Morphological and molecular characteristics of foliar nematode attacking silver birch (*Betula pendula* Roth) in Poland

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Summary

Aphelenchoides fragariae (Ritzema Bos, 1890) Christie, 1932 was isolated from leaves of silver birch (*Betula pendula* Roth) seedlings proving that the source of infection was anemones plants. This is the first report to our best knowledge showing that the source of nematode infection of a woody plant could be a perennial plant. *A. fragariae* was identified by morphometric and molecular analyses. Morphological diagnosis based on the bending shape of the tail of males and pronounced apex and rostrum proved to be the most accurate reliable characteristic. On the opposite, the high variability of the mucron shape in female tails made the identification by microscopic analyses difficult. Identification of the species was confirmed by analysis of 28S rDNA sequences. The morphometric data of adults extracted from silver birch was compared with that of nematodes isolated from *Anemone hupehensis* (Lemoine) Lemoine. Males body length varied highly in samples collected from both host plant species.

Keywords: *Aphelenchoides fragariae*; woody plant; morphometric data; 28S rDNA

Introduction

Strawberry foliar nematode (*Aphelenchoides fragariae*) (Ritzema Bos, 1891) Christie, 1932 is a widespread plant pest that has been reported on a very large number of hosts: including shrubs (e.g. *Weigela subsessilis* (Nakai) L.H.Bailey) (Khan et al., 2007a), perennials (e.g. *Hosta* sp. Tratt. (Noel & White, 1994), *Lamium maculatum* L. (LaMondia & Ocamb, 1995), *Helianthus tuberosus* L. (Khan et al., 2007a), *Stachys riederi* Cham. (Khan et al. 2008), *Anthurium andraeanum* Linden ex André (Hunter et al., 1974)) and trees (e.g. *Ficus macrophylla* Pers. (Penrose & Nikandrow, 1971)). In Poland *A. fragariae* was found only on *Fragaria* × *ananassa* Duchesne (Szczygieł, 1963; 1966; 1970). Nematodes from *Aphelenchoides* species infecting trees were detected mostly in association with stem deformations, such as dogwood cankers (Santamour & McArdle, 1987; Self & Bernard, 1994). In September of 2014

a silver birch (*Betula pendula* Roth) seedling with brown necrotic areas in proximity of the major veins of leaves was observed at the experimental field of the Research Institute of Horticulture in Skierniewice in a plot managed with overhead irrigation. The aim of our study was to identify the causative factor of these symptoms and to determine their source.

Materials and Methods

Isolation and morphological examination of nematodes
In 2014 silver birch (*Betula pendula* Roth) seedlings were grown on the experimental field of the Research Institute of Horticulture in Skierniewice with overhead irrigation, near *Anemone hupehensis* (Lemoine) Lemoine, cultivar Prinz Heinrich, infected by *Aphelenchoides fragariae*. In September of 2014 the leaves of a silver birch seedling characterized by brown necrotic areas associated

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Fig. 1. Photomicrographs of *Aphelenchoides fragariae*. A) Anterior region of male. B) Anterior region of female. C) Female posterior part of pharynx, basal bulb and pharyngeal gland. D) Female reproductive system, vulva and postvulval uterine sac. E) Male posterior region showing spicules. F) Female tail. G)-K) Female tail, variability in the mucron shape. (Scale bars: A, C, D, F = 20 μm , B, E, G-K = 10 μm).

with the major veins were collected and examined for the presence of nematodes. The plant was part of a group of seedlings grown on the experimental field of the Research Institute of Horticulture in Skierniewice with overhead irrigation, near plants of *Anemone hupehensis* (Lemoine) Lemoine cultivar Prinz Heinrich. The *A. hupehensis* plants were found to be infected by *A. fragariae* after a routine analysis performed to phytosanitary condition of plants. The nematodes were isolated from the leaves by a modified technique of Baermann method (Szczzygieł, 1966). The extracted population of nematodes was fixed partly in TAF (97 ml of formalin,

2 ml of triethanolamine and 91 ml of dH₂O) (Courtney *et al.*, 1955) for morphological observations and partly in DESS (20 % dimethyl sulphoxide, 0.25 M disodium EDTA, saturated with NaCl) (Yoder *et al.*, 2006) for molecular analyses. To determine the morphometric parameters, 60 nematodes (30 females and 30 males) were processed by Seinhorst's rapid method (Seinhorst, 1959) and mounted in dehydrated glycerin on glass slides. The observations of adults were made using a Nikon Eclipse 80i microscope equipped with a Nomarski differential interference contrast.

