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MORPHOMETRIC AND MOLECULAR CHARACTERIZATION OF *PARATYLENCHUS NANUS* COBB, 1923 (TYLENCHIDA: PARATYLENCHIDAE) ASSOCIATED WITH SOIL FROM *GLADIOLUS* PLANTS

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Abstract

Nematodes belonging to the genus Paratylenchus are widespread throughout the world and are associated with many plant species. Morphological identification of Paratylenchus species is a difficult task because it relies on many characters with a wide range of intraspecific variation. In this study we provided morphological and molecular characterisation of the species Paratylenchus nanus. In 2019, 5 soil samples were taken from Argeș county from different flowering plants to detect and identify phytoparasitic nematodes. Soil samples were taken from the following species of flowering plants: Lilium, Chrysanthemum, Gladiolus and Lisianthus. Based on laboratory tests, the nematode Paratylenchus nanus was detected in a soil of Gladiolus plants. Species were identified by morphobiometric analysis and molecular biology. To confirm the species, sequencing and phylogeny analyses were performed. Thus, following this study, we report for the first time, in Romania, the presence of the nematode Paratylenchus nanus, on another host plant, than the one previously reported.

Key words*: DNA sequencing, identification, morphometric, Romania, phylogeny, new host.*

INTRODUCTION

The pin nematode in the *Paratylenchus* genus Micoletzky, 1922 (Nematoda: Tylenchulidae) was first described based on a single female, belonging to species *P. bukowinensis* Micoletzky, 1922 coming from a flooded and sandy soil on the riverbank of Prut, once part of Romania (Ghaderi et al., 2014). The nematodes of *Paratylenchus* genus Micoletzky, 1922 feed on many species of plants and are spread throughout the world (Van den Berg et. al., 2014). As host plants, they have varied horticultural and agricultural crops, ornamental and forest plants (Raski, 1991). *Paratylenchus* genus contains over 120 species (Siddiqi, 2000). Depending on the stylet size, the pin nematode species are classified into two genera, i.e., *Gracilacus* and *Paratylenchus* (Raski, 1962). The short-stylet species are epidermal feeders whereas the long-stylet ones will go deeper into the root cortex, as they are ectoparasit-feeding nematodes (Siddiqi, 2000). *P. hamatus* is a parasite for wheat, pea (Riga et al., 2008) and celery (Lawnsbery et al., 1952); P. *neoamblycephalus* acts as a parasite for the plum saplings (Braun and Lawnsbery, 1975) and apricot saplings (Fisher, 1967); *P. projectus* is a parasite for the tobacco (Coursen and Jenkins, 1958), while *P. nanus* for the rye (Bell, 1999). *Paratylenchus nanus* has been so far reported in Europe, Australia, North America, Asia, Africa, and Antarctica (Upadhaya et al., 2019). *P. nanus* is associated with the following crops - grasses, fruit-bearing trees, vegetables, and grains (Upadhaya et al., 2019). In Romania, species *P. curvitatus* van der Linde 1938, was reported by Popovici in 1974, as having been identified on an arable land in the vicinity of Cluj-Napoca. *P. microdorus* Andrassy, 1959 was collected by Popovici and Ciobanu in 1977 and 1978 from grasslands around the Southern and Eastern Carpathians. *P. neoamblycephalus* Geraert, 1965 was reported by Popovici in 1993, as found in the grasslands with spruce-fir forests, located in Retezat Mountains and *P. (Gracilacus) aciculus* Brown, 1959 was

collected by Popovici in 1974 from grasslands and arable soil nearby the city of Cluj-Napoca and, more recently, in 1998, from meadow areas in Retezat Mountains, Cernei Mountains and Mehedinti Mountains. Ciobanu et. al, 2003, gives a short desciption of the *Paratylenchus* species present in Romania, namely *P. nanus* Cobb, 1923, *P. microdorus* Andrassy, 1959, *P. projectus* Jenkis, 1956 and *P. (Gracilacus) straeleni.* Pin nematodes are hard to be identified, due to their small size and variable features (Raski, 1962). Nevertheless, in order to be able to distinguish between the species of the pin nematode, we can use the following characteristics for the morphological identification - length of stylet, number of lateral lines, presence or absence of the vulval flap (Ghaderi et al., 2014). For a more accurate taxonomy within the species, of the nematodes in *Paratylenchus* genus, there are currently used molecular biology techniques that include contrastive analyses of the ribosomal RNA gene sequences (rARN) and phylogenetic analysis based on rARN sequences. Subotin et al., 2005, Chen et al., 2008, 2009 and van Megen et al., 2009, have provided a molecular description of the species contained in this genus, by using the D2-D3 expansion genes of the gene sequences 28S rARN, ITS rARN and gene sequences 18S rARN.

