
Andrzej Skwiercz¹, Tatyana Stefanovska², Miloslav Zouhar³, Valentina Pidlisnyuk⁴, Łukasz Flis⁵

¹ The National Institute of Horticultural Research, ul. Konstytucji 3 Maja 1/3, 96–100 Skierniewice, Poland
² Department of Entomology, Integrated Pest Management and Plant Quarantine, The National University of Life and Environmental Sciences, Heroiv Oborony St, 15, 03041 Kyiv, Ukraine
³ Department of Plant Protection, Czech University of Life Sciences, Kamýcká 129, 165 00 Praha-Suchdol, Czech Republic
⁴ Department of Environmental Chemistry and Technology, Jan Evangelista Purkyne University in Ústí nad Labem, Pasteurova 3544/1, 400 96 Ústí nad Labem-město, Czech Republic
⁵ Museum and Institute of Zoology, Polish Academy of Sciences, Wilcza 64, 00-679 Warsaw, Poland

**ABSTRACT**

During a survey of plant parasitic nematodes associated with Miscanthus × giganteus which was carried out in Ukraine in 2016–2017, an occurrence of Rotylenchus agnetis Szczygieł, 1968, Rotylenchus pumilus Perry, 1959 and Paratylenchus nanus Cobb, 1923 was found. To the best of our knowledge this is the first record of a parasite species which can damage M. × giganteus. The species were described morphologically and using molecular tools. Further profound study on pathogenicity of those species is needed.

**Key words:** plant-feeding nematodes, Miscanthus × giganteus, morphological description, molecular identification

**INTRODUCTION**

Miscanthus × giganteus J.M. Greef & Deuter ex Hodk. & Renvoize, (Poaceae = Gramineae) is a rhizomatous, perennial C4 grass species which originated in South-East Asia [Lewandowski et al. 2018]. The sterile clone M. × giganteus is a high-yielding genotype and currently the standard cultivar in commercial utilization in Ukraine [Kvak et al. 2018]. It has an adequate environmental profile with a highly adaptive potential, namely to grow in marginal soils as well as those of different anthropogenic origins, slightly contaminated by trace elements [Nebská et al. 2019, Pidlisnyuk et al. 2020]. M. × giganteus is among the most promising sources of biomass for direct energy production and bio-based products [Fradj et al. 2020]. There is a significant acreage of available marginal and abandoned lands in Ukraine where traditional agriculture is not profitable [Triboy and Dragnev 2018]. Simultaneously, the growing demands for obtaining lignocellulose biomass are good drivers for upscaling commercial production of M. × giganteus in the country. Since the first M. × giganteus plantation was established in Ukraine in 2006, the production has rapidly expanded and currently, its crop area covers up to 1,500 ha [Roik et al. 2019]. As miscanthus plots switch from
small fields to large commercial plantations in different agroecological zones, nematodes, plant pathogens and pests are beginning to emerge [Mekete et al. 2011, Stefanovska et al. 2021].

Among plant parasitic nematodes several species, including spiral Rotylenchus Steiner, 1945 and pin Paratylenchus [Micoletzky 1922] were shown as species associated with Poacea family [Háněl 2017, Ghaderi 2019]. If the area of M. × giganteus that also belongs to Poacea family increases, these nematode might parasitise the crop and cause the yield reduction. Obligate plant parasitic spiral nematodes of the genus Rotylenchus (Nematoda: Haplolaimidae) are represented by 103 valid species widely distributed across the world. In terms of Rotylenchus species numbers, Europe ranks second to Asia [Castillo and Vovlas 2005]. Several species are considered economically significant for Poacea family [Decraemer and Hunt 2006]. According to the Nematax database, R. agnetis is monophagous and known to have parasitised butcher’s broom Ruscus aculeatus L. (Asparagaceae) only. However, several studies detected this nematode species also in the rhizosphere of forest plants [Skwiercz 2012], fruit trees [Lišková et al. 2007], grapevine Vitis vinifera L. (Vitaceae) [Skwiercz et al. 2015], barley Hordeum vulgare L. (Poacea), different ornamental nursery plants and woods [Winiszewska et al. 2012].

