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DNA barcoding, phylogeny and phylogeography of the cyst nematode species of the *Schachtii* group from the genus *Heterodera* (Tylenchida: Heteroderidae)

Sergei A. SUBBOTIN [1,2], Tatiana V. ROUBTSOVA [3], Richard M. BOSTOCK [3], Zahra TANHA MAAFI [4], Vladimir N. CHIZHOV [2], Juan E. PALOMARES-RIUS [5] and Pablo CASTILLO [5,*]

[1] *California Department of Food and Agriculture, Plant Pest Diagnostic Center, 3294 Meadowview Road, 95832 Sacramento, CA, USA*
[2] *Center of Parasitology of A.N. Severtsov Institute of Ecology and Evolution of the Russian Academy of Sciences, Leninskii Prospect 33, 117071, Moscow, Russia*
[3] *Department of Plant Pathology, University of California, One Shields Ave., Davis, CA 95616, USA*
[4] *Iranian Research Institute of Plant Protection, Agricultural Research Education and Extension Organisation (AREEO), Tehran, Iran*
[5] *Instituto de Agricultura Sostenible (IAS), Consejo Superior de Investigaciones Científicas (CSIC), Campus de Excelencia Internacional Agroalimentario, ceiA3, Apdo. 4084, 14080 Córdoba, Spain*
ORCID iDs: Subbotin: 0000-0001-6648-5889; Tanha Maafi: 0000-0002-9417-0072; Palomares-Rius: 0000-0003-1776-8131; Castillo: 0000-0003-0256-876X

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Summary – Cyst-forming nematodes of the genus *Heterodera* are highly derived and economically important plant parasites. The *Schachtii* group of this genus is one of the largest ones with a total of 18 species parasitising dicotyledons. In this study, we provided comprehensive phylogenetic analyses of several hundred *COI* and ITS rRNA gene sequences of selected species from the *Schachtii* group, including *H. betae*, *H. cajani*, *H. ciceri*, *H. galeopsidis*, *H. glycines*, *H. medicaginis*, *H. mediterranea*, *H. schachtii*, *H. sonchophila* and *H. trifolii*, using Bayesian inference, maximum likelihood, and statistical parsimony. One hundred and twenty four new *COI*, 57 ITS rRNA and eight *hsp90* gene sequences from 81 nematode populations collected in 19 countries were obtained in this study. Our study showed that the ITS rRNA gene has limited discrimination power compared to the *COI* gene. However, our analysis also revealed that partial *COI* gene sequences were identical for *H. trifolii*, *H. betae* and *H. galeopsidis*. Based on the results of phylogeographical analysis and age estimation of clades with a molecular clock approach, it was hypothesised that the majority of the *Schachtii* group species originated and diversified in the Mediterranean Basin biodiversity hotspot during the Pleistocene and then dispersed from this region across the world. The Sino-Japanese Floristic Region is likely one of the centres of diversification for the soybean cyst nematode, which showed distinct population structure. The possible role of hybridisation and polyploidisation in the evolution of species of the *Schachtii* group is discussed.

Keywords – biogeography, *Heterodera betae*, *Heterodera glycines*, *Heterodera medicaginis*, *Heterodera schachtii*, Mediterranean Basin biodiversity hotspot, molecular clock, Sino-Japanese Floristic Region.

Cyst-forming nematodes of the genus *Heterodera* are highly derived and economically important plant parasites. Using morphological and molecular characteristics, the species of this genus have been divided into nine groups, namely: *Afenestrata*, *Avenae*, *Bifenestra*, *Cardiolata*, *Cyperi*, *Goettingiana*, *Humuli*, *Sacchari* and *Schachtii* (Subbotin *et al*., 2010; Handoo & Subbotin, 2018). The *Schachtii* group is one of the largest and

* Corresponding author, e-mail: p.castillo@csic.es

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contains species that parasitise dicotyledons. Presently, the *schachtii* group consists of 18 species: *Heterodera agrostis* Kazachenko, 1993, *H. betae* Wouts, Rumpenhorst & Sturhan, 2001, *H. cajani* Koshy, 1967, *H. ciceri* Vovlas, Greco & Di Vito, 1985, *H. daverti* Wouts & Sturhan, 1978, *H. dunensis* Singh, Karssen, Couvreur & Bert, 2020, *H. galeopsidis* Goffart, 1936, *H. glycines* Ichinohe, 1952, *H. lespedezae* Golden & Cobb, 1963, *H. medicaginis* Kirjanova *in* Kirjanova & Krall, 1971, *H. mediterranea* Vovlas, Inserra & Stone, 1981, *H. menthae* Kirjanova & Narbaev, 1977, *H. rosii* Duggan & Brennan, 1966, *H. schachtii* A. Schmidt, 1871, *H. sonchophila* Kirjanova, Krall & Krall, 1976, *H. spiraeae* Kazachenko, 1993, *H. swarupi* Sharma, Siddiqi, Rahaman, Ali & Ansari, 1998 and *H. trifolii* Goffart, 1932. The soybean cyst nematode, *H. glycines*, the sugar beet cyst nematode, *H. schachtii*, the yellow beet cyst nematode, *H. betae*, the chickpea cyst nematode, *H. ciceri*, the lucerne cyst nematode, *H. medicaginis*, the clover cyst nematode, *H. trifolii* and the pigeon pea cyst nematode, *H. cajani* are considered as economically important nematode pests for agriculture in some countries. Species of the *Schachtii* group are characterised by a lemon-shaped cyst having ambifenestrate cone, well-developed bullae, strong underbridge and long vulval slit and differentiated from each other in morphometrics of the second-stage juveniles and cyst structures (Subbotin *et al*., 2010; Singh *et al*., 2020).

Traditional identification of cyst nematode species is based on morphological and morphometric characteristics of cysts and second-stage juveniles, requires expert knowledge and is time consuming. During the last decades, the introduction of molecular methods has allowed the design of reliable and rapid diagnostic tools for cyst nematodes. The ITS rRNA gene sequences are used to discriminate species including some representatives of the *Schachtii* group (Subbotin *et al*., 2010). Ferris *et al*. (1993) and Subbotin *et al*. (2001) compared the ITS rRNA gene sequences from several species of the *Schachtii* group and surprisingly few differences were found between them. Moreover, intensive PCR-ITS-RFLP and ITS rRNA sequence studies of *H. schachtii*, *H. betae* and *H. trifolii* populations revealed substantial heterogeneity of the rRNA clusters (Subbotin *et al*., 2000; Wouts *et al*., 2001; Amiri *et al*., 2002).

Mitochondrial DNA analysis has proved to be a powerful tool for DNA barcoding diagnostics, assessing intraspecific genetic patterns and phylogeography in plant-parasitic nematodes. These approaches have been explored for the *Afenestrata*-, *Avenae*-, *Bifenestra*-, *Cardiolata*-, *Humuli*- and *Sacchari*-groups of the genus *Heterodera* (Subbotin *et al*., 2018, 2021, 2022). Although some species of the *Schachtii* group have been characterised using *COI* gene analysis (Vovlas *et al*., 2015; Sekimoto *et al*., 2017; Mwamula *et al*., 2018; Powers *et al*., 2019; Singh *et al*., 2020), there is no comprehensive study on DNA barcoding and phytogeography of these nematodes.