Table 1. Measurements of adults of *A. fragariae*. Length values are in μm . Average $\pm$ SD and minimum and maximum values are given.

Measurements [μm]	<i>Anemone hupehensis</i> ♀♀ n=30	<i>Anemone hupehensis</i> ♂♂ n=30	<i>Betula pendula</i> ♀♀ n=30	<i>Betula pendula</i> ♂♂ n=30
L	706.2 $\pm$ 52.6 617.2 – 814.2	605.9 $\pm$ 44.9 485.8 – 673.6	701.8 $\pm$ 38.1 609.1 – 776.9	645.4 $\pm$ 30.6 532.5 – 645.4
a	54.3 $\pm$ 3.7 47.5 – 63.9	48.6 $\pm$ 3.9 38.9 – 57.1	51.9 $\pm$ 3.5 46.4 – 60.1	45.9 $\pm$ 2.3 41.6 – 50.5
b'	4.5 $\pm$ 0.4 3.7 – 5.6	3.8 $\pm$ 0.4 2.9 – 4.4	4.5 $\pm$ 0.4 3.8 – 5.3	3.6 $\pm$ 0.2 3.3 – 4.0
c	17.6 $\pm$ 1.6 15.0 – 22.6	19.3 $\pm$ 2.2 14.9 – 25.6	17.6 $\pm$ 1.3 14.7 – 20.00	18.3 $\pm$ 1.8 15.7 – 23.3
c'	4.5 $\pm$ 0.4 3.4 – 5.5	3.0 $\pm$ 0.3 2.5 – 3.4	4.3 $\pm$ 0.3 3.7 – 4.8	3.1 $\pm$ 0.2 2.7 – 3.6
Stylet length	12.1 $\pm$ 0.5 11.1 – 12.9	11.8 $\pm$ 0.4 11.1 – 12.6	12.4 $\pm$ 0.3 11.8 – 12.9	12.1 $\pm$ 0.4 11.2 – 12.8
Pharynx length (with glands)	58.3 $\pm$ 9.7 143.00 – 174.2	161.0 $\pm$ 9.2 146.8 – 174.6	154.4 $\pm$ 12.8 133.5 – 177.5	165.2 $\pm$ 7.1 153.6 – 179.5
Excretory pore from head end	91.6 $\pm$ 4.9 85.1 – 99.9	89.9 $\pm$ 2.1 85.2 – 96.7	93.9 $\pm$ 6.2 82.3 – 102.5	91.4 $\pm$ 4.0 86.0 – 99.6
Head-vulva length	524.4 $\pm$ 45.8 437.4 – 645.7	–	518.3 $\pm$ 31.0 450.1 – 566.1	–
Vulva-anus length (v-a)	181.9 $\pm$ 15.2 153.6 – 206.0	–	182.2 $\pm$ 12.3 156.2 – 199.6	–
Maximum body diameter	13.0 $\pm$ 0.8 11.8 – 15.00	12.7 $\pm$ 0.6 11.7 – 13.7	13.5 $\pm$ 0.8 12.1 – 14.8	13.1 $\pm$ 0.8 11.7 – 15.00
Tail length	40.3 $\pm$ 2.8 35.1 – 45.4	31.6 $\pm$ 2.5 26.3 – 36.6	40.00 $\pm$ 2.7 34.7 – 44.7	33.0 $\pm$ 3.0 24.3 – 37.2
Anal body diameter	9.0 $\pm$ 0.7 7.8 – 10.8	10.4 $\pm$ 0.6 9.4 – 11.8	9.4 $\pm$ 0.7 8.3 – 10.8	10.7 $\pm$ 0.8 8.4 – 12.00
Postvulval uterine sac (PUS)	93.00 $\pm$ 4.7 84.3 – 101.6	–	94.6 $\pm$ 9.8 75.5 – 111.0	–
PUS-% v-a	50.3 $\pm$ 4.7 43.7 – 59.00	–	52.1 $\pm$ 3.6 46.2 – 60.7	–
PUS-xV	7.1 $\pm$ 0.4 6.3 – 7.9	–	7.1 $\pm$ 0.8 5.3 – 8.6	–
V%	74.2 $\pm$ 1.8 69.8 – 80.0	–	74.00 $\pm$ 1.2 71.9 – 77.3	–
Spicule length	–	18.3 $\pm$ 1.1 16.2 – 20.8	–	18.7 $\pm$ 0.8 17.4 – 20.3