At first female specimens of R. agnetis were found in strawberry Fragaria ananassa L. (Rosaceae) plantations in Poland and described by Szczygieł [1968]. The next description of isolated R. agnetis from apple Malus domestica L. Borkh (Rosaceae) was elaborated in Moldova by Nesterov [1979]. Baydulova [1981] added information on morphology of this species based on specimens isolated in Kazakhstan from Medicago sativa L. (Fabaceae). The description of R. agnetis in forest nurseries was done by Peneva and Nedelchev [1992]. These authors made the significant contribution to the clarification of taxonomic position of the species by presenting the morphological and morphometric description of R. agnetis isolated from different hosts and locations in Bulgaria. Geraert and Barooti [1996] described R. agnetis recorded for the first time at a pine tree Pinus sylvestris L. (Pinaceae) plantation in Iran and gave additional data based on a scanning electron microscope study. The latest description of R. agnetis isolated from the rhizosphere of butcher’s broom was provided by Vovlas et al. [1998] in Italy.

The available data on Rotylenchus pumilus Perry, 1959 host range related to forest plants is very limited. Yuen [1966] found a large population of R. pumilus co-occurring with Helicotylenchus vulgaris Yuen, 1964 in soil under grasses in meadows adjacent to forests. Riffle [1972] detected R. pumilus in soil under pine trees and junipers Juniperus communis L. (Cupressaceae) in South Mexico. Furthermore, in a pot experiment he found that although that nematode species parasitised the pine tree it did not lead to a significant reduction in seedling growth. Germani and La Massese [2002] also described R. pumilus which was collected in the rhizosphere of pine trees in France. Skwiercz [2012] detected the species as inhabiting the nursery of forest plants and colonizing the jerusalem artichoke Helianthus tuberosus L. (Asteraceae) [Zapalowska and Skwiercz 2018] in Poland. There are several records on R. pumilus that were observed in grapevines: by Kankina and Teben’kova [1980] in Tajikistan; Katalan-Gateva [1980] in Bulgaria and Skwiercz et al. [2015] in Poland.

The existence of a significant number of Rotylenchus species complicated the process of its pragmatic identification due to the creation of tabular and dichotomous keys following morphological features [Cantalapiedra-Navarrete et al. 2013].

Pin nematodes of the genus Paratylenchus are obligate ectoparasites. In Europe they are represented by 32 species [Castillo and Vovlas 2007]. Paratylenchus nanus Cobb, 1923 is closely related to Paratylenchus bukowinensis Micoletzki, 1922 from which it differs according to Brzeski [1998]. P. nanus has got pronounced annulation on dorsal side near terminus tail while P. bukowinensis has got indistinct annuli often small near tail end. Males P. nanus without stylet vs. males P. bukowinensis with weak stylet [Brzeski
The species of these genera are widely distributed in crops and natural vegetation worldwide [Stefanovska 1992]. P. nanus is known to be associated with perennial grasses cultivated in meadow soils [Brzeski 1998], golf courses in the rhizosphere of cereals and orchards [Viketof et al. 2005]. The species is distributed widely in perennial crops, all above grass stands, hop gardens, orchards or forest trees and shelterbelts [Wasilewska 2006, Čermák and Renčo 2010, Kozlovskyi 2012]. Italian ryegrass Lolium multiflorum L., perennial ryegrass Lolium perenne L. and cocksfoot Dactylis glomerata L. are suitable hosts for P. nanus [Watson and Bell 2001, Ciobanu et al. 2003]. Kozlovskyi [2012] recorded P. nanus in deciduous and mixed forests located in the Carpathians, showing that the species is not restricted to lowland habitats and grass vegetation. Several descriptions of P. nanus are available, however, there are some controversies about it. Morphological identification of pin nematodes is rather difficult due to their smaller size, problems associated with separation of very similar diagnostic characteristics, and high variability under influence of environmental factors [Brzeski and Hanel 2000]. Ghaderi et al. [2016] listed important morphological characteristics which facilitate the process of morphological identification accuracy, i.e. number of lateral lines, length of the stylet and the presence/absence of vulval flap. In order to avoid the complication with morphometric identification of Rotylenchus spp. and Paratylenchus spp., the alternative DNA based approach has been suggested recently [Cantalaipiedra-Navarrete et al. 2013, Van den Berg et al. 2014].