The main goals of our study were: *i*) to analyse phylogenetic relationships within selected species of the *Schachtii* group species using sequences of ITS rRNA, partial *COI* and *hsp90* genes; *ii*) to provide molecular characterisation of selected species and populations of the *Schachtii* group using sequences of the partial *COI* gene; and *iii*) to propose and test the hypotheses of the origin and distribution of the *Schachtii* group species.

Materials and methods

Nematode species and populations

Species and populations collected from different hosts, localities and countries used in this study are given in Table 1. Ten valid species and three putatively new species belonging to the *Schachtii* group were obtained for this study. A total of 81 nematode populations collected in 19 countries was analysed. Cysts were extracted from soil samples using standard flotation and sieving techniques. Species identification was made using morphological and molecular methods (Subbotin *et al*., 2010). Species delimitation of the studied populations was accomplished by integrating the results of morphological and morphometrical studies, phylogenetic and sequence analyses, as well as by analysis of nematode host-plant specificity and geographic distribution of studied samples (Subbotin *et al*., 2010).

Distribution maps

Several published and original sources were used to generate distribution maps for *H. schachtii* (Franklin, 1972; Narbaev *et al*., 1974; Lamberti & Taylor, 1986; Müller, 1999; CABI/EPPO, 2001; Amiri *et al*., 2003; Subbotin *et al*., 2010; Gracianne *et al*., 2014; Cui *et al*., 2016; Haidar *et al*., 2016; Kim *et al*., 2016; Pylypenko *et al*., 2016; Ibrahim *et al*., 2017; Shesteperov *et al*., 2017; Haque & Khan, 2021; Hussain *et al*., 2021; Peng *et al*., 2022; Wu *et al*., 2022), *H. medicaginis* (Baidulova, 1981; Subbotin *et al*., 2010) and *H. sonchophila* (Kirjanova *et al*., 1976; Subbotin *et al*., 2010).

Table 1. Species and populations of cyst nematodes of the *Schachtii* group of the genus *Heterodera* used in the present study.

Species	Location	Associated host	Sample code	*COI* haplotype	*COI* GenBank accession no.	ITS rRNA GenBank accession no.	Source and/or reference
H. betae	South Korea	*Beta vulgaris*	CD3067	Hb1	MW345308	–	J.-Y. Chun
H. betae	Germany	*B. vulgaris*	CD1462, 342	Hb1	MW345307, MW345314	MW346566	S. Amiri
H. betae	Germany, Münster	*B. vulgaris*	424	–	–	MW346570, MW346572, MW346574, MW346575	D. Sturhan
H. betae	Morocco, Berkane	*B. vulgaris*	Morocco	–	–	MW346567, MW346571	S. Amiri
H. cajani	India	*Cajanus cajan*	CD2945	Hcaj1	MW345412, MW345413	AF274389	J. Rowe; Subbotin *et al.* (2001)
H. ciceri	Syria	*Cicer* sp.	429	Hcic2	MW345320	AF274393, MW346578, MW346579	N. Vovlas; Subbotin *et al.* (2001)
H. galeopsidis	Iran, Tehran, Nivaran Park	Unknown	Zah21-1	Hgal1	MW345310	AF498390	Z. Tanha Maafi; Tanha Maafi *et al.* (2003)
H. galeopsidis	Germany	Unknown	541	–	–	MW346581, MW346582	D. Sturhan
H. glycines	USA, Iowa, Muscatine County	*Glycine max*	CD3068a, b	Hg1	MW345329, MW345336	–	G. Tylka
H. glycines	USA, Georgia, Burke County, Waynesboro (race 9)	*G. max*	CD3070	Hg1	MW345335	–	S. Finnery, G. Tylka
H. glycines	USA, Missouri, New Madrid and Pemiscot counties, Portageville (race 5)	*G. max*	CD3071	Hg1	MW345334	–	S. Finnery, G. Tylka
H. glycines	USA, Arkansas, sample 1	*G. max*	CD1474	Hg1	MW345332	–	R. Robbins
H. glycines	USA, Illinois	*G. max*	CD1554	Hg1	MW345331	–	T. Mengistu
H. glycines	Brazil	*G. max*	CD3065b	Hg1	MW345333	–	M. Doucet
H. glycines	South Korea	*G. max*	CD3066	Hg3	MW345322	–	J.-Y. Chun
H. glycines	USA, North Carolina, sample 1	*G. max*	CD3078	Hg4	MW345327	–	W. Ye
H. glycines	USA, Minnesota	*G. max*	CD1064	Hg4	MW345328	–	D. Mollov
H. glycines	USA, North Carolina, sample 2	*G. max*	CD1450	Hg6	MW345324	–	W. Ye

Table 1. (Continued.)

Species	Location	Associated host	Sample code	*COI* haplotype	*COI* GenBank accession no.	ITS rRNA GenBank accession no.	Source and/or reference
H. glycines	USA, North Carolina, sample 3	*G. max*	CD1430	Hg6	MW345325	–	W. Ye
H. glycines	Russia, Amur oblast, Blagoveshchensk	*G. max*	431a, b	Hg8, Hg9	MW345323, MW345326	–	A.S. Eroshenko
H. glycines	China, northern region	*G. max*	CD2947a, b	Hg3, Hg4	MW345414, MW345415	–	D. Peng
H. glycines	Iran	*G. max*	Zah18-8	Hg1	MW345330	–	Z. Tanha Maafi
H. medicaginis	Russia, Stavropol Krai, Mineralovodsky district	*Medicago sativa*	430a	Hme1	MW345340	–	S.A. Subbotin
H. medicaginis	Russia, Stavropol Krai, Georgiyevsky district	*M. sativa*	432	Hme2	MW345342	–	S.A. Subbotin
H. medicaginis	Russia, Stavropol Krai	*M. sativa*	540	Hme3	MW345339	MW346596	E. Krall
H. medicaginis	Russia, Rostov region, Starocherkassk	Unknown	CD3114	Hme4	MW345416	–	V.N. Chizhov
H. mediterranea	Spain, Cádiz, Vejer	Olive tree	CD3118	Hmed1	MW345393, MW345394	–	P. Castillo
H. mediterranea	Spain, Córdoba, Villaviciosa	Wild olive tree	AR108, CD3252	Hmed3	MW345405, MW345406	MW346561	P. Castillo
H. mediterranea	Spain, Cádiz, Sanlúcar de Barrameda	Wild olive tree	AR015, CD3242	Hmed1	MW345395–MW345397	MW346556, MW346565	P. Castillo
H. mediterranea	Spain, Huelva, Hinojos	Wild olive tree	AR021, CD3251	Hmed1	MW345398, MW345399	MW346558	P. Castillo
H. mediterranea	Spain, Cádiz, Tarifa	Wild olive tree	AR031, CD3254	Hmed5	MW345409–MW345411	MW346553–MW346555	P. Castillo
H. mediterranea	Spain, Cádiz, Medina Sidonia	Wild olive tree	AR060, CD3245	Hmed1	MW345402	MW346557	P. Castillo
H. mediterranea	Spain, Sevilla. Puebla de Cazalla	Wild olive tree	ST011, CD3243	Hmed1	MW345400, MW345401	MW346562, MW346563	P. Castillo
H. mediterranea	Spain, Huelva, Hinojos	Olive tree	ST007, CD3253	Hmed1	MW345403	MW346559	P. Castillo
H. mediterranea	Spain, Córdoba, Puente Genil	Olive tree	M201, CD3255	Hmed2	MW345404	MW346560	P. Castillo
H. mediterranea	Spain, Valencia, Saler	*Pistacia lentiscus*	Saler, CD3246	Hmed4	MW345407, MW345408	MW346564	J.E. Palomares-Rius
H. schachtii	Belgium, sample 1	*B. vulgaris*	CD2968a, b	Hsch1, Hsch2	MW345368, MW345379	–	M. Moens
H. schachtii	Belgium, sample 2	*B. vulgaris*	CD3083	Hsch1	MW345378	–	M. Moens