The main objectives in this study are represented by the morphological identification and the molecular charaterisation of the species *Paratylenchus nanus*, collected from Romania by making use of the D2-D3 expansion sequences of the gene sequences 28S rARN and ITS rARN. Phylogenetic trees have been built, based on both fragments of the genes and relations examined within the *Paratylenchus* species.

MATERIALS AND METHODS

In the summer of 2019, 5 soil samples were collected from a private person in Pitesti locality, Arges county, Romania and from diverse flowering plants, such as *Lilium, Chrysanthemum, Gladiolus* and *Lisianthus*. Besides the *Gladiolus* crop, which was cultivated in the open field on an acreage of 600 sqm, the other flower species were planted in protected spaces (greenhouses). Out of the 5 samples analyzed, only one was positive and phytoparasitic nematodes were detected, from *Paratylenchus* genus. The geographic coordinates (GPS) of the sampling site are 44°52'28.0"N 24°52'51.6"E (Figure 1).

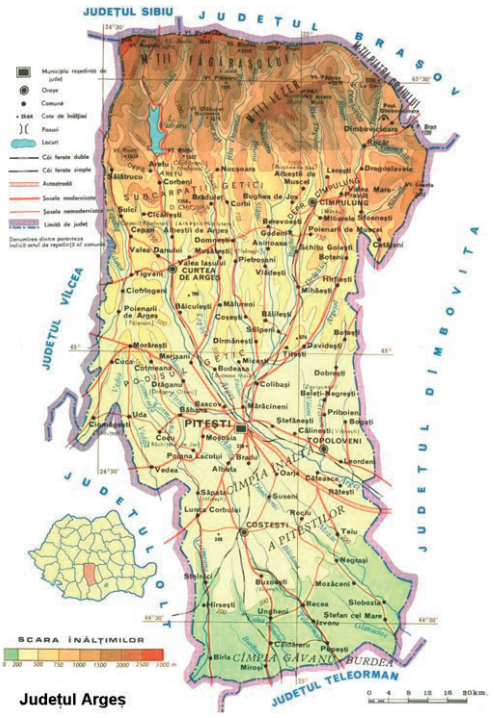

Figure 1. Geographic location of Pitesti locality, Arges county, Romania

The extraction of the nematodes from the soil was conducted by using Cobb method, 1918, with around 200 ml of soil was used for each extraction. The nematodes derived from the soil extraction procedure were collected and preserved in formaldehyde 4% for at least 10 days, in order to achieve the permanent slides (Yoder M. et al., 2006). The species *Paratylenchus nanus* was noticed and identified in a soil sample collected at the level of the radicular system, with host plants as flowering - plants in the *Gladiolus* spp. genus, mentioned above. The measurements of nematodes were done with a microscope Zeiss Axio Image 2 equipped with a digital camera Zeiss Axiocam 506 and an incorporated soft Zen 2.6 (Blue edition).

MOLECULAR ANALYSES

DNA was extracted for molecular analysis. Individual nematodes were transferred in 8 µL worm-lysis buffer (50 mM KCl, 10 mM Tris-Cl pH 8.3, 1.5 mM $MgCl_2$, 1 mM DTT, 0.45 % Tween-20) and 1 µL of proteinase K (1.2 mg ml^{-1}). The mixture was incubated for 1 h at 65°C and 10 min at 95°C followed by a centrifugation step for 1 min at 16000 g. For the amplifying of the rDNA-ITS region we used the primers TW81b (5'-GTAGGTGAACCTGCAGCTG-3', adapted TW81 primer on ILVO, not published) and AB28 (5'-ATATGCTTAAGTTCAGCGGGT-3', Joyce et al. 1994). A 50 µL PCR reaction volume contained 22.4 µL distilled water, 25 µL MyFi Mix (Bioline, Germany), 0.3 µL of each forward and reverse primer (50 µM) and 2 µL DNA. For the amplification of a part of the 28S rRNA gene the primers D2A (5'-ACAAGTACCGTGAGGGAAAGTTG-3', De Ley et al. 1999) and D3B (5'-TCGGAAGGAACCAGCTACTA-3', De Ley et al. 1999) were used. A 50 µL PCR reaction volume contained 21.4 µL distilled water, 2 µL $MgCl_2$, 25 µL 2X BIO-X-ACT (Bioline, Germany), 0.3 µL of each forward and reverse primer (50 µM) and 1 µL DNA. The thermal cycling profile was as follows: initial denaturation step at 96°C for 3 min; 35 cycles of 96°C for 30 s, X°C for 30 s, 72°C for 1 min with X being the annealing temperature of 49°C for the rDNA-ITS region and 55°C for the 28S rRNA gene; final extension step at 72°C for 10 min. The PCR products were visualized after electrophoresis (100 V, 30 min) on agarose gels (1.5 %) with Midori Green Advance stain (Nippon Genetics) using a UV_transilumminator. Purification was done following the protocol accompanying the Wizard SV Gel and PCR Clean – Up System purification kit (Promega, Belgium). The amplified fragments were sent for sequencing to GeneWiz (Germany). All fragments were sequenced in forward and reverse directions. The overlapping parts of the forward and reverse sequences of three *Paratylenchus* nematodes were removed using FinchTv version 1.4 and the contigs were compared with all DNA-sequences available in GenBank using the BLASTn tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