Molecular characterisation

Species identity and confirmation of the origin of the silver birch infection from anemone was determined by comparison of 28S rDNA sequences. Genomic DNA from eight specimens (four from silver birch and four from anemone) was extracted using the Nucleo Spin Tissue XS (Macherey-Nagel, Germany) following the manufacturer's instruction. For each sample, DNA was eluted in 30 μ l H₂O. 3 μ l of extracted DNA were used as a template for polymerase chain reactions (PCR). PCR primers for conserved regions were designed using the online PRIMER 3 software (<http://primer3.ut.ee>) (Untergrasser *et al.*, 2012). D2-D3 expansion segments of the large subunit 28S rDNA were amplified using primers Aph_F (5'-AGAGAGTGCAAGAGAACGTGA-3') and Aph_R (5'-ACCATCTTTCGGGTCTCAAA-3') (Sigma-Aldrich, Milwaukee, WI, USA). Amplification of 28S rDNA and sequencing were performed as described before (Dobosz *et al.*, 2013), but using another polymerase (12.5 μ l Taq PCR Master Mix Gdansk, Poland) and primer concentration (1 μ l of 10 μ M for each primer). Cycling was performed on Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) with the following cycling conditions: 3 min of initial denaturation at 94°C, followed by 40 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 1 min and a final extension step of 72°C for 5 min. Sequences of 28S rDNA of *A. fragariae* were aligned by ClustalW (Larkin *et al.*, 2007) in MEGA ver. 6.06 using default parameters (Tamura *et al.*, 2011). Automatic alignment was then improved manually in order to eliminate improper phylogenetic signals. For choosing the substitution model, data were analysed by jModelTest 2.1.2 (<http://jmodeltest.org>) program (Darriba *et al.*, 2012). The program showed that the best substitution model was GTR + I + G, which was then used for analysis. Phylogenetic tree was constructed for 28S rDNA using maximum likelihood method with MEGA ver. 6.06 software (Tamura *et al.*, 2011) and compared with their corresponding published gene sequences (Kanzaki *et al.*, 2008, Zhao *et al.*, 2008). New sequences for *A. fragariae* were deposited in the GenBank under the following accession numbers: KP835683-KP835687 from silver birch and KT261768-KT261770 from anemone.

Results and Discussion

Morphological data and characters of 30 females and 30 males isolated from leaves of silver birch seedling were consistent with description of *A. fragariae* given by Allen (1952), Franklin (1965, 1978), Siddiqi (1974), Chizhov *et al.*, (2006), Andrssy (2007) and Khan *et al.* (2007b, 2008).

The population of nematodes in the sample was composed of 64 % females, 11 % males and 25 % juvenile forms. No other nematode species were detected in the analyzed material. The most important diagnostic characters of females and males for species identification are reported in Table 1 and illustrated in Fig. 1. Females of *A. fragariae* from leaves of silver birch seedling are characterised by a slender body, slightly ventrally arcuate when

killed; lateral field with two incisures occupies about one-seventh of the body width; lip region almost continuous with body contour, straight to slightly rounded, without visible annulation; weakly sclerotized cephalic framework; thin stylet with small but distinct basal knobs (Fig. 1 B); ovoid, highly muscular median bulb; pharyngeal glands forming long lobe extending intestine dorsally (Fig. 1C); excretory pore level with, or close behind, nerve ring; oocytes arranged in a single row; relatively long postvulval uterine sac, taking more than half of the vulva-anus distance, often containing sperm (Fig. 1D); elongate-conoid, almost straight tail; tail end with single mucron (Fig. 1F), which shape varied, from slightly offset, simple peg to irregularly terminated, less or more enlarged in the half of the length or cut from the dorsal side with almost ventrally located tip (Fig. 1G-K).

Males lip region, stylet and pharynx as in females (Fig. 1A); body slender, ventrally curved in posterior part; tail conoid, arcuate through 45 – 90°, bearing a terminal peg; spicules prominent, ventrally arcuate, with moderately developed apex and rostrum (Fig. 1E).

Fig. 2. Phylogenetic relationships within the species of *Aphelenchoides fragariae*. Maximum likelihood tree inferred from 28S rDNA for *Aphelenchoides* species with *Aphelenchus avenae* as the outgroup.

