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Original article (Orijinal araştırma)

Occurrence and distribution of cyst nematodes, *Heterodera* spp. (Tylenchida: Heteroderidae) associated with black cabbage, *Brassica oleracea* var. *acephala* L. (Brassicales: Brassicaceae) in the Eastern Black Sea Region of Türkiye[1]

Türkiye'nin Doğu Karadeniz Bölgesi'nde karalahana, *Brassica oleracea* var. *acephala* L. (Brassicales: Brassicaceae) üretim alanlarındaki kist nematodları, *Heterodera* spp. (Tylenchida: Heteroderidae) ve dağılımları

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Abstract

This study was conducted during 2021-2022 to detect and determine distribution and population of cyst nematodes, *Heterodera* spp. (Tylenchida: Heteroderidae) in black cabbage *Brassica oleracea* var. *acephala* L. (Brassicales: Brassicaceae) production areas of the Eastern Black Sea Region of Türkiye. For it, a total of 77 samples were taken from 53 districts belonging to the Artvin, Giresun, Ordu, Rize, and Trabzon provinces in the region. Soil samples were taken from around the root of the kale plants. Nematodes were extracted by using the centrifugal flotation technique. The nematodes were identified using morphological features and molecular analysis based on Polymerase Chain Reaction (PCR) method. For molecular analysis, the ribosomal DNA region including the gene region of 28S ribosomal RNA (rRNA) (ITS1, 5.8S, ITS2) was amplified using primer sets TW81/AB28. Additionally, a species-specific primer set (Car-F/Car-R) covering the Cytochrome Oxidase I (cox1) region of mitochondrial DNA (mtDNA) was used. As a result of the analysis, cyst nematodes *Heterodera cruciferae* Franklin, 1945*, Heterodera carotae* Jones, 1950 and *Heterodera fici* Kirjanova, 1954 species were identified in the kale production areas in the region. *Heterodera carotae* is the first record of the cyst nematode species in Türkiye. *Heterodera cruciferae, H. carotae,* and *H. fici* were detected from the total collected soil samples at 16.9%, 3.9%, and 1.3% relative frequency, respectively. Among all, Giresun was the most infected province with 35.3% infection rate, followed by Trabzon with 26.3%, Ordu with 21.1% and Rize with 13.3%.

Keywords: Black cabbage, *Heterodera*, ITS, PCR, taxonomy, Türkiye

Öz

Bu çalışma, Türkiye'nin Doğu Karadeniz Bölgesi lahana, *Brassica oleracea* var. *acephala* L. (Brassicales: Brassicaceae) üretim alanlarında kist nematodlarını *Heterodera* spp. (Tylenchida: Heteroderidae) tespit etmek ve dağılımları ve popülasyonlarını belirlemek amacıyla 2021-2022 yıllarında yürütülmüştür. Bu amaçla, bölgedeki Artvin, Giresun, Ordu, Rize ve Trabzon illerine ait 53 ilçeden toplam 77 örnekleme gerçekleştirilmiştir. Toprak örnekleri karalahana bitkilerinin kök çevresinden alınmıştır. Nematodlar santrifüj yöntemi kullanılarak elde edilmiştir. Nematodlar, morfolojik özellikler ve Polimeraz Zincir Reaksiyonu (PCR) yöntemine dayanan moleküler analiz kullanılarak tanımlanmıştır. Moleküler analiz için, 28S ribozomal RNA (rRNA) gen bölgesini (ITS1, 5.8S, ITS2) içeren ribozomal DNA bölgesi TW81/AB28 primer setleri kullanılarak çoğaltılmıştır. Ayrıca, mitokondriyal DNA'nın (mtDNA) Sitokrom Oksidaz I (cox1) bölgesini kapsayan türe özgü primer seti (Car-F/Car-R) kullanılmıştır. Analiz sonucunda bölgedeki karalahana üretim alanlarında *Heterodera cruciferae* Franklin, 1945*, Heterodera carotae* Jones, 1950 ve *Heterodera fici* Kirjanova, 1954 kist nematod türleri teşhis edilmiştir. *Heterodera carotae* türü Türkiye için ilk kayıt niteliğindedir. Toplanan toplam toprak örneklerinde *H. cruciferae, H. carotae* ve *H. fici* sırasıyla %16.9, %3.9 ve %1.3 oranlarında tespit edilmiştir. Çalışmada, %35.3 ile Giresun ili en çok bulaşık olan il olurken, bunu %26.3 ile Trabzon, %21.1 ile Ordu ve %13.3 ile Rize illeri takip etmiştir.

Anahtar sözcükler: Karalahana, *Heterodera*, ITS, PCR, taksonomi, Türkiye

[1] This study was derived from the first authors' MSc thesis.

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Introduction

Black cabbage, scientifically classified as *Brassica oleracea* var. *acephala* L., is a prominent member of the Brassicaceae family (Öztürk, 2005). A biennial vegetable, its cultivation spans the entire year in European nations, with exceptions during one or two months in specific locales (Vural, 2008). Globally, cabbage production yields a substantial 104 million tons, with China commanding a notable one-third of this output. The other important production countries are India, Russia, South Korea, Ukraine, Indonesia, Japan, Vietnam, the United States of America, Poland, and Kenya (FAO, 2020). Notably, Türkiye has registered an annual black cabbage production of 819.000 tons, cultivated across 4939.8 hectares as of 2021. Within Türkiye, the epicenter of this cultivation lies in the Black Sea region, spanning an impressive 4104.9 hectares. Among the provinces in the region, Giresun ranks first, followed by Samsun, Trabzon and Ordu (TUIK, 2021).

Despite its esteemed status as a globally significant crop, black cabbage cultivation is not resistant to losses from diseases, pests, and invasive vegetation. Cyst nematodes is one of the most important plant parasitic nematodes negatively affecting cabbage production (Pehlivan et al., 2020). Cyst nematodes are species of the *Heterodera* and *Globodera* genera that are extremely resistant to adverse conditions and cause economic losses in many cultivated plants. It is known that among these species, only *Heterodera cruciferae* Franklin 1945 and *Heterodera schachtii* Schmidt 1871 can feed on cabbage plants. Cabbage infected with *H. cruciferae*, also known as the cabbage cyst nematode, usually shows wilting, chlorosis between the veins, or a reddish color on the leaves (Thorne, 1961). It is stated that the presence of 20 cysts/100 g of soil is sufficient to cause severe wilt in cabbage plants (McCann, 1981). Jensen (1972) and McCann (1981) indicated that *H. schachtii* and *H. cruciferae* generally occur together in cabbage production areas.