PHYLOGENETIC ANALYSES

Sequenced fragments were edited and assembled using DNA Dynamo software and deposited into the GenBank database. Subsequently the obtained sequences were aligned with the sequences of the D2-D3 expansion segments of the 28S rDNA or sequences of ITS rDNA, respectively, of other *Paratylenchus* species published in the GenBank using the nucleotide BLAST program in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).
Multiple alignments of sequences were done using MUSCLE integrated in MEGA software (Kumar et al., 2008). The evolutionary history was inferred by using the Maximum Likelihood method and the robustness of the ML trees was inferred using 1000 bootstrap replicates (Felsenstein J., 1985). The initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura 3 parameter model (Tamura ,1992), for D2-D3 expansion segments of the 28S rRNA sequences, and Kimura 2-parameter model (Kimura, 1980) for ITS rRNA sequences respectively, and then selecting the topology with superior log likelihood value, a discrete Gamma distribution being used to model evolutionary rate differences among sites. Evolutionary analyses were conducted in MEGA X (Kumar , 2018).

RESULTS AND DISCUSSIONS

***Paratylenchus nanus* Cobb, 1923**
(Figure 2)

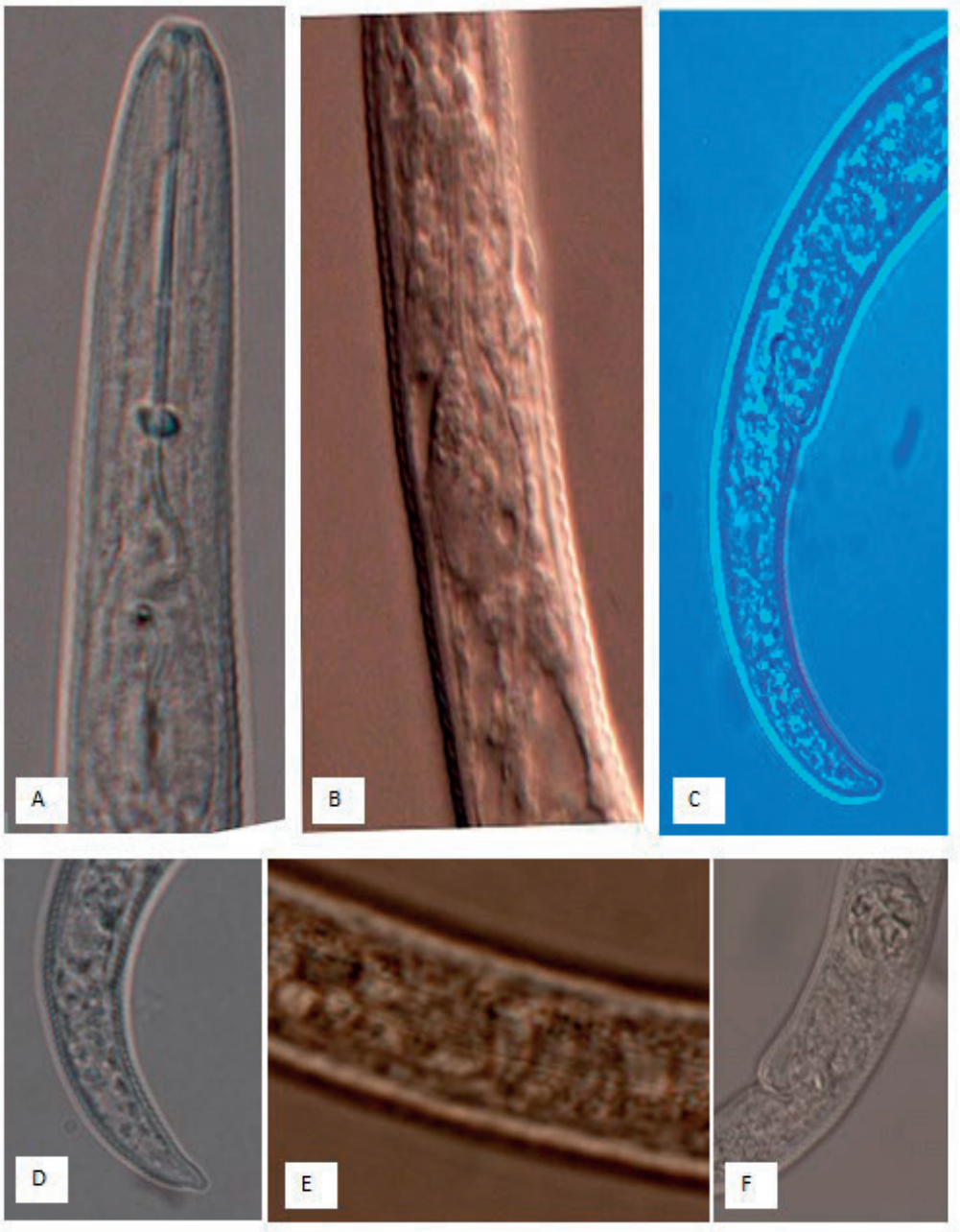

Figure 2. *Paratylenchus nanus*, Romanian population, (A-F) Female. A: Anterior region; B: Excretory por; CD: Posterior region; E: Lateral field at mid-body; F: Vulvar area.

MEASUREMENTS (See Table 1)

Table 1. Morphometric data for *Paratylenchus nanus* Cobb, 1923, female, from Romania

Species:	*P. nanus*	
Site location: Characters / ratios	After Brzeski, 1998	Pitești - Argeș Plant host: *Gladiolus* spp.
