Morphological, Morphometrical, Molecular Characterization, and Phylogenetic Relationship of *Paratylenchus holdemani* from Kale (*Brassica oleracea* var. *acephala*) Cultivation Areas in Turkey

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https://dx.doi.org/10.4314/tjpp.v20i2.5 **(*Turkey*)**

ABSTRACT

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Pin nematodes (*Paratylenchus* spp.) have been recorded in association with a wide range of economically significant crops worldwide, including various cereals, vegetables, and ornamental plants. In October 2021, soil samples were collected from kale (*Brassica oleracea* var. *acephala*) growing field in the Giresun province of Turkey. In this study nematodes were extracted from the soil using a modified baermann funnel method. Standard morphological characters were measured and compared to those reported in previous studies. For molecular characterization DNA was extracted from immature females and the D2-D3 expansion region of the 28S rRNA gene was amplified using primer pair D2A (5-ACA AGTACCGTGAGGGAAAGTTG-3) and D3B (5- TCGGAAGGAACCAGCTACTA-3). PCR product (750 bp) was sequenced and then compared with sequences of *Paratylenchus holdemani* available in the GenBank database. The NCBI BLAST analysis of the Turkish population sequences showed 100% similarity with *Paratylenchus holdemani* sequences registered in GenBank. The results obtained from morphological, morphometrical, molecular and phylogenetic relationship studies showed that the pin nematode population was *P. holdemani*.

Keywords: D2-D3, Kale, morphological characters, *Paratylenchus holdemani*, Phylogeny, Turkey

Nematodes, which cause significant damage to cultivated plants worldwide, constitute an important pest group that causes approximately 12% crop loss, and the financial value of this loss is estimated to be around 100 billion dollars (Sasser and Freckman 1987). Pin nematodes (*Paratylenchus* spp.) are obligate ectoparasites that attack many plant species including herbaceous plants, shrubs, and trees that are distributed worldwide (Singh et al. 2021). The genus *Paratylenchus* currently includes more than 100 plant parasitic species (Munawar et al. 2021). Some of those are *P. bukowinensis, P. hamatus, P. sarissus, P. alleni, P. holdemani, P. nainianus* and *P.*

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nanus. Particularly, *Paratylenchus holdemani* Raski, 1975 (Tylenchida: Tylenchulidae) is one of the crucial species from this genus (Singh et al. 2021). It has been reported in many countries such as Spain, El Salvador, Czech Republic, Belgium and Turkey (Clavero-Camacho et al. 2021). In Turkey, it has been reported for the first time on peach (Kepenekci 2001).

Kale crop (*Brassica oleracea* var. *acephala*) has an important place as a nutritional raw material in Turkey. It suffers from crop losses due to diseases, pests and weeds. In particular, nematodes such as *P. holdemani*, are among the important plant pests responsible for some of these losses. Accurate identification of those plant-parasitic nematodes is crucial for effective nematode management in agriculture (Devran and Söğüt 2009). However, the high intraspecific variability within this species presents challenges for identification based solely on morphological characteristics (Palomares-Rius et al. 2018). Consequently, molecular approaches are essential for precise identification (Palomares-Rius et al. 2021). In this study, morphological, morphometric, and molecular characteristics, along with phylogenetic analyses, were utilized to identify *Paratylenchus* populations collected from a kale field in Giresun Province, Turkey.

MATERIALS AND METHODS

Soil sampling and nematode extraction.

Soil samples were collected from kale field at the bases of the plants on October 2021, from Giresun province (40°57'27.0"N 38°48'18.0"E) in the Black Sea region of Turkey (Fig. 1).