During a nematological survey conducted at six locations and four soil types across Ukraine in 2016–2017, soil samples under M. × giganteus grown in 1–10 age plantations were collected. The study of nematode specimens extracted from these samples revealed the presence of 53 plant-feeding nematode species. Among frequently occurring in this energy crop species, R. agnetis, R. pumilus, P. nanus were recorded. Because these nematodes were recorded for the first time in M. × giganteus plantations in Ukraine, characterisation of populations from Ukraine was needed. The objective of this study was to combine the morphological and molecular approaches in order to identify R. agnetis, R. pumilus, and P. nanus. Connecting the morphological and molecular species concepts might facilitate species identification within the genus Rotylenchus, Paratylenchus.

**MATERIAL AND METHODS**

Nematodes were isolated from soil samples collected to a depth of about 20–40 cm and taken randomly in autumn from different plots of M. × giganteus. Plants did not have symptoms of nematode infection. The map with locations where R. agnetis, R. pumilus and P. nanus were detected is featured in Figure 1. Samples were collected at locations described in each species data list (Tab. 1). Nematodes were isolated using the modified Baermann and centrifugal flotation techniques [Hooper et al. 1962]. Extracted nematodes for the further morphological study were heat-killed, fixed in TAF and processed to glycerol. Identification and measurements of specimens were made on prepared temporary slides by using light microscopy. For the identification and comparison with original descriptions of the nematode species detected in this study, several diagnostic keys were used, i.e.: Perry et al. [1959], Sher [1961], Hooper [1962], Geraert [1965], Decraemer [1995], Brzeski [1998] and Andrássy [2007].

Extraction of DNA from fixed nematodes was done by Exgene Genomic DNA micro kit. The specimens were removed from the fixative solution and were individually kept in 10 µm of extraction buffer and processed in accordance with the manufacturer’s instructions. Total DNA from single nematodes was amplified by the protocol described by Douda et al. [2013], using two prepared fragments, i.e.: the first one using the primers D2A (5´-ACAAGTACCCT-GAGGGAAAGTTG-3´) and D3B (5´-TCGGAAGGAAACAGCTACTA-3´) [Ellis et al. 1986, Courtright et al. 2000] for the 28S large sub-unit rRNA, S18 (5´-TTGATTAGTCCCTGCCCCTTT-3´) and S28 (5´-TTTCACTCGCCGTTACTAAGG-3´) situated inside the cistron rDNA including region ITS1-5.8S-ITS2 rDNA (S18 S28) [Ellis et al. 1986]. The PCR products were separated by 2% agarose gel electrophoresis and purified by GeneJET Gel Extraction Kit (Thermo Fisher Sci.). Three PCR products were prepared from every single nematode and sequenced. The DNA sequencing was performed in GATC Biotech (Germany). Obtained sequences were processed by free avail-

---

able software BioEdit and consensual sequences were assembled. The BLAST algorithm for screening of obtained data was used. [Sayers et al. 2011].