n		16 ♀
L (µm)	340 (270-460)	370 ± 19.56 (325-397)
a	22 (17-28)	20 ± 1.49 (17-23)
b	3.8 (3.2-4.4)	4 ± 0.18 (3.6-4.3)
c	13.8 (9.6-18.9)	14.3 ± 1 (13-17)
c'	2.6 (1.9-4.4)	2.4 ± 0.18 (2–2.7)
V%	83 (79-86)	83 ± 1.01 (82 - 85)
Stilet length	28.5 (23-34)	27 ± 1.41 (25 – 30)
Pharynx	91 (83-104)	92.69 ± 3.77 (86 – 101)
Anterior end to excretory pore	77 (65-103)	76.8 ± 3.13 (72 -82)
Head – vulva	-	308 ± 15.57 (276-334)
Tail length	26 (18-36)	26 ± 2.42 (22-31)
Max. body diam.	-	18 ± 0.83 (16.5-19)
Anal body diam.	-	10.7 ± 0.70 (10-12)

Note: All measurement are in µm and in the form of mean ± s.d. (range).

DESCRIPTION

Female

When relaxed, the female and the juveniles are arcuate ventrally and their cuticle has distinct annulations.

The lateral lines are four in number, taking up approx. 1/5 of the body width. The head is round, in a continuous shape, with the lip or labial plate not protruding.

The stylet has an average length of 27 µm (25-30µm), with the stylet cone holding 63-70% of its length, knobs with the width of 4-5 µm, while DEGO is posteriorly located compared to the knobs, with the length ranging from 4.5 to 5 µm.

Spermatecha is full, round in shape, with the advulval flaps distinct, in a round form. In comparison to the head, the excretory pore is located about 76.8 µm length, with a average between 72 and 82 µm (Table 1).

The median bulb is unexpectedly well developed, occupying half of the oesophagus length. Vulva has a massive transversal slit, with strong lateral membranes.

The tail is variable, in most cases slightly indented on the dorsal side close to the top, annulated towards the tail end, with a rounded terminus (Figure 2).

Male not found.