Table 1. (Continued.)

Species	Location	Associated host	Sample code	*COI* haplotype	*COI* GenBank accession no.	ITS rRNA GenBank accession no.	Source and/or reference
H. schachtii	Ukraine, Vinnitsa region, Kryzhopil district, Zabolotnoe	*B. vulgaris*	CD1530	Hsch1	MW345364	–	V.N. Chizhov
H. schachtii	The Netherlands	*B. vulgaris*	168a, b	Hsch1	MW345357, MW345373	–	M. Moens
H. schachtii	USA, California	Unknown	CD436	Hsch1	MW345367	–	S.A. Subbotin
H. schachtii	USA, California, Santa Barbara	Unknown	CD1436	Hsch1	MW345358, MW345365	–	S.A. Subbotin
H. schachtii	Iran, Fars, Marvdasht	*B. vulgaris*	Zah32	Hsch1	MW345362, MW345363, MW345369, MW345375, MW345377	AF498389	Z. Tanha Maafi; Tanha Maafi *et al.* (2003)
H. schachtii	Iran, Ardabil, Ardabil	*B. vulgaris*	Zah39	Hach1	MW345360, MW345371, MW345372	–	Z. Tanha Maafi; Tanha Maafi *et al.* (2003)
H. schachtii	Iran, Western Azerbaijan, Khoy	*B. vulgaris*	Zah40	Hsch1	MW345359, MW345361, MW345366	–	Z. Tanha Maafi; Tanha Maafi *et al.* (2003)
H. schachtii	Iran, Khorasan, Chenaran	*B. vulgaris*	Zah31	Hsch1	MW345355, MW345370	–	Z. Tanha Maafi; Tanha Maafi *et al.* (2003)
H. schachtii	Spain, Murcia	Cauliflower	CD3119	Hsch1	MW345374, MW345376	–	P. Castillo
H. schachtii	Spain, Lebrija	*B. vulgaris*	CD3120b, c	Hsch5	MW345387, MW345389	–	P. Castillo
H. schachtii	Spain, Posadas	*B. vulgaris*	CD3121b	Hsch5	MW345388	–	P. Castillo
H. schachtii	Spain, Valladolid	*B. vulgaris*	AIM18, CD3244a, b	Hsch1, Hsch6	MW345353, MW345390, MW345391	MW346545, MW346551	J.E. Palomares-Rius
H. schachtii	Spain, Cullera, Valencia	*Brassica napus* var. *napobrassica*	Brosquil, CD3248	Hsch1	MW345348	MW346552	J.E. Palomares-Rius
H. schachtii	Poland, Kijewice	Corn field	IAS98730, CD3241	Hsch1	MW345344, MW345346, MW345349, MW345354	MW346547–MW346550	J.E. Palomares-Rius

Table 1. (Continued.)

Species	Location	Associated host	Sample code	*COI* haplotype	*COI* GenBank accession no.	ITS rRNA GenBank accession no.	Source and/or reference
H. schachtii	Poland, Cetyn	Corn field	IAS97770, CD3250	Hsch1	MW345345, MW345350, MW345351	–	J.E. Palomares-Rius
H. schachtii	The Netherlands, Merselo	Corn field	IAS84582, CD3247	Hsch1	MW345347, MW345352	MW346544, MW346546	J.E. Palomares-Rius
H. schachtii	Australia	*B. vulgaris*	CD3749	Hsch1	OQ969979–OQ969981	–	I. Riley
H. schachtii	Spain, Sevilla, Los Palacios y Villafranca	*Rumex* sp.	CD3260a, b, d	Hsch5, Hsch7	MW345381, MW345384, MW345385	–	J.E. Palomares-Rius and P. Castillo
H. schachtii	Spain, Sevilla, Palmar de Troya	Fallow	CD3262a, b, c	Hsch5	MW345380, MW345382, MW345386	–	J.E. Palomares-Rius and P. Castillo
H. schachtii	Spain, Sevilla, Los Palacios y Villafranca	*B. vulgaris*	CD3263	Hsch1	MW345343	–	J.E. Palomares-Rius and P. Castillo
H. schachtii	Germany	*B. vulgaris*	Germany	–	–	MW346583–MW346594	D. Sturhan
H. schachtii	Germany, Münster	*B. vulgaris*	568	Hsch1	MW345356	–	D. Sturhan
H. schachtii	Spain, Malaga, Antequera	Onion field	CD3264b	Hsch5	MW345383	MW346543	J.E. Palomares-Rius and P. Castillo
H. sonchophila	Estonia, Pärnu County, Tahkuranna	*Sonchus arvensis*	543	Hso1	MW345341	MW346595, MW346599	E. Krall
H. trifolii	Australia, Victoria	*Trifolium repens*	604	Ht6	MW345319	–	I. Riley
H. trifolii	USA, California	Unknown	CD1083	Ht1	MW345316	–	S.A. Subbotin
H. trifolii	USA, California, Sacramento	*T. repens*	CD2474	Ht3	MW345303	–	S.A. Subbotin
H. trifolii	Russia, Nizhny Novgorod region	Unknown	CD1438	Ht2	MW345304	–	L. Nasonova
H. trifolii	Australia, Western Australia, Manjimup	*T. repens*	605a, b	Ht1	MW345298, MW345306	–	I. Riley, W. Wouts
H. trifolii	Estonia	*Plantago* sp.	CD539	Ht1	MW345312	MW346569	E. Krall
H. trifolii	New Zealand	*T. repens*	315	Ht1	MW345305	MW346576, MW346577	W. Wouts
H. trifolii	Iran, Mazandaran	*T. repens*	Zah15	Ht1	MW345309	–	Z. Tanha Maafi
H. trifolii	Kyrgyzstan	Unknown	CD3111b	Ht1	MW345313	–	S.A.Subbotin
H. trifolii	Russia, Moscow region	*T. repens*	225	–	–	MW346568	V.N. Chizhov

Table 1. (Continued.)