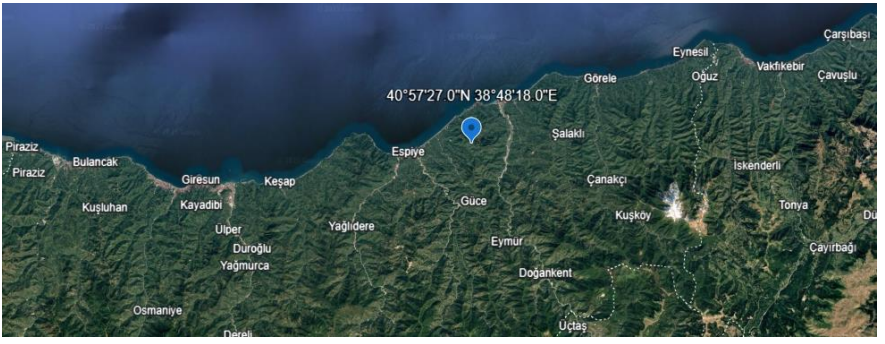

Fig. 1. A map of the location where kale are produced in Giresun

Soil samples were collected in polyethylene bags and transported to the laboratory for analysis. The samples were stored at 4°C until the nematode extraction process. Nematodes were isolated from 100 cm³ of soil using the centrifuge sugar water method (Jenkins 1964). Identification and enumeration of

nematodes were performed under an inverted microscope at 40× magnification.

Morphological characterization.
Standard morphological characters were studied on *P. holdemani*. For morphometric characters, 10 females were used. Nematodes were placed in a drop of pure water on a clean glass slide and killed by heat for 4-6 s. Then, the specimens were examined for morphological characters and morphometric measurements using a camera (AXIOCam 105) mounted on the ZEISS primo Vert microscope (ZEISS,) at a size of 400X. Microsoft Excel was used for the analysis of female morphometric measurements.

DNA extraction.
For molecular studies the genomic DNA were extracted as follows: Each single specimen was collected into 10 µl of extraction buffer (10 mM Tris-HCl, pH 8.8; 1 mM EDTA; 1% Triton X-100 (v/v); 20 mg/ml Proteinase K) in a 1.5 ml Eppendorf tube. Sample tubes were kept at -20°C for 1 night (Pagan et al. 2015). The samples were digested using a glass capillary tube and incubated at 56°C for 1 h, followed by an additional incubation at 95°C for 10 min. The resulting mixture was then utilized as a DNA template for PCR analysis.

PCR amplification.
For the amplification of the 28S rDNA (D2/D3 region of the large subunit, LSU), the primers D2A (5´-ACA AGT ACC GTG AGG GAA AGT TG-3´) and D3B (5´-TCG GAA GGA ACC AGC TAC TA-3´) were used (De Ley et al. 1999). PCR thermocycling was performed using a Mastercycler Veriti (Singapore). The thermocycling conditions included an initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 2 min, with a final extension at 72°C for 7 min.

Electrophoresis.
DNA fragments were separated by electrophoresis in a 1.5% agarose gel using 1X TAE buffer. The gel was stained with ethidium bromide, and visualization was performed using an ErBiyotek GEN-BOX imagERFx system, followed by documentation through photography. The PCR products were then sent to Metabion International AG (Germany) for sequencing analysis.

Phylogenetic analysis.
For the purpose of conducting the phylogenetic analysis, the acquired raw sequences were manually reviewed and edited with BioEDIT version 7.2.5 (Hall 1999). The consensus sequences that were generated were then compared with those stored in the GenBank database utilizing the BLAST search engine to assess sequence homology. The D2/D3 sequences collected in this research, along with those accessed from the GenBank databases (Table 1), were aligned using Clustal W, which facilitated multiple alignments for 31 nucleotide sequences employing MEGA 11.0 software (Kumar et al. 2016). Subsequently, the alignment was examined to determine the model of base substitution for these sequences, again using MEGA 11. Finally, a phylogenetic tree was constructed by applying the Maximum Likelihood Model (ML) with 1000 bootstrap replicates, executed in MEGA 11.0 (Kumar et al. 2016), as illustrated in Fig. 3.

RESULTS
The *Paratylenchus* populations from Turkey that were examined in this research were taxonomically classified and found to possess a phylogenetic

connection to *P. holdemani*. High densities of *P. holdemani* individuals, specifically measuring 130 nematodes per 100 cm³ of soil, were identified in the rhizosphere of kale. Additionally, the morphological, morphometrical, molecular characteristics and phylogenetic associations of the female specimens extracted from soil samples were scrutinized.

