for base-calling errors.

Results and Discussion

Morphological study
(Figs. 1, 2; Tables 1, 2)
Male. Habitus J-shaped when heat killed. Cuticle 2 µm in onchiostyle region and 2 – 2.5 µm along the body, slightly thickened on

Fig. 2. Photomicrographs of *Trichodorus similis* Seinhorst, 1963, female and male.
A: Lips, onchiostyle and ventromedian pores of male; B: Lip and onchiostyle region (female); C: Amphid; D, E – Variations in vagina; F: Male tail and spicules; G, H: Variations in female tail.

tail (3 – 6 µm). Anterior to sensillium pouch 15 µm. Three ventromedian cervical papillae, anterior to secretory-excretory pore (S-E) pore (in one specimen 4, the fourth after S-E pore, Fig. 1C); the first one in the onchiostyle region; in one specimen CP2 opposite to posterior end of pharyngostom. S-E pore opposite the isthmus. Pharyngeal bulb offset. One dorsal and two pairs of ventrosublateral glands, nuclei of the first pair smaller. Spicule markedly and regularly curved; manubrium offset from the shaft, knob like, widened; shaft conical, smooth with few bristles. Gubernaculum slightly curved, hooked proximally and knobbed distally. Sperm large (5 – 6 × 8 – 10 µm) with sausage-shaped nucleus (2 – 3 × 5 – 6 µm). Ventromedian precloacal supplements three, the first just posterior the level of proximal end of retracted spicules.

Codes according to the polytomous key by Decraemer and Baujard (1998): F3 D3 P2 A210 B21 C11 E12 G0 H22 I11 J500 K22 L11 M16 N11 O1.

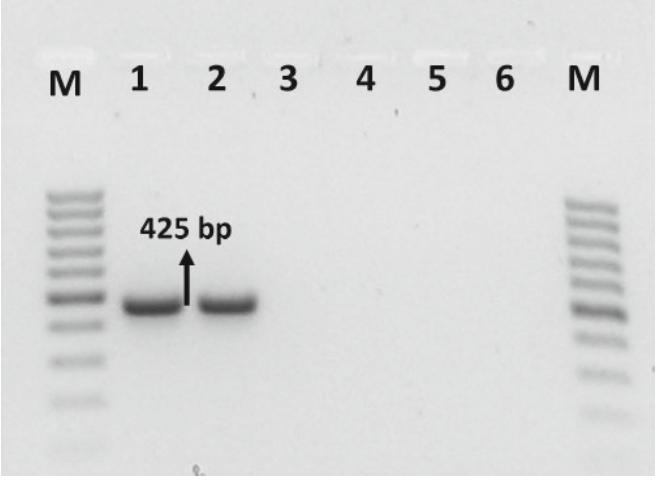

Fig. 3. Electrophoresis of the amplified products of *Trichodorus similis*: lane M - 100bp DNA ladder (Fermentas); lane 1 – female1; lane 2 – female2; lane 3 – *T. pakistanensis*; lane 4 – *T. variopapillatus* 4; lane 5 - *T. viruliferus* and lane 6: negative control.

Female. Body slightly curved ventrally when heat killed. Cuticle 2 – 2.5 µm in onchiostyle region, 2 – 3 µm along the body, slightly thickened at tail (4 – 6 µm). Anterior to sensillium pouch 14 – 16 µm. Position of S-E pore as in males, pharyngeal bulb offset. Genital system didelphic, ovaries reflexed. Vulva a narrow transverse silt, post-equatorial (aver. V=55.8 %). Vagina length 48 – 59 % of corresponding body width. Vagina rhomboid, *pars distalis* 2 – 3 µm, *pars proximalis* 11 – 13 µm. Vaginal sclerotized pieces triangular, oblique (tips directed towards vulva), close to each other, up to 2 µm long. Proximal of vagina end surrounded by constrictor muscles. Sperm located in spermathecae and rarely in uterus.

Codes following Decraemer and Baujard (1998): D1 C1 L1 K300 A210 B210 E 200 F100 G2 H2 I32 J11 M1 N1 O12 P11 Q? R? S1.