Species	Location	Associated host	Sample code	*COI* haplotype	*COI* GenBank accession no.	ITS rRNA GenBank accession no.	Source and/or reference
H. trifolii	Russia, Moscow	*Polygonum* sp.	CD3112	Ht1	MW345296, MW345315	–	V. N. Chizhov
H. trifolii	Spain, Cordoba	*Rumex* sp.	CD3261	Ht1	MW345302, MW345318	–	P. Castillo
H. trifolii	Germany	*Rumex* sp.	542c	Ht1	MW345297	MW346573	D. Sturhan
H. trifolii	Belgium	*T. repens*	288	–	–	MW346580	S.A. Subbotin
H. trifolii	Iran, Gilan, Bandar Anzali	Unknown	Zah19-2, 4 5,	Ht1, Ht3	MW345299–MW345301, MW345317	–	Z. Tanha Maafi
H. trifolii	Russia, Russia, Sakhalin Island	Unknown	422b	Ht1	MW345311	.–	A.S. Eroshenko
Heterodera sp.1	Belgium, Zwin, nature reserve	*Atriplex littoralis*	345, CD3117	HspA1	MW345337, MW345338	MW346597, MW346598	S.A. Subbotin
Heterodera sp.2	Morocco	Unknown	674a	Hsp2–1	MW345392	–	S. Amiri
Heterodera sp.3	Spain, Cazorla	Undetermined grasses	818	Hsp3–1	MW345321	–	P. Castillo

DNA EXTRACTION, PCR AND SEQUENCING

DNA extraction, PCR and sequencing were performed as described by Subbotin *et al.* (2018). Several primer sets were used in the present study: *i*) the forward Het-coxiF (5′-TAG TTG ATC GTA ATT TTA ATG G-3′) and the reverse Het-coxiR (5′-CCT AAA ACA TAA TGA AAA TGW GC-3′) primers or the forward JB3 (5′-TTT TTT GGG CAT CCT GAG GTT TAT-3′) and the reverse JB4 (5′-TAA AGA AAG AAC ATA ATG AAA ATG-3′) primers for amplification of the partial *COI* gene; *ii*) the forward TW81 (5′-GTT TCC GTA GGT GAA CCT GC-3′) and the reverse AB28 (5′-ATA TGC TTA AGT TCA GCG GGT-3′) primers for amplification of the ITS1-5.8S-ITS2 rRNA gene (Subbotin *et al.*, 2018); and *iii*) the forward U831 (5′-AAY AAR ACM AAG CCN TYT GGA C-3′) and the reverse L1110 (5′-TCR CAR TTV TCC ATG ATR AAV AC-3′) primers for amplification of the partial *hsp90* gene (Skantar & Carta, 2005). New sequences were deposited in the GenBank database under accession numbers: MW346543-MW346599 (ITS rRNA gene), MW345296-MW345416, OQ969979-OQ969981 (*COI* gene) and ON934497-ON934501, ON934504, ON934505, OQ993341 (*hsp90* gene) as indicated in Table 1 and phylogenetic networks and trees.

PHYLOGENETIC, SEQUENCE AND PHYLOGEOGRAPHIC ANALYSIS

Alignments with the ITS rRNA, *COI* and *hsp90* gene sequences were created using ClustalX 1.83 (Chenna *et al.*, 2003) with default parameters. New sequences were aligned with corresponding published gene sequences (Subbotin *et al.*, 2001; Madani *et al.*, 2004, 2007; Toumi *et al.*, 2013; Vovlas *et al.*, 2015; Cui *et al.*, 2016; Guesmi-Mzoughi *et al.*, 2018; Escobar-Avila *et al.*, 2019; Kim *et al.*, 2019; Powers *et al.*, 2019; Sekimoto *et al.*, 2019; Handoo *et al.*, 2020; Jain *et al.*, 2022; Peng *et al.*, 2022 and others). Several alignments were created: *i*) ITS rRNA gene alignment containing only reference sequences of 13 species of the *Schachtii* group; *ii*) ITS rRNA gene alignment containing new and published sequences of the *Schachtii* group species published, except for *H. cajani* and *H. dunensis*; *iii*) *COI* gene alignment containing only reference haplotype sequences of valid and undescribed species of the *Schachtii* group; *iv*) several *COI* gene sequence alignments containing sequences of nine species: *H. betae*, *H. ciceri*, *H. daverti*, *H. galeopsidis*, *H. glycines*, *H. medicaginis*, *H. mediterranea*, *H. schachtii* and *H. trifolii*; and *v*) *hsp90* gene alignment containing new and published sequences of the *Schachtii* group species. Pairwise divergence between taxa was calculated as the absolute distance value and the percent of mean distance, with adjustment for missing data, using PAUP* 4b10 (Swofford, 2003).

The ITS rRNA, *COI* and *hsp90* gene sequence alignments were analysed with maximum likelihood (ML), maximum parsimony (MP) using PAUP* and Bayesian inference (BI) using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) as described by Subbotin *et al.* (2018). The best fit models of DNA evolution were obtained using the program jModeltest 2.1.1 (Posada, 2008) with the Akaike Information Criterion. Bootstrap support (BS) values for ML and MP trees were calculated by a heuristic search from 1000 replicates.

The alignments for ITS rRNA and *COI* gene sequences were used to construct phylogenetic networks using statistical parsimony (SP) as implemented in POPART software (http://popart.otago.ac.nz) (Bandelt *et al.*, 1999). The estimation of divergence time with BEAST 2.4.5 (Bouckaert *et al.*, 2014) was performed as described by Subbotin *et al.* (2018). The tree prior a lognormal relaxed clock with uncorrelated rates were assigned to the Yule model with the mitochondrial substitution genome rate equal to 7.2×10^{-8} per site per generation as calculated by Howe *et al.* (2010) for *Caenorhabditis briggsae*. The life cycle with two generations per year was considered for the *Schachtii* group species (Subbotin *et al.*, 2010).

The *COI* gene polymorphism was estimated for some species. Several parameters – number of haplotypes, number of segregating sites, haplotype and nucleotide diversities and the Tajima's D values for neutrality test – were calculated using DnaSP v.5.10 (Librado *et al.*, 2009).

Ancestral area reconstruction was done with RASP 4.2 (Yu *et al.*, 2015), which implements Statistical Dispersal Vicariance Analysis (S-DIVA). The distribution of species was divided into 14 geographical regions (A-N), where these species were naturally found and/or have unique haplotypes. The number of maximum areas for each node was kept at two. The most likely ancestral regions for each node were mapped on the 50% majority rule consensus BI tree inferred from the analysis of the *COI* gene sequence alignment.