Note: In 1965, Geraert made a comment concerning the species in the group of *P. curvitatus,* more exactly that they are difficult to be distinguished from one another. Among the species in this group, we can enumerate *P. nanus, P. neoamblycephalus, P. dianthus, P. hamatus, P. amblycephalus, P. nainianus, P. projectus.* In 1965, Fisher considered the species *P. nanus* synonymous with *P. projectus.* Five years earlier, Tarjan was upholding the idea that species *P.nanus* and *P. neoamblycephalus* are similar, whereas species *P. projectus* is close to species *P. nanus* and *P. curvitatus.* Other researchers did not accept these synonyms later.

MOLECULAR PROFILES AND PHYLOGENETIC STATUS

The species was molecularly characterized using partial gene D2-D3 of 28S and ITS field of rDNA gene and deposited in the GenBank. The genus contains over 120 species but only a dozen species have been molecularly characterized. *Paratylenchus nanus* was reconstructed and is shown in (Figures 3-4). Ghaderi et al., 2014 has used the most stable characters to identify the species in *Paratylenchus* genus, namely the length of stylet, number of lateral lines and the presence or absence of the advulval flaps. Based on these three characters, the species of nematodes in *Paratylenchus* genus were classified into 11 groups.

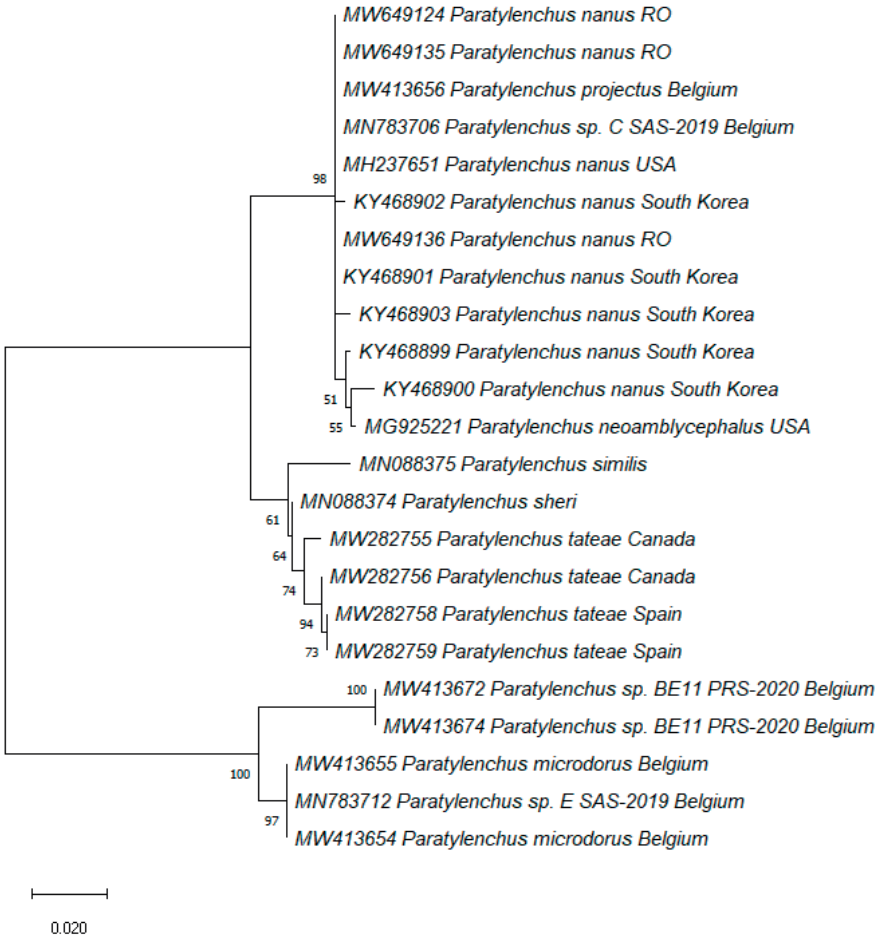

Figure 3. Phylogenetic relationships of *Paratylenchus nanus* isolate collected from Romania and isolates from other geographical regions based on the sequence alignment of the D2-D3 expansion segments of the 28S rDNA. The phylogenetic tree was inferred by using Maximum Likelihood method and Tamura 3-parameter model with 1000 bootstrap replication. The tree with the highest log likelihood (-1628.43) is shown. Bootstrap values are indicated at the nodes. The analysis involved 23 nucleotide sequences and there was a total of 686 positions in the final dataset. The scale bar indicates 0.020 nucleotide substitutions per nucleotide position

The obtained sequences of the ITS rDNA and the D2 - D3 of the 28S rDNA regions of each of the six nucleotides were 709 - 714 - 727 bp and 735 - 774 - 787 bp long, respectively.