Remarks. Male and female morphometrics were compared with the type population from the Netherlands (Seinhorst, 1963), one from Germany (Wyss, 1974), two from Italy (Roca & Lamberti, 1984) and three populations from Bulgaria (Peneva, 1988). Male body is shorter L = 730 (658 – 783) µm *vs* L = 810 (740 – 910) µm, and L = 887 (803 – 976) µm and L = 973 (880 – 1059) µm in the German and Italian populations, respectively, and is longer compared to two Bulgarian populations (L = 678.3 (601 – 782) and L = 666.6 (576 – 771) µm). Onchiostyle is shorter 38.9 (37 – 40) µm compared to the Italian populations (43 (40 – 47) and 45.5 (43 – 49) µm). Average distance CP1-CP2 20.2 (17 – 22) µm is close to all three Bulgarian populations but lower range of two of them is lower 19.9 (8 – 33) and 18.7 (10 – 24) µm. Distance CP2-CP3 (14.8 (11 – 16)) is shorter than in Bulgarian populations (19.1 (11 – 23) and 18.1 (7 – 32) µm). Ratio 'a' 26 (23.6 – 29) is higher compared to the Bulgarian populations 22.3 (19.5 – 24.5) and 23.4 (20.3 – 25.8); ratio 'b' 5.3 (4.8 – 5.7) is smaller compared to the Italian specimens 10 (9.3 – 11.3) and 9.3 (8 – 10.3); ratio 'c' is smaller than all other seven populations.

Female body is shorter compared to German and Italian materials, (av 758 µm vs avs. 820 µm, and 855 and 952 µm) and longer compared to the Bulgarian populations (692 and 644 µm). Onchiostyle is shorter compared to one Italian and one Bulgarian population (39.3 (37.7 – 41.6) µm vs 45 (43 – 47) µm and 46.5 (42 – 52)) µm. Ratio 'a' 24.7 (21.3 – 28.1) is somewhat higher compared to the Bulgarian populations 20.9 (17.9 – 24.1) and 20.9 (19.2 – 23) and 'b' ratio 5.5 (4.7 – 6.3) is lower than in Italian specimens b=9.6 (8.4 – 11) and 9.5 (8.7 – 11).

Molecular study

Morphological identification of this population was reliably verified by PCR of ribosomal DNA from single specimens using species-specific primers (Boutsika *et al*., 2004b). A single fragment of approximately 452 bp was amplified for all studied individuals (Fig. 3). No PCR products were obtained in the negative controls lacking DNA template or containing DNA of *T. pakistanensis*, *T. variopapillatus* and *T. viruliferus* (data not shown).

Identical sequences were obtained from two individuals of the Kaliště population processed, thus two sequences were deposited in the NCBI (National Center for Biotechnology Information) database with accession numbers KX522761 (D2–D3 segment of 28S gene) and KX522760 (ITS1). After a BLASTN search tool D2–D3 sequence showed 771/775 nucleotides identity to *T. similis* from United Kingdom (accession number AM180730, Holeva *et al*., unpublished) and 753/755 nucleotides identity to *T. similis* from deciduous forest in the Czech Republic (DQ832183, Kumari *et al*., 2007). Similarly, the ITS1 showed 1237/1255 identity to *T. similis* from the UK (AJ439523, Boutsika *et al*., 2004a) and 1359/1364 identity to *T. similis* from the Czech Republic (JN123317, Kumari and Subbotin, 2012). ITS1 showed polymorphisms at three sites.

Concluding remarks

Taking into account the agricultural importance of trichodorids and tobraviruses as pathogens of potato (Taylor & Brown, 1997), a study has been initiated in the Czech Republic. The damage-level threshold is in the case of virus vector species equivalent to a single nematode. Therefore, the information on *T. similis* would be useful for developing nematode management strategies in the country. In this study, both taxonomical and molecular analysis verified the presence of *T. similis* in association with potato in the Czech Republic and extends the knowledge on morphological variability and genetic diversity of the species.

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