Table 1. Species of nematodes used in the phylogenetic analysis, including GenBank accession numbers and origin

Species	GenBank Accession No	Country
Paratylenchus holdemani	MW413636.1	Belgium
Paratylenchus holdemani	MW413639.1	Belgium
Paratylenchus holdemani	MW413637.1	Belgium
Paratylenchus holdemani	MW413634.1	Belgium
Paratylenchus holdemani	MW413640.1	Belgium
Paratylenchus holdemani	MW413642.1	Belgium
Paratylenchus holdemani	MW413641.1	Belgium
Paratylenchus holdemani	MW413635.1	Belgium
Paratylenchus holdemani	MW798300.1	Spain
Paratylenchus holdemani	MZ265107.1	Spain
Paratylenchus holdemani	MZ265106.1	Spain
Paratylenchus holdemani	OQ749960.1	USA
Paratylenchus sp.	ON873243.1	Spain
Paratylenchus sp.	MW413667.1	Belgium
Paratylenchus sp.	MW413665.1	Belgium
Paratylenchus sp.	MW413670.1	Belgium
Paratylenchus sp.	MW319821.1	Belgium
Paratylenchus sp.	OR177652.1	South Korea
Paratylenchus sp.	MN535544.1	Belgium
Paratylenchus tenuicaudatus	OL884401.1	Spain
Paratylenchus tenuicaudatus	OL884411.1	Spain
Paratylenchus tenuicaudatus	OL884403.1	Spain
Paratylenchus tenuicaudatus	OL884421.1	Spain
Paratylenchus tenuicaudatus	MW798307.1	Spain
Paratylenchus nanus	PQ859461.1	USA
Paratylenchus nanus	MH237651.1	USA
Paratylenchus nanus	KY468900.1	South Korea
Paratylenchus bukowinensis	MN088372.1	Iran
Paratylenchus bukowinensis	MN783703.1	Belgium
Paratylenchus caravaquenus	MW798272.1	Spain
Xiphinema simile	ON500623.1	Russia

Morphological and morphometrical characterization.

The nematodes exhibited a transformation from a C-shape when subjected to heat. The head rounded, the pharynx is well-developed, constituting approximately one-fourth of the total body length. The secretory-excretory pore is typically located between the mid-isthmus and the level of the end bulb. The vagina is oriented obliquely, extending to two-thirds of the body width. The tail measures between 25 to 30 µm in length and has a conical shape, characterized by a consistently fine terminal roundness that may occasionally appear blunt. Male is not found.

The female nematodes body length was characterised (387.9±14.4 µm) (Fig. 2A). Stylet length 26±0.9 µm, stylet knobs width 3.5±0.2 µm, stylet knobs height 1.8±0.2 µm on average (Fig. 2B). The maximum body diameter is 17±1.1 µm. The vulva is located close to the posterior parts of the body and its distance from the anterior part is 314±15 µm. The tail is 27.6±1.1 µm on average and the tail ends rounded (Fig. 2C). Table 2 showed comparison of the morphobiometric characteristics of *P. holdemani* females with previously recorded world populations.

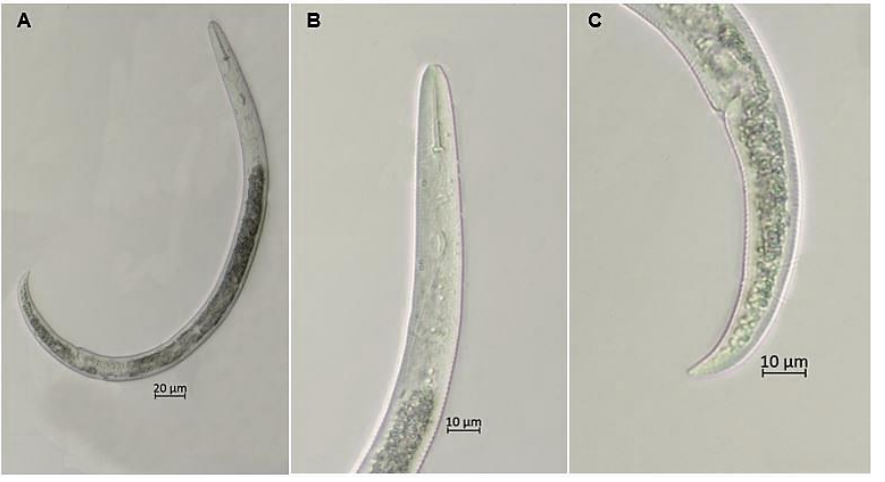

Fig. 2. Light micrographs of female *Paratylenchus holdemani* from Turkey: A: Whole body, B: Female anterior region, C: Tail region.