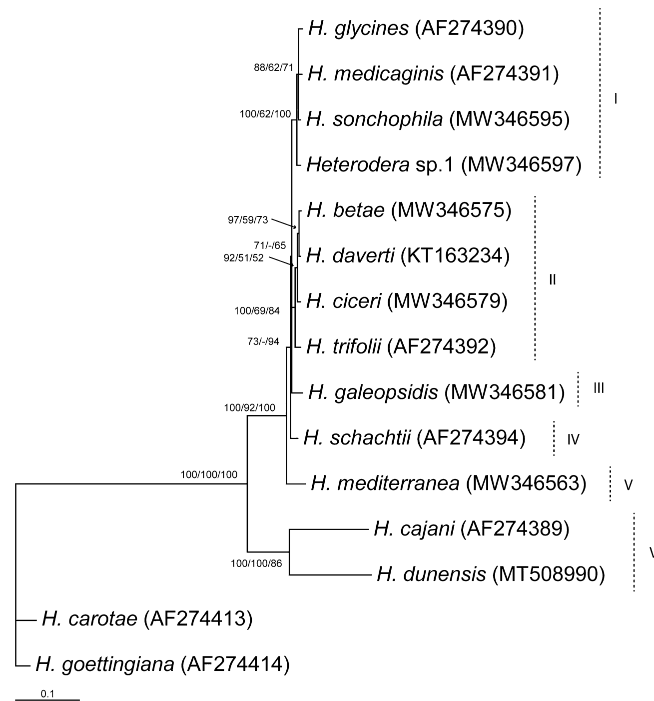


Fig. 1. Phylogenetic relationships between *Heterodera* species from the *Schachtii* group as inferred from maximum parsimony (MP) analysis of the ITS rRNA gene sequences. *Heterodera carotae* and *H. goettingiana* are outgroup taxa. Posterior probability and bootstrap support values for BI, ML and MP analysis are given for appropriate clades, respectively. Values less than 50% are not indicated.

Results

Phylogenetic and sequence analysis with ITS rRNA gene

Phylogenetic relationships within 12 valid and one undescribed species of the *Schachtii* group species as inferred from MP, BI and ML analyses of the ITS rRNA gene reference sequences are given in Figure 1. The alignment was 957 bp in length. Phylogenetic analyses revealed the presence of several clades within the *Schachtii* group: *i*) *H. glycines* + *H. medicaginis* + *H. sonchophila* + *Heterodera* sp.1; *ii*) *H. daverti* + *H. betae* + *H. ciceri* + *H. trifolii*; *iii*) *H. galeopsidis*; *iv*) *H. schachtii*; *v*) *H. mediterranea*; and *vi*) *H. cajani* and *H. dunensis*.

A total of 479 ITS rRNA gene sequences, including 57 new ones of ten valid species and one undescribed species of the *Schachtii* group were analysed in this study. The alignment was 947 bp in length and included 306 sequences of *H. glycines*, 82 sequences of *H. schachtii*, 22 sequences of *H. trifolii*, 22 sequences of *H. betae*, 19 sequences of *H. mediterranea*, 12 sequences of *H. medicaginis*, five sequences of *H. daverti*, four sequences of *H. ciceri*, 3 sequences of *H. galeopsidis*, two sequences of *H. sonchophila*, two sequences of *Heterodera* sp.1. Maximal intraspecific sequence variation for *H. mediterranea* was 2.6%, *H. schachtii* 2.6%. *H. glycines* 2.3% and for *H. medicaginis* 0.9%. The SP network showing the phylogenetic relationships among ITS rRNA gene sequences of species listed above are given in Figure 2. Sequences of *H. glycines* belonged to the central Hg1 haplotype and numerous peripheral haplotypes, whereas sequences of *H. schachtii* distributed among four groups (Fig. 2). Some ITS rRNA gene sequences of *H. schachtii* belonging to the fourth group from Turkey were similar to those of *H. ciceri*. The SP analysis revealed several similar haplotypes for: *i*) *H. glycines* and *H. medicaginis*; and *ii*) *H. betae*, *H. daverti*, *H. trifolii* and *H. schachtii*.

Phylogenetic and sequence analysis with *COI* gene

A total of 352 sequences, including 124 new ones, was included in the analysis. The *COI* gene alignment was

Fig. 2. Statistical parsimony network showing the phylogenetic relationships between ITS rRNA gene sequences of some *Heterodera* species from the *Schachtii* group. The sequences of each species are marked by different colours. Pies (circles) represent sequences of each species with the same haplotype and their size is proportional to the number of these sequences in the samples. Numbers of nucleotide differences between the sequences are indicated on lines connecting the pies. Small black circles represent missing haplotypes. New sequences are indicated by bold font. Four ITS groups of *H. schachtii* are also marked.