A few researchers have performed studies on cyst nematodes in cabbage in Türkiye, but sufficiently comprehensive studies on these issues are still needed. In a study conducted by Muşdağı & Gözel (2015) on cabbage in Türkiye in Çanakkale province, 76 soil samples were taken on 5 different cabbage varieties to determine the prevalence and density of cyst nematodes. As a result of the survey, they reported that *Heterodera avenae* Wollenweber, 1924 (7.9%), *H. cruciferae* (7.9%), and *H. schachtii* (2.7%) were among the cyst nematodes detected. In addition, Mennan & Aydınlı (2007) found that approximately 45% of cabbage cultivated areas in Samsun province were infected with *H. cruciferae*. In another study, Mennan et al. (2009) determined that 45 of 101 fields were infected with cyst nematodes in their surveys conducted in cabbage cultivation areas in Samsun between 2002 and 2006. They reported that the most common species were *H. cruciferae* (77.70%) and *Heterodera mediterranea* Inserra, Vovlas & Stone, 1981 (20.00%). In another study, Aydınlı (2009) aimed to reveal the effects of *H. cruciferae* on the development of cabbage plants in cabbage production areas in Samsun. As a result of the research on the factors affecting larval emergence from *H. cruciferae* cysts, it was reported that the optimum temperature for egg opening was 10ºC and leaf cabbage root secretions promoted egg opening. Aydınlı & Mennan (2012), found that sixty percent of *acephala* (Kale) varieties were partially susceptible, while 40% were resistant. The studies conducted generally include other cabbage varieties, and it seems that not enough studies have been conducted on kale. There are no studies on cyst nematode populations in the Eastern Black Sea region, where kale production is intense. For this reason, the study aimed to reveal the cyst nematode species and their distribution in the cabbage cultivation areas of Artvin, Giresun, Ordu, Rize and Trabzon provinces.

The first objective of this study is to detect cyst nematodes in kale production areas within the provinces of Artvin, Giresun, Ordu, Rize and Trabzon in the Eastern Black Sea region of Türkiye, based on morphological and molecular characteristics. Secondly, the study aims to reveal the distribution and population of the nematodes obtained in the region.

Materials and Methods

Description of study sites

The Black Sea region is located in the north of Türkiye. It covers 18% of the Turkish territory and extends east-west for 1.400 km resembling a strip. The Eastern Black Sea region, which is the most mountainous and receives the highest amount of rainfall (average annual 842.6 mm) among the regions of the Black Sea, is characterized by humidity levels. There are significant climatic differences between the coastal and inland areas, leading to variations in the types of crops cultivated. In the Eastern Black Sea region, the highest rainfall occurs in autumn, while the lowest rainfall is observed in spring. The average yearly temperature ranges from 13 to 15ºC. Due to its geographical location and mostly rainy days, the Black Sea region has the lowest sunshine time. The soil structure in the provinces of the region is generally fine textural class, acidic reaction, non-saline, low lime content, and sufficient organic matter content (Ay & Kızılkaya, 2021). The primary crop in the region, particularly in its eastern areas, is hazelnuts. In addition, black cabbage (kale), corn, kiwi, rice, beans, and potatoes are among the important agricultural products in the region. Among these, kale is a cold climate plant. It is resistant to drought and difficult production conditions and has a wide production area in the world. It has dark green and broad leaves surrounding the stem and veins. Its leaves contain chlorophyll pigment, beta carotene, ascorbic acid and calcium. It contains plenty of vitamins and minerals (Anonymous, 2024a). In this study, seventy-seven black cabbage production fields from five provinces were surveyed during the September-November of 2021-2022.

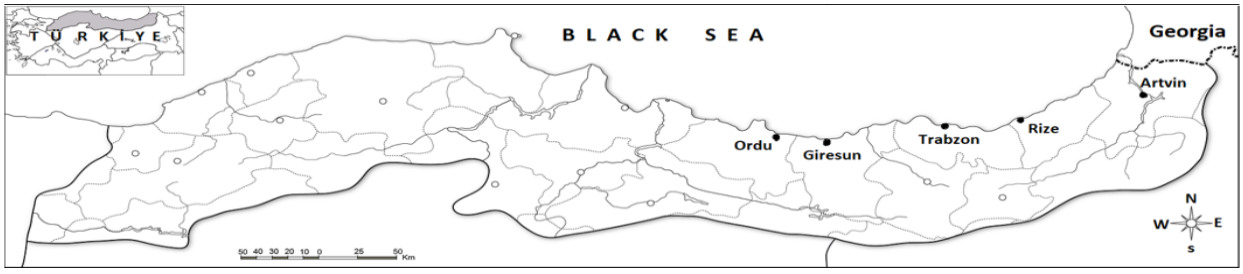

Figure 1. Map indicating location of sample locations within the five Eastern Black Sea region provinces (Anonymous, 2023a).

Soil sampling

During the September-November of 2021-2022, surveys were conducted in53 districts, including 5 from Artvin, 15 from Ordu, 10 from Giresun, 11 from Rize, and 12 from Trabzon provinces in the Eastern Black Sea region of Türkiye. Samples were taken from a total of 77 locations including 7, 17, 19, 15, 19 from Artvin, Giresun, Ordu, Rize, and Trabzon provinces, respectively (Table 1). Soil-root samples were collected from the rhizosphere of black cabbage plants to a depth of approximately 20 cm (Figure 7a). Sampling was taken to represent the field, according to the field size. Soil samples were taken using a hand shovel and were obtained by combining samples from 5 places within 1 da area in each field. The latitude and longitude of each sampling field were recorded using the global positioning system (GPS) (Table 1). All subsamples were mixed well and a sample of 1kg of soil and roots. The collected samples were immediately placed in labeled plastic bags and transported to the laboratory. The samples were kept in the refrigerator at +6ºC until examined.

Extraction of nematodes

Infective second stage juveniles (J_2) were extracted from the soil using the centrifugal flotation technique (Jenkins, 1964). Cysts were extracted from each soil sample using the sieving and flotation method (Shepherd, 1986). Cysts remaining on the 60-mesh sieve were collected with a brush using a stereomicroscope (Leica, S8APO) at 40x magnification on Whatman filter paper. A total of 17 cyst-forming nematode populations were collected from 77 samples. All cysts were preserved in laboratory conditions for molecular and morphological identification in this study.