Based on the 28S gene (Figure 3), the three nucleotide sequences in Romania, two are grouped *P. nanus* (MW649124; MW649135), and the third (MW649136) is at a distance of four nucleotides from the first because it is shorter but it is in the same clade.

P. nanus (MW649124; MW649135) are nearly identical with *P. projectus* (MW413656) and *Paratylenchus* sp. (MN783706) from Belgium, *P. nanus* (MH237651) from USA and *P. nanus* (KY468902) from South Korea.

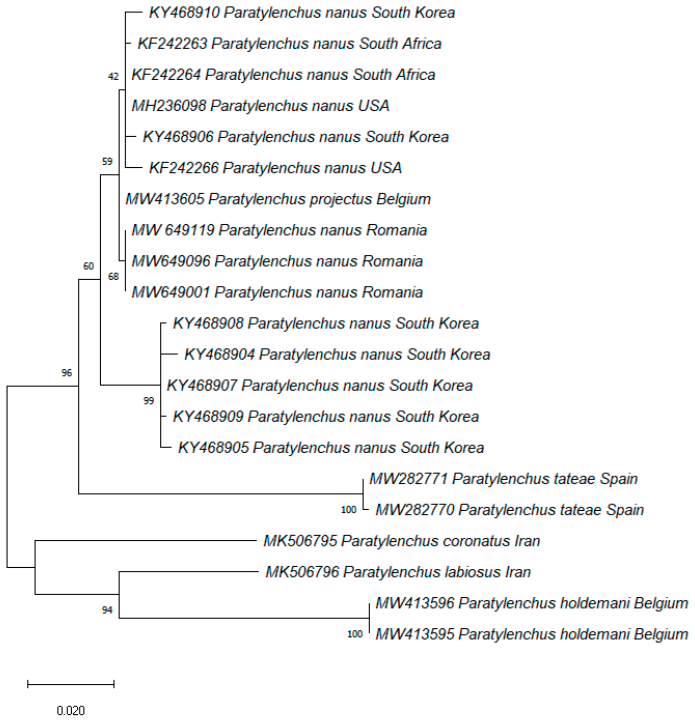

Figure 4. Phylogenetic relationships of *Paratylenchus nanus* isolate collected from Romania and isolates from other geographical regions based on the sequence alignment of the ITS rDNA. The phylogenetic tree was inferred by using Maximum Likelihood method and Kimura 2-parameter model with 1000 bootstrap replication. The tree with the highest log likelihood (-2096.95) is shown. Bootstrap values are indicated at the nodes. The analysis involved 21 nucleotide sequences and there was a total of 749 positions in the final dataset. The scale bar indicates 0.020 nucleotide substitutions per nucleotide position

The other nucleotide sequence in Romania *P. nanus* (MW649136) is almost identical to *P. nanus* in South Korea (KY468902; KY468901; KY468903) and *P. nanus* (MH237651) from USA, *Paratylenchus* sp., (MN783706) *P. projectus* (MW413656) from Belgium and *P. nanus* (MW649124; MW649135) from Romania. The analysis involved 23 nucleotide sequences and there was a total of 686 positions.

Based on the ITS gene (Figure 4), the three sequences belonging to the *P. nanus* species from Romania (MW649119; MW649096; MW649001) are clearly differentiated from those of other species.

The three sequences belonging to the species *P. nanus* from Romania are very close to the species *P. projectus* (MW413605) from Belgium.

They are sisters to *P. nanus* (KF242266; MH236098) from the USA, *P. nanus* (KY468906; KY468910) from South Korea and *P. nanus* (KF242263; KF242264) from South Africa.

Paratylenchus nanus from Romania is sister to the five species of *P. nanus* (KY468908; KY468904; KY468907; KY468909; KY468905) from South Korea and *P. tateae* (MW282771; MW282770) from Spain.

The analysis involved 21 nucleotide sequences and there was a total of 749 positions.

All species that are part of the phylogenetic tree for both 28S and ITS genes are part of G3 according to Ghaderi et al., 2014.

CONCLUSIONS

The species is present in Romania and it was described and morphobiometrically identified by Ciobanu et al., 2003, in Fagaras Mountains, at an altitude of circa 740-900 m, in mixed deciduous forests (Carpino-Fagetum).

Besides the description and the morphobiometrical identification, a molecular characterization has been completed.
The species *Paratylenchus nanus* was found on a new host plant (*Gladiolus* sp.), in a different area.

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