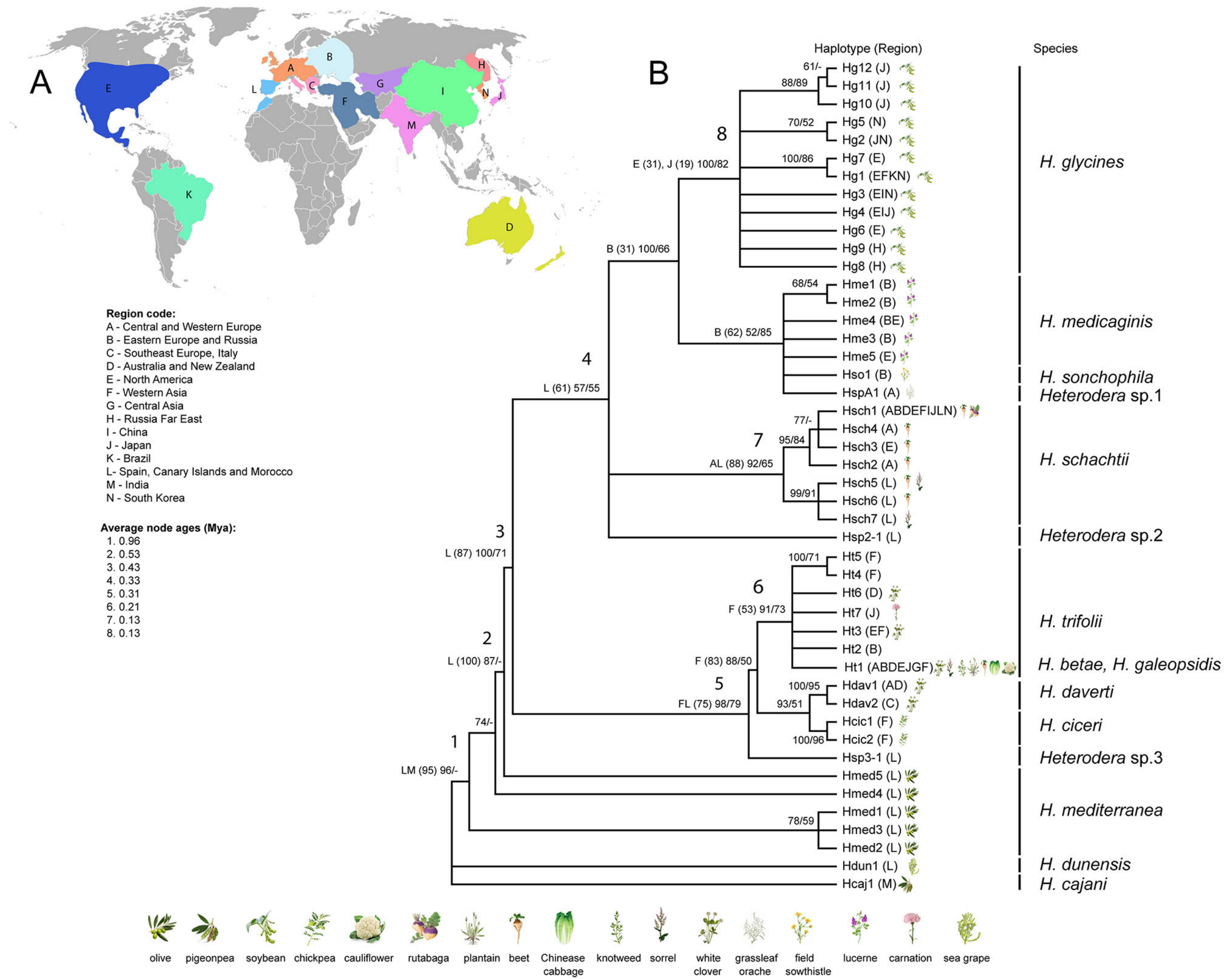


Fig. 3. Phylogenetic relationships between *COI* haplotypes of the *Schachtii* group species as inferred from Bayesian analysis with mapping of regions, plant-hosts and indication of node ages. A: World map with region codes; B: Phylogenetic tree. Codes with most probable ancestral regions, posterior probability values for BI analysis and bootstrap values for ML analysis are given to appropriate clades. Mya = million years ago.

434 bp in length. The majority of nematode populations contained only one *COI* gene haplotype, whereas two haplotypes were found in six populations. Phylogenetic relationships within the *Schachtii* group species, containing 45 reference haplotype sequences and two sequences of outgroup species as inferred from BI and ML analyses, are presented in Figure 3. The parameters of *COI* gene polymorphism for some species are given in Table 2. Negative values of Tajima's *D*-test were obtained for all studied species, but they were insignificant.

Heterodera schachtii

A total of 115 sequences of *H. schachtii* were analysed. Seven haplotypes were revealed. The geographical distribution of the *COI* haplotypes in *H. schachtii* is illustrated in Figure 4A and the haplotype phylogenetic network is given in Figure 4B. The Hsch1 haplotype (94 sequences) was most distributed. The Hsch2, Hsch3 and Hsch4 haplotypes differed in one change from the Hsch1 haplotype. The Hsch3 haplotype was found in Wyoming, USA, and the Hsch2 and Hsch4 haplotypes were reported in Bel-

Table 2. Polymorphism of the *COI* gene fragment for some cyst nematode species from the *Schachtii* group and the neutrality test.

Species	Number of sequences	Number of haplotypes	Number of variable sites	Haplotype diversity and standard deviation	Nucleotide diversity and standard deviation	Tajima's *D*
Heterodera glycines	131	12	17	0.615 ± 0.044	0.00589 ± 0.00067	−1.1413 ($P > 0.10$)
Heterodera medicaginis	18	5	7	0.405 ± 0.143	0.00335 ± 0.01320	−1.2029 ($P > 0.10$)
Heterodera mediterranea	19	5	46	0.649 ± 0.108	0.03183 ± 0.00886	−0.1401 ($P > 0.10$)
Heterodera schachtii	115	7	11	0.376 ± 0.055	0.00685 ± 0.00144	−0.4478 ($P > 0.10$)
Heterodera daverti	4	3	3	0.833 ± 0.049	0.00384 ± 0.00140	−0.7544 ($P > 0.10$)
Heterodera ciceri	2	2	6	1.000 ± 0.500	0.01942 ± 0.00971	–
Heterodera betae	17	1	0	0	0	–
Heterodera trifolii	41	7	5	0.428 ± 0.096	0.00168 ± 0.00044	−1.3210 ($P > 0.10$)

gium. The unique regional haplotypes Hsch5, Hsch6 and Hsch7 were found in Spain only. Maximal intraspecific sequence diversity was 4.1%.

Heterodera medicaginis*,* H. sonchophila *and* Heterodera *sp.1*

A total of 18 sequences of *H. medicaginis* were analysed. Five haplotypes were revealed. Hme1, Hme2, Hme3 haplotypes were represented by one sequence each. The geographical distributions of the *COI* haplotypes in *H. medicaginis* and two related species are illustrated in Figure 5A and the haplotype phylogenetic network is given in Figure 5B. Highest haplotype diversity was found among Russian populations. Three haplotypes: Hme1, Hme2 and Hme3, were found in Russia, Stavropol Krai. Hme4 haplotype (14 sequences) was reported in the USA and Russia and a unique Hme5 haplotype was only found in Utah, USA. Maximal intraspecific sequence diversity of *H. medicaginis* was 1.3%. *Heterodera sonchophila* from Estonia was represented by only one sequence, which differed in three or more nucleotides from *H. medicaginis* (Fig. 5B). *Heterodera* sp.1 from Belgium was also represented by only one sequence, which differed at least in one nucleotide from *H. medicaginis* (Fig. 5B).