Table 1. Locations and coordinates of surveys for the detection of cyst nematodes in kale plants in the Eastern Black Sea region in this study

		Districts	latitude	longitude			Districts	latitude	longitude
Ordu	1	Perşembe 1	41º02'22.6"N	37º41'41.3"E	Giresun	39	Tirebolu 1	40º58'28.2"N	38º45'31.7"E
	2	Perşembe 2	41º00'58.7"N	37º49'41.2"E		40	Tirebolu 2	40º57'25.6"N	38º47'42.0"E
	3	Kabataş	40º44'49.2"N	37º23'56.8"E		41	Tirebolu 3	40º57'27.0"N	38º48'18.0"E
	4	Çatalpınar	41º06'28.6"N	37º15'10.1"E		42	Tirebolu 4	40º57'15.5"N	38º48'54.0"E
	5	Kumru	40º53'00.5"N	37º16'50.5"E		43	Tirebolu 5	40º57'16.6"N	38º48'44.3"E
	6	Gürgentepe 1	40º46'46.0"N	37º36'37.7"E		44	Güce	40º54'50.4"N	38º47'29.4"E
	7	Gürgentepe 2	40º46'46.2"N	37º36'50.7"E		45	Dereli	40º44'16.7"N	38º27'21.8"E
	8	Ulubey	40º53'01.0"N	37º46'48.4"E		46	Eynesil 1	41º03'23.8"N	39º08'42.0"E
	9	Gölköy	40º41'36.5"N	37º36'33.2"E		47	Eynesil 2	41º02'23.5"N	39º09'03.0"E
	10	Çaybaşı	41º01'23.6"N	37º06'51.3"E		48	Görele	40º55'07.7"N	38º57'28.1"E
	11	Mesudiye	40º27'14.4"N	37º46'28.6"E		49	Görele 2	41º01'49.9"N	39º00'52.0"E
	12	Korgan	40º51'53.6"N	37º27'12.2"E		50	Keşap 1	40º54'57.8"N	38º31'15.3"E
	13	Altınordu 1	40º58'28.9"N	37º57'51.8"E		51	Keşap 2	40º53'51.5"N	38º31'30.7"E
	14	Altınordu 2	40º58'35.5"N	37º57'35.5"E		52	Çanakçı	40º55'48.4"N	39º01'15.6"E
	15	Gülyalı	40º58'03.4"N	38º03'04.7"E		53	Espiye	40º56'34.4"N	38º45'25.9"E
	16	Fatsa 1	40º54'21.2"N	37º31'28.6"E		54	Piraziz	40º57'00.0"N	38º09'06.5"E
	17	Fatsa 2	40º58'00.5"N	37º30'16.6"E		55	Bulancak	40º56'09.5"N	38º11'23.3"E
	18	Ünye	41º07'09.7"N	37º16'08.3"E	Rize	56	Çamlıhemşin	41º04'58.1"N	41º02'01.0"E
	19	Aybastı	40º42'22.6"N	37º24'43.1"E		57	Güneysu	40º59'46.0"N	40º35'52.4"E
Trabzon	20	Çarşıbaşı	41º05'32.6"N	39º23'33.7"E		58	Çayeli 1	41º03'18.0"N	40º37'10.6"E
	21	Arsin 1	40º57'12.2"N	39º54'27.7"E		59	Çayeli 2	41º03'49.0"N	40º43'02.3"E
	22	Arsin 2	40º57'13.1"N	39º55'39.6"E		60	Fındıklı	41º14'59.3"N	41º06'49.7"E
	23	Beşikdüzü	41º02'48.8"N	39º14'37.7"E		61	Pazar	41º10'15.6"N	40º50'08.9"E
	24	Yomra	40º57'16.9"N	39º52'16.3"E		62	Merkez 1	41º01'29.6"N	40º32'33.7"E
	25	Vakfıkebir 1	41º02'49.6"N	39º15'10.1"E		63	Merkez 2	41º01'50.9"N	40º33'32.4"E
	26	Vakfıkebir 2	41º00'23.0"N	39º19'59.5"E		64	Merkez 3	41º02'59.3"N	40º36'32.8"E
	27	Araklı	40º54'18.4"N	40º03'21.2"E		65	Derepazarı	41º01'15.6"N	40º25'20.6"E
	28	Sürmene 1	40º54'33.8"N	40º06'39.6"E		66	Kalkandere 1	40º57'06.1"N	40º25'21.4"E
	29	Sürmene 2	40º54'45.4"N	40º09'32.4"E		67	Kalkandere 2	40º56'00.6"N	40º26'07.8"E
	30	Hayrat	40º54'43.9"N	40º20'58.9"E		68	Hemşin	41º03'19.4"N	40º53'58.6"E
	31	Yomra 2	40º57'26.4"N	39º51'05.4"E		69	İyidere	40º59'20.4"N	40º20'00.2"E
	32	Of 1	40º55'35.4"N	40º13'40.8"E		70	Ardeşen	41º11'15.4"N	40º59'06.0"E
	33	Of 2	40º54'00.0"N	40º16'37.9"E	Artvin	71	Arhavi	41º21'02.5"N	41º18'00.0"E
	34	Of 3	40º49'39.7"N	40º15'55.1"E		72	Borçka	41º26'51.0"N	41º42'11.9"E
	35	Dernekpazarı	40º47'32.3"N	40º16'19.6"E		73	Hopa	41º23'31.2"N	41º25'37.2"E
	36	Akçaabat 1	41º05'29.4"N	39º28'48.0"E		74	Merkez 1	41º10'44.4"N	41º49'26.4"E
	37	Akçaabat 2	41º02'07.5"N	39º33'24.0"E		75	Merkez 2	41º10'56.5"N	41º49'43.2"E
	38	Çaykara	40º45'10.6"N	40º14'53.7"E		76	Kemalpaşa 1	41º29'33.4"N	41º32'02.8"E
						77	Kemalpaşa 2	41º28'34.8"N	41º32'21.7"E
	Total					**77**			

Morphological studies

For microscopical examination of morphological characters and using them in diagnosis, second stage juveniles (J_2), males and cysts were used. Nematodes transferred to a drop of pure water on a clean glass slide on the hot plate were killed in 4-6 seconds at 60ºC. The head structures, stylet and tail structures of the second instar larvae were examined. The morphological characters and preparing the microphotographs were performed using a light microscope (Carl Zeiss Axio) equipped with a ZEISS Axiocam 105 digital camera. The vulval cones region of cysts were examined on permanent slides including the main characters as vulval slit, underbridge, and fenestra structures. For the permanent preparations, the vulval cone regions of cyst were cut with 45% lactic acid and cleaned with a fine tip brush, then transferred into glycerin, and mounted on slides under a Leica S8APO stereo microscope (Taylor & Netscher, 1974; Hartman & Sasser, 1985).

Molecular analyses

DNA extraction

In this investigation, the genomic DNA extraction procedure adhered to the protocol elucidated by Pagan et al. (2015). Specifically, five second-stage nematode samples obtained from hatched eggs in the cysts were collected and transferred to 1.5 ml Eppendorf tubes., each containing 10 µl of extraction buffer (1M Tris, 0.1M EDTA, pH 8), composed of 10 mM Tris-HCl (pH 8.8), 1 mM EDTA, 0.1% Triton X-100 (v/v), and 20 mg/ml Proteinase K. Subsequently, the tubes were subjected to overnight storage at a temperature of −20ºC. Following this, each sample underwent grinding using a micropestle and was incubated at a temperature of 56ºC for a duration of 1 hour, followed by an additional incubation at 95ºC for 10 minutes. This extraction process yielded genomic DNA from the five specimens, which subsequently served as the template for the ensuing PCR reaction.