RESULTS AND DISCUSSION

Rotylenchus agnetis Szczygieł, 1968

Female, n: 12. L = 800 (640–960) µm, c = (46–64); c' = 0,7 (0,5–0,9) µm stylet = 32 (28–34) µm, pharynx = 112 (106–118) µm. V = 59 (56–62); tail = 13 (10–16) µm; tail annuli = 7 (6–8), phasmids at 4 annuli anterior to 2 annuli posterior anus. Body is loosely spiral, head offset by constriction with 5–7 annuli. Excretory pore is opposite to the pharynx and intestine junction. Pharyngeal lobe 0,8–1,0 µm body width long. In the female reproductive system spermatheca is filled with sperm. Phasmids are 4 annuli anterior to 2 annuli posterior anus. Tail hemispherical bearing 6–8 annuli, on the tip two widely annuli.

The female length was significantly less than recorded by Geraert and Barooti [1996] but it was almost in agreement with Szczygieł [1968], Nesterov [1979] and Peneva and Nedelchev [1992]. The stylet was longer in comparison with those reported by Szczygieł [1968], Geraert and Barooti [1996], Peneva and Nedelchev [1992], however fitted with measurements of R. aceri – a species synonymized with R. agnetis was done by Berezina [1985]. The tail length was in the correspondence with Peneva and Nedelchev [1992] and Geraert and Barooti [1996]. A compilation of the numbers of specimens of this species detected in the samples is presented in Table 1.

Fig. 1. The map of Ukraine with locations where Rotylenchus agnetis Szczygieł, 1968, Rotylenchus pumilus Perry, 1959 and Paratylenchus nanus Cobb, 1923 were detected
Rotylenchus pumilus Perry, 1959

Female, n: 8. L = 622 (495–750) µm, c' = 0.9 (0.9–1.0); stylet: 32 (24–28) µm, pharynx: 102.5 (95–110) µm, V = 59 (58–60); tail = 14 (12–16) µm. Body is small, tightly spiral, location of the lateral field is irregular. Head bearing 4–5 annuli, not offset, with strongly refractive constriction. Pharyngeal lobe overlapped intestine for 0.6–1.0 µm body width. Spermatheca is filled with sperm. Tail rounded, convex on the dorsal side, equal of anal body diameter.

The three morphological features of *R. pumilus* are not in agreement with measurements of those species detected in the rhizosphere under pine trees [Gernani and La Massese 2002], namely: female length is smaller; the stylet and tail are slightly larger. However, if compared to the description of *R. pumilus* by Perry et al. [1959] and Sher [1961], in our records the body length and stylet are shorter, but the tail is longer.

A compilation of the numbers of specimens of this species detected in the samples is presented in Table 1.

Paratylenchus nanus Cobb, 1923

Female, n: 15. L = 370 (320–420) µm, stylet: = 26 (24–28) µm, pharynx = 94 (90–98) µm, tail = 22 (18–26) µm, c = 15 (12–18), c' = 2.7 (2.0–3.4); V = 83 (82–84). Female bent ventrad, head rounded, excretory pore is 21–25% of body length. Spermatheca with sperm, vulval claps rounded, distinct.

Morphological features values in our description of *P. nanus* fitted with original description presented in Table 1.

Table 1. Habitat and number of investigated species from the plantation of Miscanthus × giganteus J.M. Greef & Deuter ex Hodk. & Renvoize