Table 2. Comparison of the morphobiometric characteristics of *Paratylenchus holdemani* females with previously recorded world populations (All measurements are in µm (except L in µm) and expressed as means ± standard deviation (Min-Max range).

Characters	Turkey Present Study	Belgium Singh et al. (2021)	Spain Clavero-Camacho et al. (2021)	USA Raski (1975)
n	**10**	**31**	**4**	**7**
L	**387.9**±14.4 (359.2-410.3)	**359**±47 (285-475)	**392.3**±30.2 (364-435)	**320** (290-350)
a	**22.9**±1.2 (20.8-24.1)	**20.9**±1.9 (16.4-25.2)	**24.9**±1.6 (23.5-27.2)	**22** (19-24)
b	**4.1**±0 (3.8-5.3)	4.1±0.7 (2.2-5.1)	**3.9**±0.3 (3.6-4.1)	**4** (3.7-4.7)
c	**14.1**±1 (13-15)	14.8±1.4 (12.4-17.7)	**13.3**±1 (12.2-14.6)	**17** (16-19)
c'	**3.1**±0.2 (2.6-3.3)	**2.5**±0.3 (2.1-3.2)	**3**±0.4 (2.7-3.5)	-
Maximum body width	**17**±1.1 (15.4-19.3)	**17.3**±3 (11.3-23.8)	**15.8**±0.6 (15-16.5)	-
Stylet length	**26**±0.9 (24.6-27.4)	**22.5**±2 (19-26.1)	**26.8**±0.6 (26-27.5)	**22** (21-23)
Stylet knobs height	**1.8**±0.2 (1.5-2.2)	-	-	-
Stylet knobs width	**3.5**±0.2 (3.2-3.9)	**3.3**±0.4 (2.9-4.2)	-	-
Pharynx length	**95.8**±8.9 (77.1-103.9)	**89.7**±21.5 (66.1-161)	**100.3**±5.1 (93-105)	-
Anterior end to excretory pore	**84.5**±4 (77.3-89.7)	**74.8**±9.1 (60.1-99)	**85.5**±5.2 (79.5-82)	**73** (67-77)
Anterior end to vulva	**314**±15 (274.9-330.2)	**303**±40.9 (238-391)	-	-
V%	**80.9**±1.6 (76.5-82.3)	**84.3**±1.8 (81.3-90.5)	**81**±0.8 (79.9-81.9)	**85** (84-86)
Body width at anus	**9**±0.6 (8-10)	**10**±1.3 (7.2-12.3)	**9.8**±0.3 (9.5-10)	-
Tail length	**27.6**±1.1 (26.1-29.6)	**25.2**±2.8 (20-29.5)	**29.6**±3.4 (26.5-33.5)	-

Abbreviations: n: number of nematodes measured; L: body length; a: body length/maximum body width; b = body length / pharyngeal length; c: body length/tail length; c': tail length/body width at anus; V%: distance of the vulva from anterior end expressed as a percentage of body length.

Sequence results

The amplification of the ribosomal region 28S rDNA (D2/D3 region of the large subunit, LSU) gene expansion segments produced a single 750 bp fragment, as confirmed by gel electrophoresis (Fig. 3). Comparing the sequences of the ribosomal region 28S rDNA (D2/D3 region of the large subunit, LSU) gene obtained from PCR products of

P. holdemani in Turkey with those present in the GenBank database revealed a whole similarity (100%) with the species *P. holdemani* (MW413637.1). The phylogenetic relationships of the Turkey population of *P. holdemani* with other populations using the 28S rDNA region are also depicted in Fig. 3.