PCR amplification

Polymerase Chain Reaction (PCR) amplification of the Internal Transcribed Spacer (ITS1, 5.8S, ITS2) gene was undertaken utilizing the designated primers TW81 (5'-GTT TCC GTA GGT GAA CCT GC-3´) and AB28 (5´-ATA TGC TTA AGT TCA GCG GGT-3´) (Joyce et al., 1994). Additionally, the Cytochrome Oxidase I (cox1) region of mitochondrial DNA (mtDNA) was targeted with the species-specific primer set Car-F (5´-CTTTGGTTTAATTAGTTTAAGAG-3´) Car-R (5´-GAAAAATATCTAAACTAGCG-3´) for the purpose of *Heterodera carotae* Jones 1950 identification (Madani et al., 2018). The PCR reactions were executed in a final volume of 25 µl, comprising 8.5 µl of distilled water, 12.5 µl of DreamTaq Green Master mix (2X) (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 1.25 µl of each primer (10 pMol/µl), and 1.5 µl of DNA template. For the ITS primers, PCR was conducted using a thermal cycler (96-Well, Veriti™ Singapore), employing the following program: denaturation at 95ºC for 4 min, followed by 40 cycles of 30 s at 95ºC, 45 s at 56ºC, 2 min at 72ºC, with a final extension at 72ºC for 10 min. The thermocycling reactions for the species-specific primer set (Car-F/Car-R) were performed following the protocol recommended by Madani et al. (2018).

The amplification products were subsequently segregated through electrophoresis in a 1% TAE (Tris-acetate-EDTA) buffer, 1.5% agarose gel, under a voltage of 100 V for a duration of 28 minutes. Following electrophoresis, the products were treated with ethidium bromide staining, and subsequently visualized through UV illumination using ErBiyotek GEN-BOX imageER Fx, employing the methodology as described by Sambrook et al. (1989). For the purpose of sequence analysis, the PCR products were forwarded to the STAB VIDA company located in Portugal. Sequencing was conducted using an ABI 3730xl DNA Analyzer. The acquired sequences were BLASTed to ascertain sequence similarity with those archived within the National Center for Biotechnology Information (NCBI) database.

Results and Discussion

In this study conducted on cyst nematodes in the kale production areas of the Eastern Black Sea region in 2021-2022, 77 samples covering Artvin, Giresun, Ordu, Rize and Trabzon provinces were examined. As a result of the morphological characteristics and molecular analysis of the cyst nematode populations obtained from the surveyed areas, their species were determined. The cyst nematode species *Heterodera carotae* Jones, 1950, *H. cruciferae*, and *Heterodera fici* Kirjanova, 1954 belonging to the *Heterodera* genera were identified from the soil samples in the study. The consequence of this survey indicated that cyst nematode *H. cruciferae* was found to be the common species (Figure 2).

Figure 2. Map of soil sampling points and distribution of cyst nematode *Heterodera* spp. on the Eastern Black Sea region of Türkiye in this study (Placemarks are marked on google earth) (Anonymous, 2024b).

Survey studies

In the region, only 17 of the 77 sampling areas surveyed were found to be infected (22%) with cyst nematodes species. Soil samples collected from four provinces including Ordu, Giresun, Trabzon and Rize were found as contaminated with cyst nematodes species. However, cyst nematodes were not found in the soil samples taken from Artvin province. *Heterodera cruciferae, Heterodera carotae* and *Heterodera fici* were detected from the total infected soil samples at 16.9%, 3.9%, and 1.3% relative frequency, respectively. Of the 17 soils detected as infected, 13 (76.5%) were found to be infected with *H. cruciferae*, 3 (17.6%) with *H. carotae* and 1 (5.9%) with *H. fici*. The most common species was *H. cruciferae* present in all provinces except Artvin; The highest population density of *H. cruciferae* was detected in Altınordu district with 38 cysts/100 cm^3 soil and 18 J2s/100 cm^3 soil. *Heterodera carotae* was found in Ordu and Giresun provinces. The highest density was 48 cysts/100 cm^3 soil in Gülyalı district and 40 J2s/100 cm^3 soil in Fatsa district in Ordu (Table 2). *Heterodera fici* was found only in Ordu. It was only detected in Mesudiye district with 2 cysts/100 cm^3 soil and 8 J2s/100 cm^3 soil. In the study, as a result of the surveys conducted in Ordu province, 4 out of 19 soil samples taken from 15 districts were found to be infected (21.1%) with cyst nematodes. The detection of second stage juvenile and cysts from the soil was found only in 4 districts. In these districts of Ordu province, 29 J2s/ 100 cm^3 soil and 48 cysts/100 cm^3 soil were obtained and the highest population was found in Gülyalı district (Figure 3). Additionally, white females and brown cysts were found on kale root samples taken from Gülyalı (Figure 7 b,c). This was followed by Fatsa district with 20 infective puppies and 16 cysts/100 cm3 soil. In Altınordu district, 9 J2s and 19 cyst/100 cm^3 soil populations were detected. The minimum density was 2 J2s and 8 cysts/100 cm^3 soil populations in Mesudiye district. Cyst nematodes were not found in the soils taken from other 11 surveyed districts. In Giresun province, cyst nematode was found in 6 of 17 soil samples taken from 10 districts.

As a result of the evaluation, 35.3% of the soils taken from Giresun province where kale is grown were found to be contaminated with cyst nematode. The detection of second stage juveniles and cysts from the soil was found only in 5 districts. The highest population was found in Dereli district with 12 J2s and 16 cysts / 100 cm^3 soil, which was followed by Eynesil district with 7 J2s and 5 cysts / 100 cm^3 soil (Figure 3). In Keşap district, 2 J2s and 5 cysts were detected in 100 cm^3 soil. In Tirebolu district, 3 J2s and 3 cyst/ 100 cm^3 soil were detected. The lowest density was found in Piraziz district, where only 2 cysts /100 cm^3 soil were detected. Cyst nematodes were not found in the soils taken from other 6 surveyed districts. In Trabzon Province, cyst nematode was found in 5 of 19 soil samples taken from 13 districts. As a result of the evaluation, 26.3% of the black cabbage grown soils from Trabzon province were found to be contaminated with cyst nematode. The detection of second stage larvae and cysts from the soil was found only in 4 districts. In these districts of Trabzon province, the highest population was found in Arsin district with 10 J2s and 6 cysts per 100 cm^3 soil. Arsin district was followed by Akçaabat district and 9 J2s and 4 cysts/100 cm^3 soil were found (Figure 3). In Vakfıkebir district, 4 J2s and 6 cysts were detected in 100 cm^3 soil. The lowest density was detected in Yomra district, where only 2 cysts populations were detected in 100 cm^3 soil. Cyst nematodes were not found in the soils taken from other 9 surveyed districts. In Rize province, cyst nematode was found in 2 of 15 soil samples taken from 10 districts. As a result of the evaluation, 13.3% of the black cabbage cultivated soils taken from Rize province were found to be contaminated with cyst nematode. The detection of second stage juvenile and cysts from the soil was found only in 2 districts. In these districts of Rize province, 1 infective juvenile and 5 cysts were obtained in 100 cm^3 soil and the highest population was found in Çamlıhemşin district. The lowest density was detected in Ardeşen district with only 3 cysts per 100 cm^3 soil (Figure 3). Cyst nematodes were not found in the soils taken from other 8 surveyed districts.