<table>
<thead>
<tr>
<th>Species</th>
<th>Locality</th>
<th>Age of plantation (years)</th>
<th>Soil type</th>
<th>Number of samples</th>
<th>Number of samples with the investigated species</th>
<th>% of samples with the investigated species</th>
<th>Number of specimens of plant parasitic nematodes</th>
<th>Number of specimens of investigated species</th>
<th>Number of specimens of investigated species/unit of soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rotylenchus agnetis</td>
<td>Szepetivka</td>
<td>50°07′53.5″N 27°13′58.3″E</td>
<td>Chernozem</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>25</td>
<td>558</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>Batyeva Gora</td>
<td>50°25′04.9″N 30°29′55.0″E</td>
<td>Clayic podzoluvisoil</td>
<td>7</td>
<td>4</td>
<td>1</td>
<td>25</td>
<td>1080</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>Veremyivka</td>
<td>49°36′51.9″N 33°15′09.8″E</td>
<td>Chernozem</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>25</td>
<td>738</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Kalynivka</td>
<td>50°13′45.8″N 30°13′09.0″E</td>
<td>Chernozem</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>25</td>
<td>871</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>Kurakhove</td>
<td>47°58′30.5″N 37°19′28.7″E</td>
<td>Haplic chernozem</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>25</td>
<td>465</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>Dolyna</td>
<td>48°58′14.8″N 23°58′59.9″E</td>
<td>Clayic cambisols</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>25</td>
<td>715</td>
<td>150</td>
</tr>
<tr>
<td>Rotylenchus pumilus</td>
<td>Szepetivka</td>
<td>50°07′53.5″N 27°13′58.3″E</td>
<td>Chernozem</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>25</td>
<td>558</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>Batyeva Gora</td>
<td>50°25′04.9″N 30°29′55.0″E</td>
<td>Clayic podzoluvisoil</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>25</td>
<td>738</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>Batyeva Gora</td>
<td>50°25′04.9″N 30°29′55.0″E</td>
<td>Clayic podzoluvisoil</td>
<td>7</td>
<td>4</td>
<td>2</td>
<td>50</td>
<td>1080</td>
<td>206</td>
</tr>
<tr>
<td>Paratylenchus nanus</td>
<td>Szepetivka</td>
<td>50°07′53.5″N 27°13′58.3″E</td>
<td>Chernozem</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>50</td>
<td>558</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>Szepetivka</td>
<td>50°07′53.5″N 27°13′58.3″E</td>
<td>Chernozem</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>50</td>
<td>1074</td>
<td>300</td>
</tr>
</tbody>
</table>
by Thorne and Smolik [1971] and Nouri et al. [2004] with the exception of body length. It is shorter in comparison with measurements made by Torn and Smolik [1971].

A compilation of the numbers of specimens of this species detected in the samples is presented in Table 1.

**Molecular characterization**

It should be noted that not all cases obtained sufficient quality DNA to amplify all the desired regions, which may be due to nematode fixation and also a small amount of biological material because the DNA was isolated from a single nematode at a time.

In the case of *R. pumilus* and *P. nanus*, the obtained sequences of the small and large ribosomal DNA subunit sections were compared using the BLAST algorithm with the NCBI database and the sequences showed more than 99% similarity with the sequences listed in the database. The species affiliation determined using morphological data was confirmed by analysis of the nucleotide sequences obtained. In the case of *R. agnetis*, it was not possible to confirm the morphological data by molecular biological sequence analysis because the only DNA segment available for comparison in the NCBI database, the 28S ribosomal RNA gene, could not be amplified.

**CONCLUSION**

Three nematode species: *R. agnetis*, *R. pumilus* and *P. nanus* have been recorded for the first time in *M. × giganteus* plantations located in two agroecological zones: Forestry Steppe and Steppe in Ukraine and characterized by four types of soil. Those three species were characterized morphometrically and two species using molecular methods. The description prepared of *R. agnetis* specimens detected on *M. × giganteus* (perennial grass of Poacea family) extends the body of morphological data as previously it covered the forest plants only. The detection of *P. nanus* on this perennial grass confirmed a preference displayed by this species for inhabiting the rhizosphere of grasses. There is a likelihood that this species could have been distributed while planting *M. × giganteus* stands as the acreage was increasing. The morphological and molecular descriptions of the three plant-feeding nematode species will facilitate the overcoming of the current nematode threats through their identification and contribute to the spiral and pin nematodes’ biodiversity information.

**SOURCE OF FUNDING**

This research was partly supported by NATO SPS G4687.

**REFERENCES**