KT261768.1	ACCTGTAAAAMYTTWWKRACGGATG-AATTT	samples from
KT261769.1	ACCTGTAAAAC TTTATGA-CGGATG-AATTT	<i>A. hupehensis</i>
KT261770.1	ACCTGTAAAAC TTTATGA-CGGRWKGAAATTT	
KP835683.1	ACCTGTAAAAC TTTATGA-CGGATG-AATTT	samples from
KP835684.1	ACCTGTAAAAC TTTATGA-CGGATG-AATTT	<i>B. pendula</i>
KP835685.1	ACCTGTAAAAC TTTATGA-CGGATG-AATTT	
KP835686.1	ACCTGTAAAAC TTTATGA-CGGATG-AATTT	
KP835687.1	ACCTGTAAAAC TTTATGA-CGGATG-AATTT	

Fig. 3. Comparison of 28S rDNA sequences of *Aphelenchoides fragariae* samples isolated from *A. hupehensis* and *B. pendula* (only partial 28S rDNA sequences with visible nucleotide insertions/deletions are shown).

This species resembles other *Aphelenchoides* species occurring in leaves. However, due to the two incisures in lateral fields, the shape of the mucron, the distinctive construction of the head and more prominent apex and rostrum in male spicules, can be differentiate from *A. ritzemabosi* and *A. besseyi*.

For the population of *A. fragariae* obtained from silver birch the value of the mucron as a reliable differentiating character was doubtful because mucron of some females had been confusingly similar to other *Aphelenchoides* species, including *A. ritzemabosi*. The same conclusion were drawn by Franklin (1978). Proper identification required analysis of other morphological features of the specimens. The most accurate diagnosis was based on bending the tail of males and pronounced apex and rostrum, forming the proximal part of spicules in ear-like shape.

The length of sequenced 28S rDNA D2-D3 region ranged from 516 bp to 521 bp. Phylogenetic trees generated from this region showed that sequences of samples isolated from silver birch and from anemone grouped together with sequences of *A. fragariae* deposited in the GenBank by Kanzaki *et al.* (2008) as well as Zhao *et al.* (2008) (Fig. 2). The comparison of 28S rDNA sequences of nematodes isolated from silver birch and anemone revealed high similarity and differed only by two nucleotide substitution (Fig. 3), which probably reflect sequence variability in the population. Results of molecular analysis confirmed that nematodes feeding on anemone were the source of infection for the birch.

A. fragariae infects the leaves of plants by migrating up the stem and entering their stomata. The nematodes spread by migrating through films of water over plant surfaces (Klingler, 1970). Plants moistened by dew, rain or overhead irrigation are the most susceptible to infection, because the nematodes move by splash dispersal from plant to plant (Lehmann, 1996; Kohl *et al.*, 2010). Reports about nematodes found in the above-ground part of hardwood trees are scant. Santamour and McArdle (1987) isolated a species of *Aphelenchoides* resembling *A. fragariae* from burls of red maple (*Acer rubrum* L.), Higan cherry (*Prunus subhirtella* Mig.), red oak (*Quercus rubra* L.), black locust (*Robinia pseudoacacia* L.), Siberian elm (*Ulmus pumila* L.) and from *Cornus florida* L. cankers. McCuiston *et al.* (2007) and Kohl *et al.* (2010) reported isolation

of *A. fragariae* from woody ornamental plants *Lantana camara* L. and *Buddleja davidii* Franch. Morphometric comparison of nematodes from silver birch seedlings and anemone showed subtle dissimilarities (Table 1), mainly in the length of males body. Males isolated from *B. pendula* had a longer body length. The differences between the compared populations may arise from a variety of external factors i.a. species of host. Such differences were also noted by Wu (1960) in his study during the *Ditylenchus destructor* Thorne, 1945 survey. He observed that parameters such as the body width and length, pharynx and stylet length are dependent on the host plant. Similar results were obtained by El-Sherif (1972) with respect to *A. sacchari* Hooper, 1958 and *A. rutgersi* Hooper & Myers 1971.

Detection of *A. fragariae* in a new plant – silver birch – extends the wide host range of this nematode. The results show that nematodes can infect woody plants from perennials located in their vicinity. This is the first report to our best knowledge showing that the source of nematode infection of a woody plant could be a perennial plant. This observation highlights the importance of controlling this foliar nematode in nurseries. Both the morphological and molecular analysis are crucial to properly identify *A. fragariae*, because the characteristic symptoms of the feeding of this species observed on infected leaves are not only specific for this foliar nematode.

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