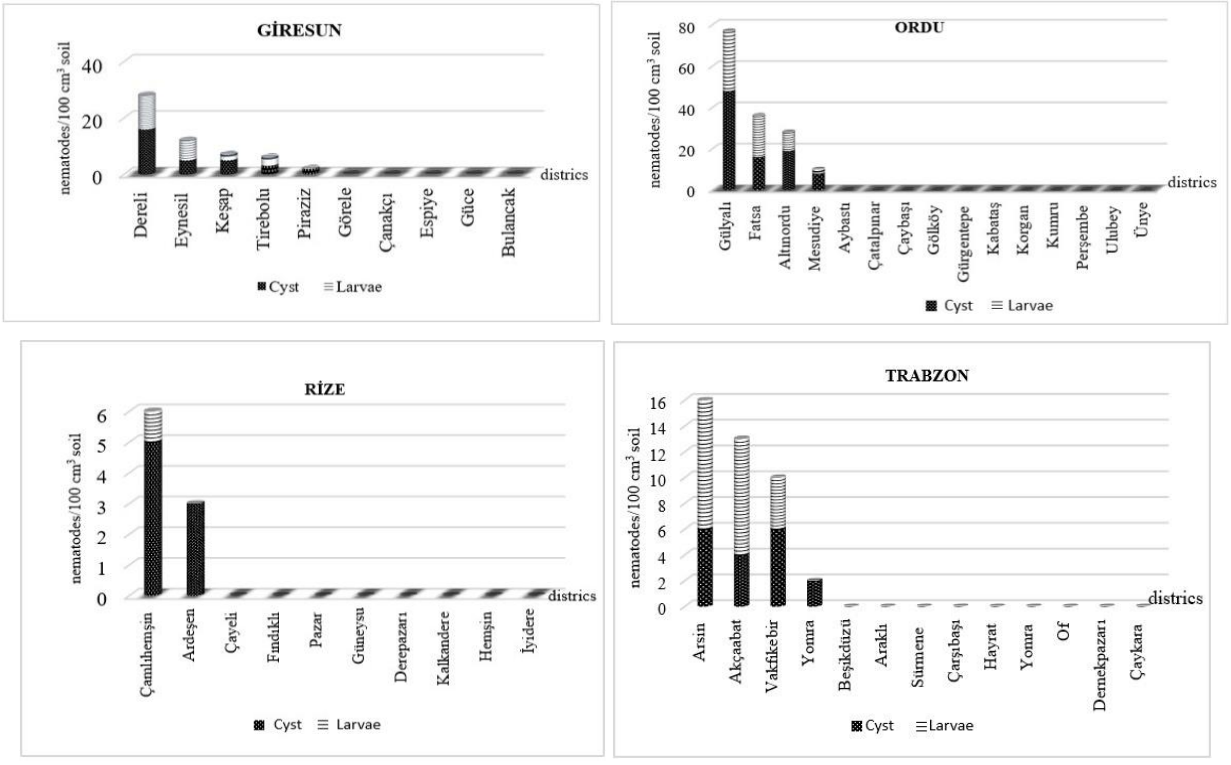

Figure 3. Population abundance of *Heterodera* spp. cysts and larvae in 100 cm^3 soil in districts of Giresun, Ordu, Rize and Trabzon, provinces in this study.

Table 2. Detected cyst nematode species and their abundance and incidence of the cyst and infective juveniles in kale production areas in Artvin, Giresun, Ordu, Rize, and Trabzon provinces in the Eastern Black Sea region

Provinces	Districts	Number of positive samples	Cysts/100 cm^3 soil	Infective Juvenile /100 cm^3 soil	Incidence (%)	Species
			Abundance			
Ordu	Altınordu	1	38	18	50	H. *cruciferae*
	Fatsa	1	31	40	50	H. *carotae*
	Gülyalı	1	48	29	100	H. *carotae*
	Mesudiye	1	8	2	100	H. *fici*
Giresun	Tirebolu-5	1	18	18	16.6	H. *cruciferae*
	Dereli	1	16	12	100	H. *cruciferae*
	Keşap-2	1	9	3	50	H. *cruciferae*
	Eynesil-1	1	4	13	100	H. *carotae*
	Eynesil-2	1	5	2	100	H. *cruciferae*
	Piraziz	1	2	0	100	H. *cruciferae*
Trabzon	Arsin	1	6	10	50	*H. cruciferae*
	Yomra	1	2	0	100	*H. cruciferae*
	Vakfıkebir-1	1	8	5	100	*H. cruciferae*
	Vakfıkebir-2	1	3	3	100	*H. cruciferae*
	Akçaabat	1	7	18	50	*H. cruciferae*
Rize	Çamlıhemşin	1	5	1	100	*H. cruciferae*
	Ardeşen	1	3	0	100	*H. cruciferae*
Artvin	Arhavi	1	0	0	0	Not found
	Borçka	1	0	0	0	Not found
	Hopa	1	0	0	0	Not found
	Merkez 1	1	0	0	0	Not found
	Merkez 2	1	0	0	0	Not found
	Kemalpaşa 1	1	0	0	0	Not found
	Kemalpaşa 2	1	0	0	0	Not found

In Artvin province, no cyst nematode was found in any of the 7 soil samples taken from 5 districts. Considering the districts of the other provinces where cyst nematodes were found in the study, it is noteworthy that they are districts located on the coastline, but cyst nematodes are not found in high-altitude districts. As a result of the study, it was determined that the soils taken from Artvin province where kale is grown are not found with cyst nematodes. The absence of cyst nematode in the soils of this province, even though it is a host, highlights the effect of soil conditions. Several studies have established a correlation between nematode population densities and environmental conditions, particularly variations in soil properties. Chowdhury et al. (2020) stated that soil properties like soil texture, pH, and organic matter are considered the main variables of the nematode. Similarly, one of the most influential environmental factors affecting nematode development is soil temperature. It is also, key factors such as soil texture, moisture levels, and temperature have been identified as important in influencing the presence of plant parasitic nematodes (Wallace, 1959; Schmidt et al., 1993; Avendaño et al., 2004). Fenwick (1951) reported that environmental factors such as soil temperature influence the number of eggs and larvae in cysts of some species. Abd-Elgawad (2021) stated that soil organic matters have presented as an important suppressor of plant-parasitic nematodes. Hbirkou et al. (2011) stated that soil texture has an indirect effect on the living conditions of nematodes. In the light of these explanations, the reasons why cyst nematodes are not encountered in kale fields in Artvin province can be listed.

Morphological characters

The morphological details of cysts nematodes obtained from black cabbage fields in Türkiye were observed using second stage juveniles, males and cysts. The morphological characteristics of cyst nematode stages were examined using light microscope in this study (Figure 4).