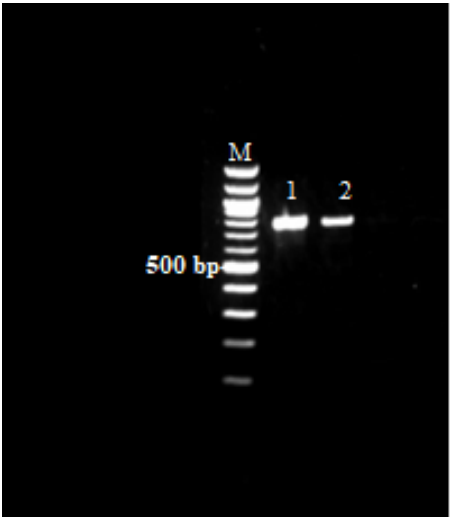

Fig. 3. PCR products of *Paratylenchus holdemani* species. (a) 28S rDNA (D2/D3 region of the large subunit, LSU) gene using D2A/D3B primer pair (Line1-2).

Phylogenetic analysis

Phylogenetic analysis was conducted to determine the evolutionary relationships of *P. holdemani*, as presented in Figure 4. The phylogenetic tree was reconstructed using 31 sequences, including 12 sequences from the *P. holdemani* group, 7 from the *Paratylenchus* spp. group, 5 from the *P. tenuicaudatus* group, 3 from *P. nanus*, 2 from *P. bukowinensis*, and 1 from *P. caravaquenus*, with *Xiphinema simile* used as the outgroup taxon. Alignment and phylogenetic analysis of the D2-D3 sequences revealed multiple clades, which were distinguished by varying bootstrap support (BS) values in the Maximum Likelihood (ML) analysis.