Heterodera glycines

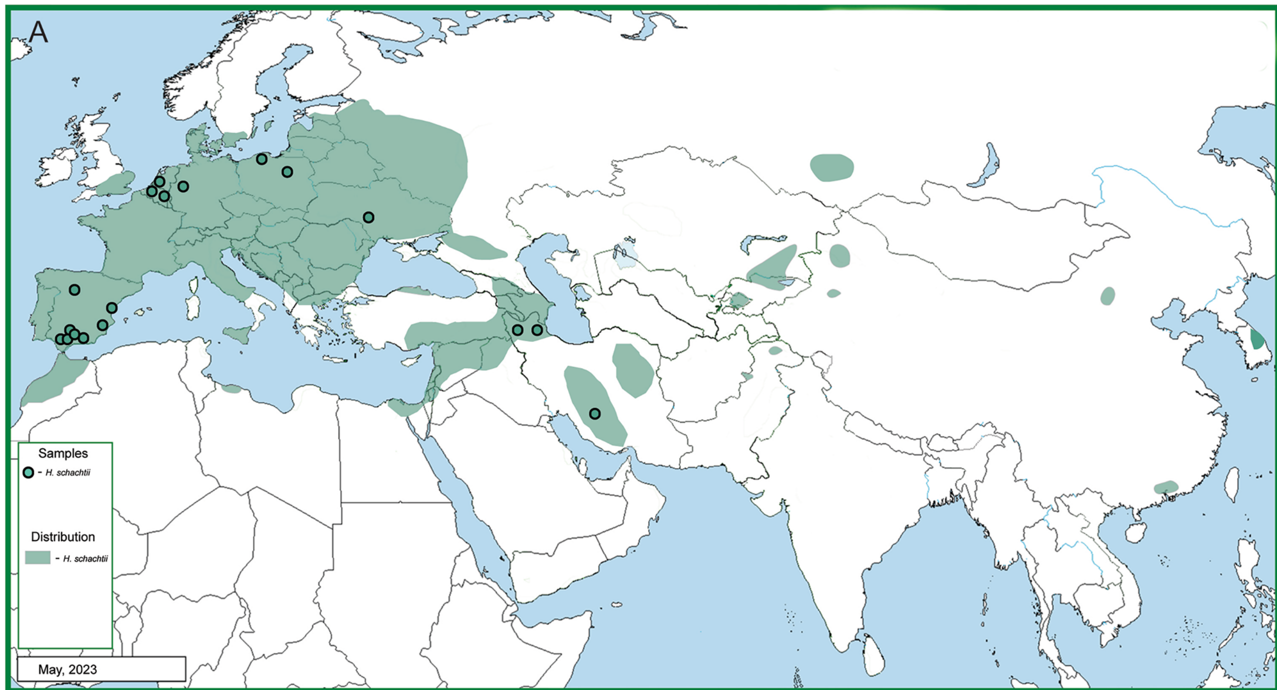
A total of 131 sequences of *H. glycines* were analysed. Twelve haplotypes were revealed. The haplotype phylogenetic network is given in Figure 6A. The Hg1 haplotype (78 sequences) was found in South Korea, the USA, Iran and Brazil. Other haplotypes were distributed the following ways: central Hg2 haplotype in Japan and South Korea, Hg3 in South Korea and USA (Georgia), Hg4 in Japan and the USA, Hg5 in South Korea, Hg6 and H7 in the USA, unique regional H8 and Hg9 haplotypes in Russia, Amur Oblast, and unique regional Hg10, Hg11 and Hg12 haplotypes in Japan. Maximal intraspecific sequence diversity was 2.0%.

Heterodera trifolii*,* H. betae *and* H. galeopsidis

A total of 41 sequences of *Heterodera trifolii*, 17 sequences of *H. betae* and one sequence of *H. galeopsidis* were analysed. The haplotype phylogenetic network is given in Figure 6B. Seven haplotypes were revealed within the group. *COI* sequences obtained from cysts infesting *Trifolium repens*, *Plantago* sp. *Polygonum* sp. and *Dianthus caryophyllus* were similar and they were identified as representatives of *H. trifolii*. Sequences of *H. betae* (Hb1 haplotype) were identical to those of the Ht1 haplotype of *H. trifolii* and Hgal1 haplotype of *H. galeopsidis*. Maximal sequence diversity for these three species was 1.2%.

Heterodera mediterranea

Nineteen sequences of Spanish *H. mediterranea* were analysed, revealing five haplotypes. The haplotype phylogenetic network is given in Figure 6C. Maximal intraspecific sequence diversity was 8.1%.



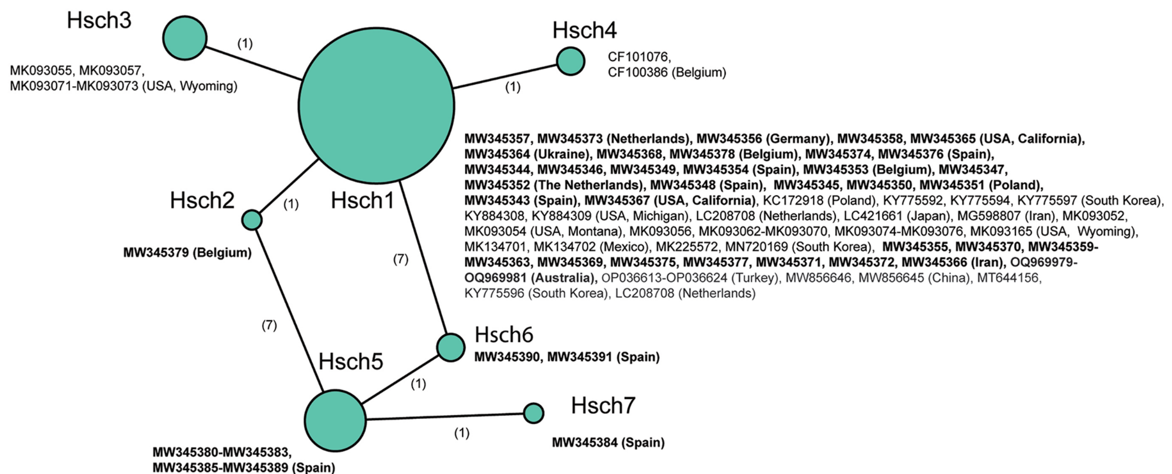
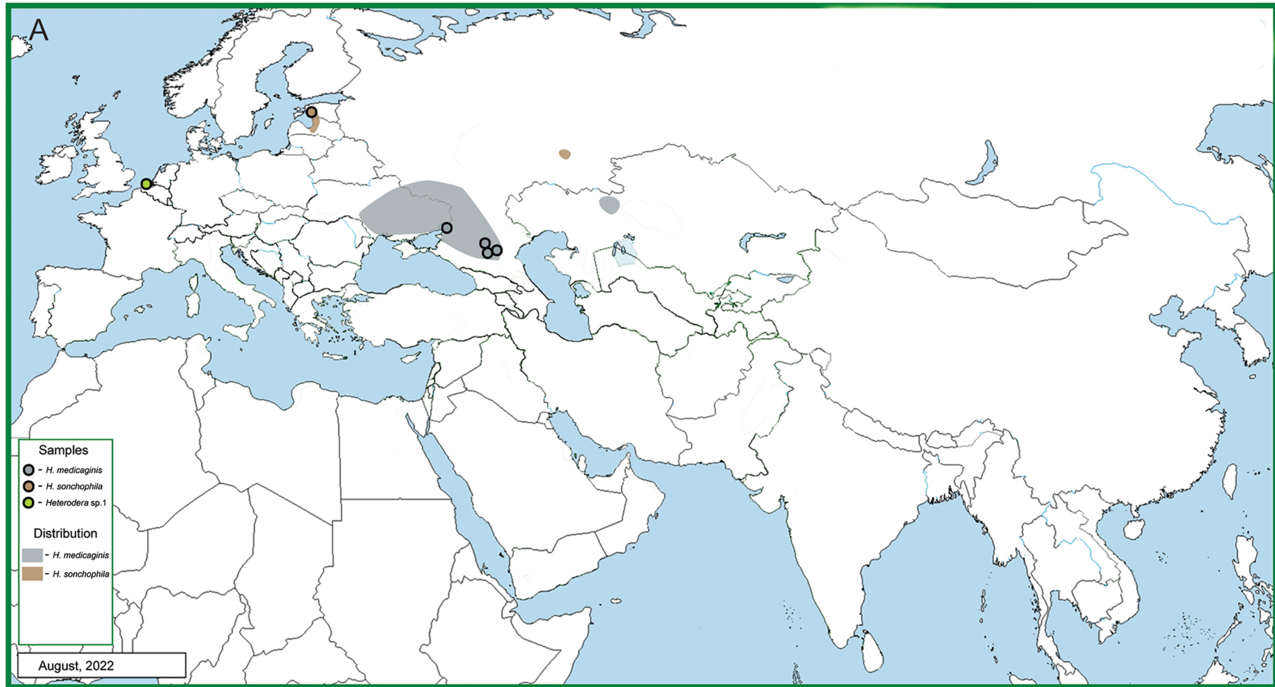


Fig. 4. A: Distribution map of *Heterodera schachtii* with indication of the studied samples; B: Statistical parsimony network showing the phylogenetic relationships between *COI* haplotypes. Pie (circle) sizes are proportional to the number of samples with a particular haplotype. New sequences are indicated in boldface.