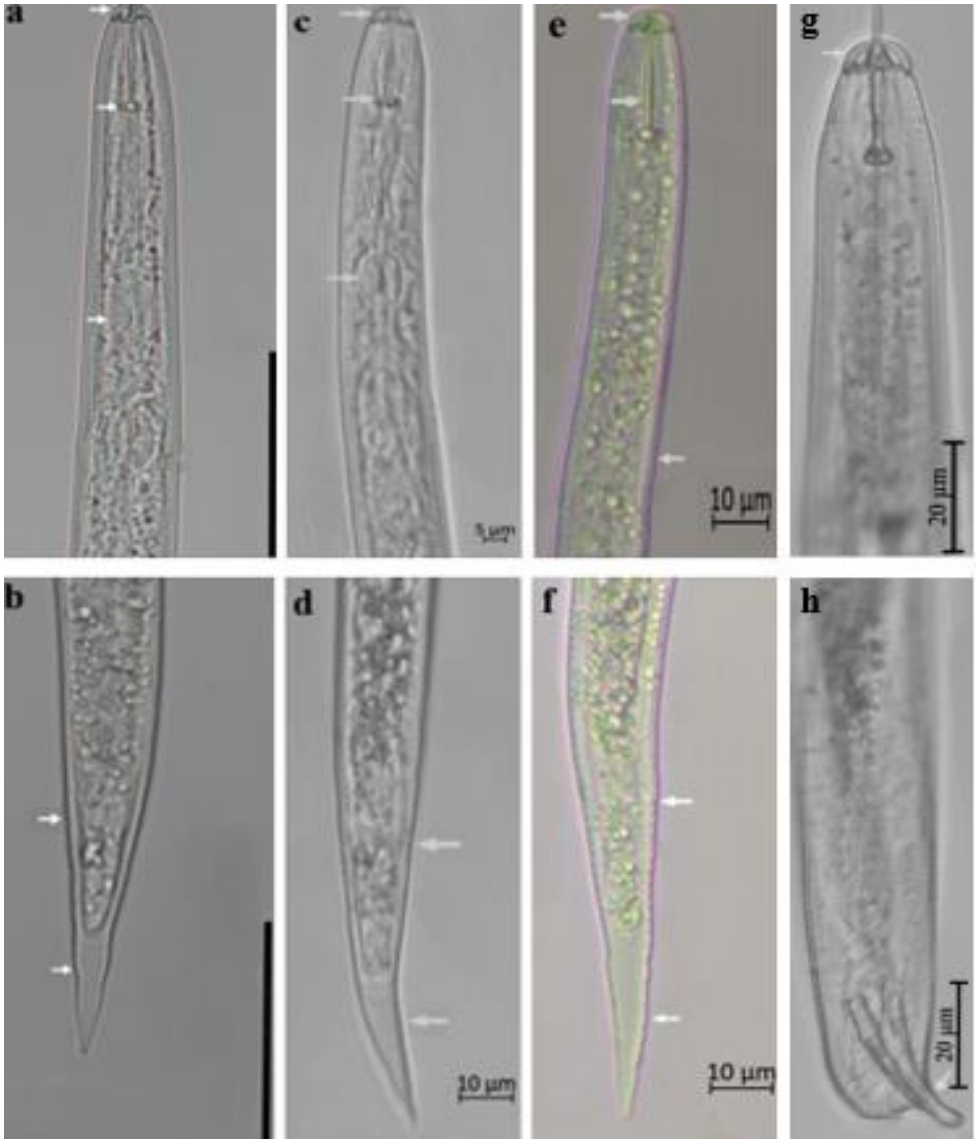

Figure 4. Photomicrographs of second stage juvenile structure of: a, b) *Heterodera carotae,* c, d) *Heterodera cruciferae,* e, f) *Heterodera fici* and male of g, h) *Heterodera carotae* isolated from kale production areas in Eastern Black Sea region of Türkiye. a, c, e) anterior regions showing head, stylet, and median bulb, b, d, f) posterior regions showing tail, anus, hyaline portion, g) anterior region showing head framework and stylet of *H. carotae* male, h) posterior region showing spicula and tail.

Heterodera carotae: Second stage juvenile body structure was vermiform. The head is slightly offset, and cephalic framework is well developed and heavily sclerotized (Figure 4a). The stylet is remarkably robust, with round stylet knobs (Figure 4a). The median bulb is oval, featuring a distinct valve. The pharyngeal glands are elongated, tapering posteriorly, and overlapping the intestine ventrally (Figure 4a). The tail is conical and has a prominent terminal hyaline part (Figure 4b). Male body is vermiform, the head is offset, and cephalic framework is robust (Figure 4g). The stylet is strong, characterized by well-developed knobs. The spicules are arcuate, the gubernaculum is slightly curved (Figure 4h). The tail short. Cysts lemon-shaped with distinct neck and color changes from white to russet brown (Figure 5a). Vulval bridge broken in some specimens (Figure 6a). Bullae absent. Underbridge poorly developed, vulval slit long. *H. carotae* is most closely related to *H. crucifera*. It has been identified as a belonging to the *Goettingiana* group. It differs from *H. cruciferae* by a longer average hyaline part of tail region in J2 and a longer average vulval slit in cysts (Subbotin et al., 2010).

Heterodera cruciferae: Second-stage juveniles body vermiform, head rounded. Cephalic framework strongly developed (Figure 4c). Stylet well developed and stylet knobs rounded (Figure 4c). Cysts slightly lemon shaped, lighyt to dark brown (Figure 5b). Body has zigzag cuticular surface pattern. The vulva semifenestrate ambifenestrate without bullae (Figure 6b). Underbridge a very weak. Male not found. *Heterodera cruciferae* is placed in the Goettingiana group (Handoo & Subbotin, 2018) and has been detected in various regions of Türkiye.

Heterodera fici: Second-stage juveniles' body vermiform, head slightly set off, rounded. Cephalic framework moderate, stylet well developed, basal knobs rounded in second stage larvae (Figure 4e). Esophageal lobe overlaps anterior part of intestine. Hyaline terminal about 1/2 tail length (Figure 4f). Cysts lemon shaped (Figure 5c), the fenestrae in some cysts are small and appearing biffenestrate. Ballue small and dome-shaped scattered about the level of underbridge. Underbridge weakly developed (Figure 6c), with furcate ends. Vulval slit about same length as bridge (Figure 6d). Male not found. Golden et al. (1988) stated that *H. fici* properly belongs in the "schachtii group" of species. *H. fici* is most closely related to *H. schachtii*, *Heterodera glycies* Ichiohe, 1952, and *Heterodera cajani*, 1967. It differs from these species by the presence of a weakly developed underbridge and small, scattered bullae (Golden et al.,1988).