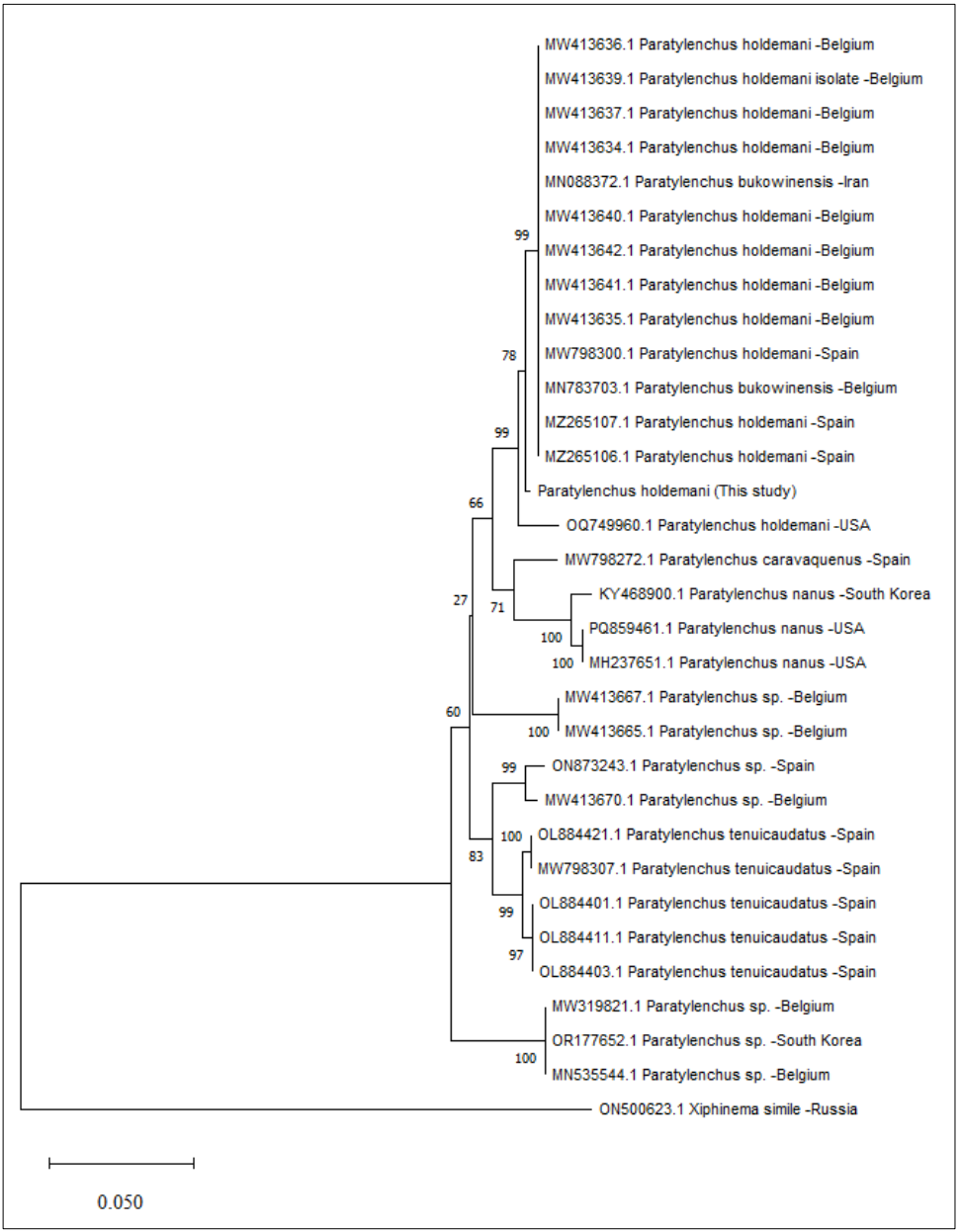

Fig. 4. Maximum likelihood (ML) phylogenetic tree of *Paratylenchus holdemani*, inferred from D2 expansion segment of LSU rDNA. The analysis was using 1000 bootstrap replicates. *P. holdemani* (Turkey) obtained from this study. *Xiphinema simile* sequences were used as out group for the construction of phylogram.

DISCUSSION

Although morphological identification methods are important in nematology, molecular methods are increasingly applied in nematode diagnostics due to their ability to provide precise identification. As a result, ribosomal DNA has emerged as the preferred marker for identifying nematodes. The ribosomal ITS regions of nematodes are highly variable, making them particularly useful for diagnostic purposes (Subbotin et al. 2011). Precise identification of plant-parasitic nematode species that adversely impact crop productivity is essential for the development of effective control strategies.

Previous molecular studies have reported identical D2–D3 expansion segment sequences for populations identified as *P. holdemani* from Belgium and Spain. The molecular results obtained in the present study are very similar to those reports. These findings support the view that the sequences belong to *P. holdemani* and confirm the usefulness of molecular markers for reliable species identification in different geographical regions (Singh et al. 2021).

P. holdemani has been reported on almonds in Spain, on coffee in El Salvador, on unidentified host in USA and in wild olive in the southern Iberian Peninsula (Clavero-Camacho et al. 2021, Álvarez-Ortega et al. 2023). Turkish population of *P. holdemani* has a slightly shorter mean body length than that of specimens from Spanish population (387 µm vs. 392.3). However, compared with that of the Belgium and USA (359 µm vs. 320 µm), it is slightly longer, respectively. The stylet of the Turkish population is longer than that of Belgium and USA specimens (24.6-27.4 µm vs. 19-26.1 µm and 21-23 µm) but compared with that of the Spain stylet (24.6-27.4 vs. 26-27.5 µm), it is slightly shorter. The stylet knobs width of the Turkish population are furthermore almost the same than that of Belgium (3.5 µm vs. 3.3 µm). The pharynx length of the Turkish population is furthermore longer than that of Belgium (95.8 µm vs. 89.7 µm) but compared with that of the Spain pharynx length (95.8 µm vs. 100.3 µm), it is shorter. The variations observed in the measurements are believed to be influenced by climatic and regional conditions.

In conclusion, *P. holdemani* was discovered in kale soil in the Giresun provinces in Turkey. The identification presents a significant opportunity for future nematological studies, such as the identification of different species. As a result, *P. holdemani* in kale fields offers valuable insights that can fuel research aimed at enhancing control measures for *Paratylenchus* species, ultimately leading to increased kale yield. We underscore the continued need for further investigation into the genus *Paratylenchus* to mitigate potential yield losses in kale production.