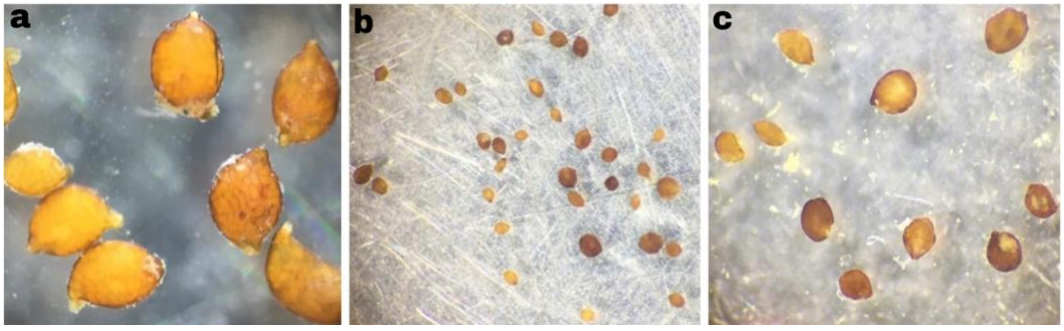

Figure 5. Photomicrograph of cyst forming females of *Heterodera* spp. extracted from kale production areas in Eastern Black Sea region of Türkiye: a) *Heterodera carotae,* b) *Heterodera cruciferae,* c) *Heterodera fici*.

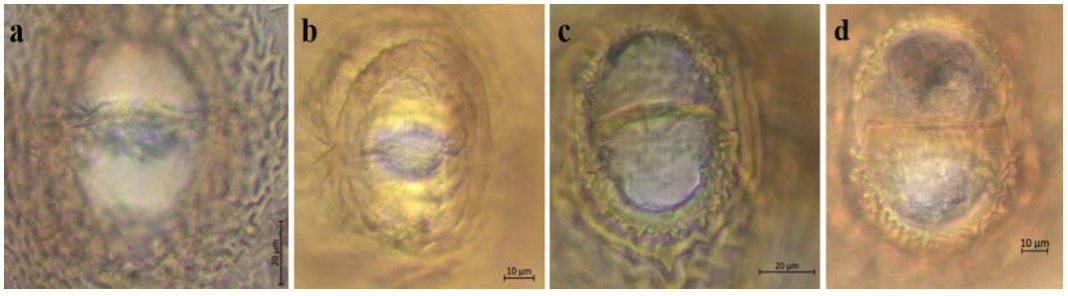

Figure 6. Photomicrograph of perineal pattern structure of *Heterodera* spp. isolated from kale production areas in Eastern Black Sea region of Türkiye: a) *Heterodera carotae,* b) *H*eterodera *cruciferae* and c, d) *Heterodera fici.*

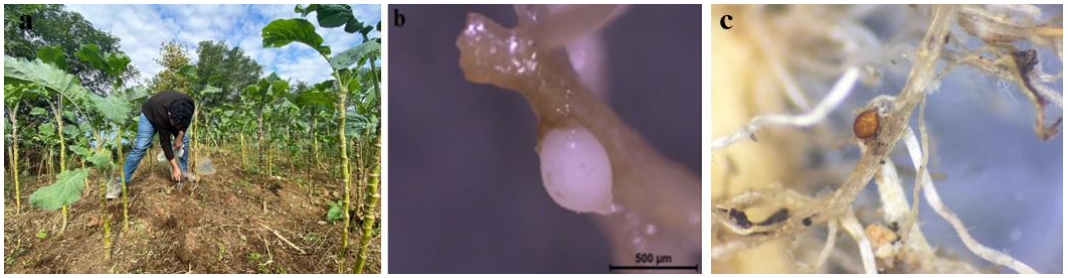

Figure 7. Photomicrograph of *Heterodera carotae*: a) soil sampling rhizosphere of kale, b) white females on kale (black cabbage) roots, c) brown cyst on the kale roots.

Molecular analyses

Genomic DNAs of the populations obtained in this study were amplified by PCR and then visualized by gel electrophoresis. Internal transcribed spacer (ITS) gene expansion segments produced a single 1020 bp fragment for *all* three species (Figure 8 a). The amplification of the expansion segments utilizing the specific primer Car-F/Car-R for *H. carotae* yielded a fragment measuring 350 base pairs, as determined through gel electrophoresis analysis (Figure 8 b). The sequences of the ribosomal region spanning the ITS gene obtained from PCR products of *Heterodera* populations in this study were compared with those present in the GenBank database using BLAST revealed a high similarity. The sequence results obtained were found to be similar to *H. carotae* (e.g., GenBank accession nos. MG976790.1), *H. cruciferae* (e.g., GenBank accession nos. MG848393.1) and *H. fici* (e.g., GenBank accession nos. KY635987.1) with a similarity rate of over 99% in the similarity query in the NCBI gene bank.

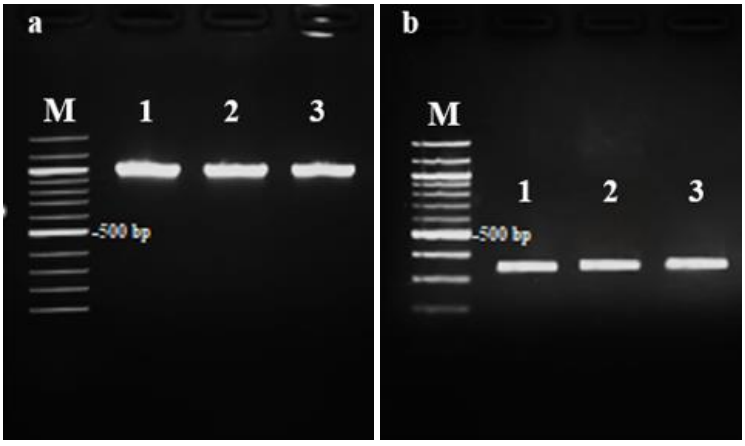

Figure 8. PCR products of *Heterodera carotae, H. cruciferae* and *H. fici* species. a: Fragments of internal-transcribed spacer (ITS) (ITS1-5.8S) region of rRNA using TW81/AB28 primer pair (Line1-4); b: Fragments of cytochrome oxidase I of mitochondrial DNA (coxI) using Car F/Car R primer pair for H. *carotae* (Line1-3); M, 100 bp DNA marker ladder.

Discussion

Molecular approaches are increasingly used in nematode diagnosis as they provide accurate diagnosis. For this reason, ribosomal DNA has become the preferred option for nematode diagnosis. Ribosomal ITS regions of nematodes are highly variable and consequently useful for diagnosis (Subbotin et al. 2011). Most *Heterodera* species identified to date have been identified using morphological and molecular data, particularly based on the rDNA-ITS region (ITS + 5.8 S + ITS2). Accurate identification of plant parasitic nematode species that cause plant yield losses is important for effective control against them. This research aims to conduct a comprehensive review focusing on the taxonomic identification of *Heterodera* species cultivating kale. During this research study, it was determined that kale cultivation areas harbor a community of *Heterodera* species such as *H. carotae, H. cruciferae* and *H. fici*. The scope of nematode parasitism on kale covers a spectrum of three *Heterodera* species. The most prominent among these is *H. cruciferae*, which occurs in twelve separate locations, followed by *H. carotae*, which occurs in four different locations. In contrast, the relatively rare *H. fici* was detected in a single location.