RESUME

Güvercin B., Akyazı F. et Yiğit U. 2025. Caractérisation morphologique, morphométrique et moléculaire, et relations phylogénétiques de *Paratylenchus holdemani* dans les zones de culture du chou frisé (*Brassica oleracea* var. *acephala*) en Turquie. Tunisian Journal of Plant Protection 20 (2): 85-95.

Les nématodes des pins (*Paratylenchus* spp.) sont fréquemment associés à une grande variété de cultures importantes à travers le monde, notamment diverses céréales, légumes et plantes ornementales. En Octobre 2021, des échantillons de sol ont été prélevés dans un champ de chou frisé (*Brassica oleracea* var. *acephala*) de la province de Giresun en Turquie. Dans cette étude, les nématodes ont été extraits du sol par la méthode de l'entonnoir de Baermann modifiée. Leurs caractères morphologiques standards ont été mesurés et comparés à ceux rapportés dans des études antérieures. Pour la caractérisation moléculaire, l'ADN a été extrait de femelles immatures et la région d'extension D2-D3 du gène de l'ARNr 28S a été amplifiée à l'aide des amorces D2A (5'-ACA AGTACCGTGAGGGAAAGTTG-3') et D3B (5'-TCGGAAGGAACCAGCTACTA-3'). Le produit de PCR (750 pb) a été séquencé puis comparé aux séquences de *Paratylenchus holdemani* disponibles dans la base de données GenBank. L'analyse BLAST du NCBI des séquences de la population turque a révélé une similarité de 100% avec les séquences de *P. holdemani* enregistrées dans GenBank. Les résultats des études morphologiques, morphométriques, moléculaires et phylogénétiques ont confirmé que la population de nématodes appartenait à l'espèce *P. holdemani*.

Mots-clés: D2-D3, caractères morphologiques, chou frisé, *Paratylenchus holdemani*, phylogénie, Turquie

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ملخص

الخصائص المورفولوجية والمورفومترية والجزيئية والعلاقة التطورية لنماتودا Paratylenchus holdemani في مناطق زراعة الكرنب الأجعد (*Brassica oleracea* var. *acephala*) في تركيا.

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تم تسجيل وجود النيماتودا الصنوبريات (*Paratylenchus* spp.) في مجموعة واسعة من المحاصيل ذات الأهمية الاقتصادية حول العالم، بما في ذلك أنواع مختلفة من الحبوب والخضراوات ونباتات الزينة. في أكتوبر 2021، جُمعت عينات من التربة من حقل زراعة الكرنب الأجعد (*Brassica oleracea* var. *acephala*) في محافظة غيرسون بتركيا. في هذه الدراسة، عُزل النيماتودا من التربة باستخدام طريقة قمع بايرمان (Baermann) المُعدلة. قِيست الصفات المورفولوجية القياسية وقورنت بتلك المذكورة في الدراسات السابقة. وللتحليل الجزيئي، استُخلص الحمض النووي من الإناث غير الناضجة، وجرى تضخيم منطقة التوسع D2-D3 من جين S rRNA28 باستخدام زوج البادئات D2A (5-ACA AGTACCGTGAGGGAAAGTTG-3) و D3B (5-TCGGAAGGAACCAGCTACTA-3). تم تنفيذ التسلسل الناتج تفاعل البوليميراز المتسلسل (750 زوجًا قاعديًا) ثم مقارنته بتسلسلات *Paratylenchus holdemani* المتوفرة في قاعدة بيانات GenBank. أظهر تحليل NCBI BLAST لتسلسلات العينة التركية تطابقًا بنسبة 100% مع تسلسلات *P. holdemani* المسجلة في GenBank. وأظهرت نتائج الدراسات المورفولوجية والمورفومترية والجزيئية ودراسات العلاقات التطورية أن مجموعة النيماتودا هي *P. holdemani*.

الكلمات المفتاحية: تركيا، صفات مورفولوجية، علم النشوء والتطور، كالي، D2-D3، *Paratylenchus holdemani*

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