Heterodera carotae, a member of the Heteroderidae family, was originally described by Jones (1950). The present study identifies *H. carotae* in kale plants, indicating a novel host association. This discovery underscores the adaptability of *H. carotae* to kale. Notably, this nematode exhibits a restricted host range, primarily impacting carrots, *Daucus carota* (L.) (Apiales: Apiaceae) and *Daucus pulcherrimus (*Willd.) W. D. Koch ex DC.1830 (Apiales: Apiaceae)). Its detrimental effects on plants include uneven growth, yellowing leaves, chlorosis, stunted growth, wilting, taproot rot, and premature lignification, rendering affected carrots

unfit for market (Anonymous, 2023b). In the context of carrot production in Italy, *H. carotae* is responsible for considerable yield losses, ranging from 20% to 90% (Greco et al., 1993). While Jones (1950) initially documented *H. carotae* in carrot soils in Spain, subsequent reports have expanded its host range to include *Daucus carota* (Yu et al., 2017), *Daucus pulcherrimus* (Goodey et al., 1965), *Torilis arvensis (*Huds.) Link, 1821 *(*Apiales: *Apiaceae*) and *Torilis leptophylla* (L.) Reichb. (Apiales: Apiaceae) (Escobar-Avila et al., 2018). Beyond Spain, *H. carotae* has been reported in various regions, encompassing Europe, India, Cyprus, South Africa, North America, and Mexico, with documented occurrences on carrots by Berney & Bird (1992), Subbotin et al. (2010), Escobar-Avila et al. (2018), and Shubane et al. (2021). Taxonomically, *H. carotae* is classified within the Goettingiana group. Its distinctions from *H. cruciferae* include a longer average hyaline region in the tail of J2 and a lengthier average vulval slit in cysts (Subbotin et al., 2010).

Heterodera cruciferae represents a distinct species within the taxonomic confines of the Heteroderidae family, an attribution initially proposed by Franklin in 1945, as chronicled by Winslow in 1955. The first recorded instance of identifying *H. cruciferae* on cabbage dates back to the year 1963, within the locale of Erzurum in Türkiye (Yüksel, 1973). In the context of the broader *Heterodera* genus, *H. cruciferae* stands apart for its comparatively circumscribed geographic distribution, which has been documented across diverse global locales. The species' occurrence spans multiple regions, encompassing Europe, the United States most notably California-Australia, Iran, and Azerbaijan (Franklin, 1945; Stone & Rowe, 1976; Sturhan & Lišková, 2004; Jabbari & Niknam, 2008; Chizhov et al., 2009; Mennan & Handoo, 2012). Regarded as a prominent taxon within the realm of *Heterodera*, *H. cruciferae* assumes a position of economic salience due to its capacity to impose substantial agrarian detriment, with a pronounced predilection for cruciferous crops, notably cabbage and Brussels sprouts (Ravichandra, 2014; Mennan & Handoo, 2012). The ecological imprint of this species reverberates across an array of crops, embracing cabbage, broccoli, cauliflower, radish, turnip, pea, and rapeseed. Empirical findings by Turner and Subbotin underscore that *H. cruciferae*'s maturation trajectory culminates within 30 days at a temperature of 20ºC, facilitating the succession of up to three discrete generations. Moreover, its ubiquity persists seamlessly throughout the seasonal panorama in the European milieu (Turner & Subbotin, 2013).

Toktay et al. (2022) used ribosomal DNA region (rDNA-ITS) and cytochrome oxidase subunit 1 (mtDNA-COI) sequences to identify cyst nematodes in cabbage production areas in Niğde province with molecular methods. For the first time in Türkiye, *H. cruciferae* were used for identification. Jabbari and Niknam (2008) investigated plant parasitic nematode biodiversity in vegetable fields in Tabriz city of East Azerbaijan province of Iran between 2004 and 2005. They identified 25 species of 16 nematode genera from 88 soil and root samples, including a large population of cyst nematodes, *H. cruciferae*, in most of the sampling sites. During a nematological survey conducted in Russia, *H. cruciferae* was detected in cabbage-growing areas along the Oka River, Ozery and Serpukhov regions in the Moscow region of Russia. They recorded the first report of this nematode in the Moscow region. Rapeseed, rutabaga and radish have been identified as additional host plants for this nematode (Chizhov et al., 2009).

Heterodera fici, a constituent of the Heteroderidae family, was originally characterized by Kirjanova (1954). Fig cyst nematode, *H. fici*, was first described by Kirjanova in 1954 from rubber plant (*Ficus elastica* Roxb. Ex Hernem (Rosales: Moraceae) roots in Harbin, People's Republic of China (Kirjanova, 1954). A study conducted in the Aegean region of Türkiye reported for the first time that *H. fici* parasitized *Ficus carica* (L.) (Rosales: Moraceae) and *F. domestica* (Yuksel, 1981). Later, Mulvey and Golden identified this cyst nematode from California, Florida and Virginia in the United States; They summarized its known spread from Brazil, Australia, Germany, Italy, Poland, South Africa, Spain, Türkiye, USSR and Yugoslavia. During a study conducted in 1986 in an orchard in Saryab, Quetta, Pakistan, it was reported that *H. fici* was heavily parasitized on the roots of fig (*F. carica*) plants, and these plants showed signs of growth retardation and yellowing of the leaves (Mulvey, 1972; Mulvey et al., 1983). *Heterodera fici* is a harmful species on fig plants and heavy infestation has been reported to cause growth retardation and yellowing of leaves (Maqbool et al., 1987).

Di Vito & Sasanelli (1990) investigated the emergence of offspring and cysts of *H. fici* in a growth chamber at 24ºC for a period of 7 weeks in 2% natural and artificial incubation materials. Cysts were collected from commercial fig roots and incubated in batches of 100 each in ornamental or commercial fig roots, picrolonic acid, sodium metavanadate, zinc chloride, zinc sulfate or distilled water. They reported that more juvenile cysts appeared in commercial fig root juice (97%) compared to ornamental fig root juice (45%). They reported that the yield in sodium metavanadate was 64%, in zinc chloride 40% and in zinc sulfate 27%, and in picrolonic acid the yield was very low (5%).

Conclusion

The objectives of this study are to understand the yield losses caused by the *Heterodera* genus in the Eastern Black Sea region and to focus on reducing these losses, especially in kale cultivation. Consequently, there is an imperative for further investigations to formulate effective strategies aimed at the control of *Heterodera* species, ultimately augmenting yield in cabbage fields. It is crucial to underscore that ongoing and future research endeavors directed towards the *Heterodera* genus remain imperative for the prevention of yield losses specifically in black cabbage cultivation.

Acknowledgments

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