Heterodera daverti and H. ciceri

Four sequences of *H. daverti* and two sequences of *H. ciceri* were analysed. The haplotype phylogenetic network is given in Figure 7. Two haplotypes of *H. daverti*, one from Germany and Australia and a second from Italy, and two haplotypes of *H. ciceri*, both from Syria, were revealed. The Australian sequence of *H. daverti* differed in one nucleotide from the sequence of the German population and in two nucleotides from that of the Italian population. Maximal sequence diversity for *H. daverti* was 0.7% and for *H. ciceri* was 1.9%.



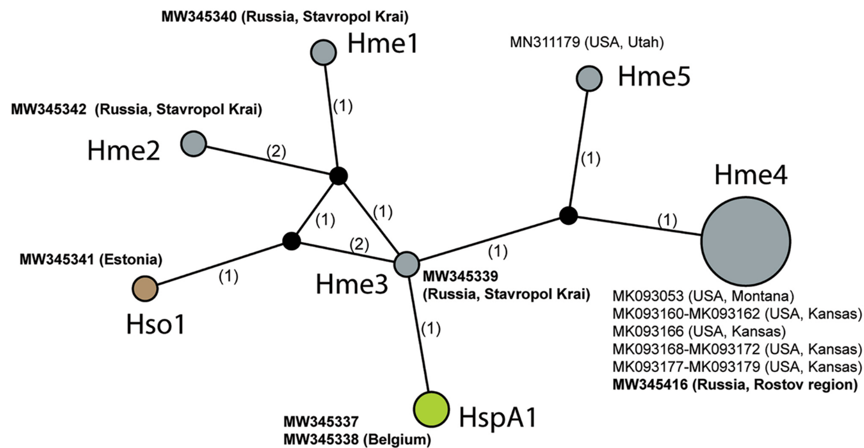


Fig. 5. A: Distribution map of *Heterodera medicaginis* with indication of the studied samples; B: Statistical parsimony network showing the phylogenetic relationships between *COI* haplotypes. Pie (circle) sizes are proportional to the number of samples with a particular haplotype. New sequences are indicated in boldface.

Heterodera cajani

This species was represented by two identical sequences obtained from a population from India.

Phylogenetic and sequence analysis with *hsp90* gene

Fifty-eight sequences of the *Schachtii* group species belonging to *H. glycines*, *H. medicaginis*, *H. schachtii*, *H. trifolii* and *H. mediterranea* were analysed. Eight new

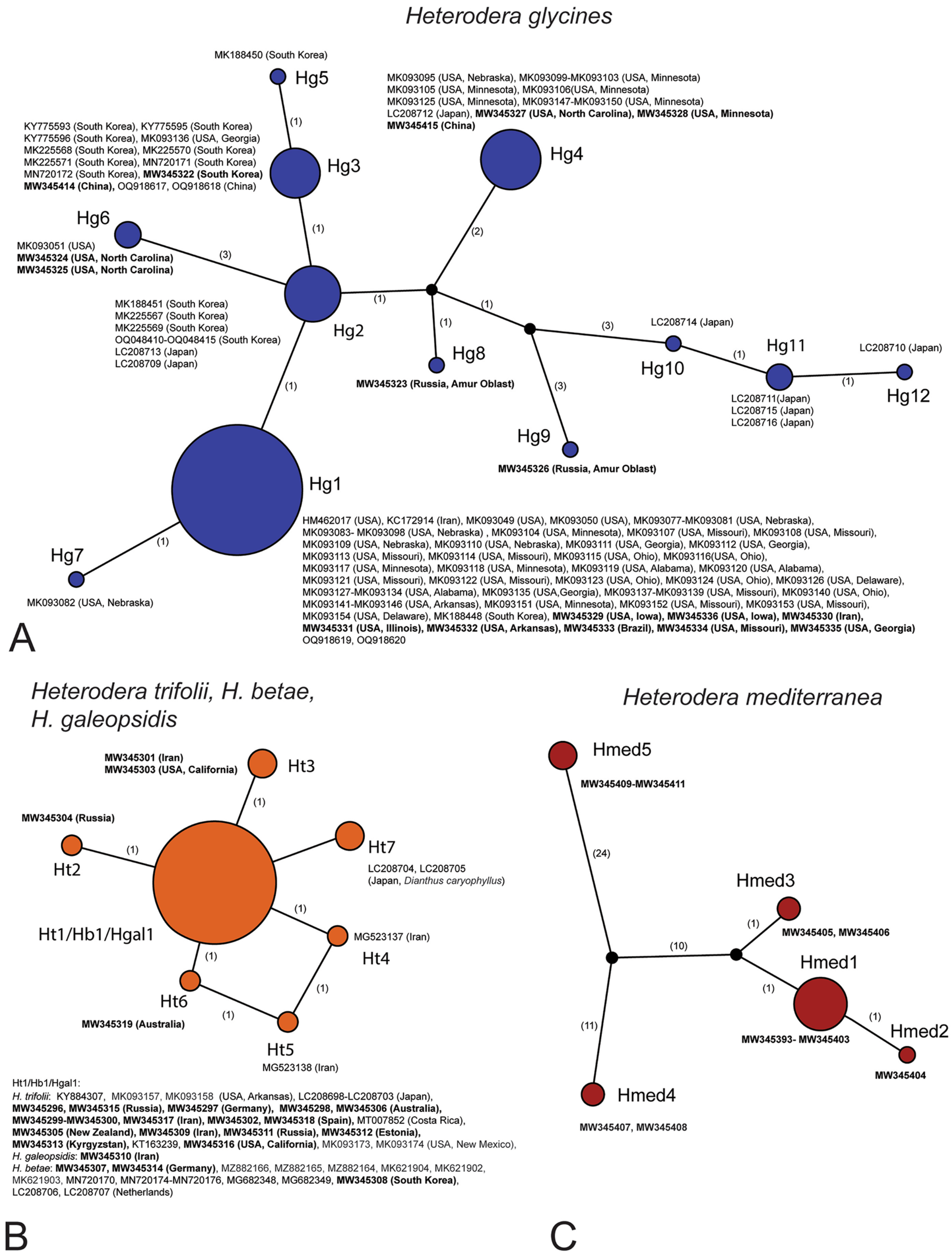


Fig. 6. Statistical parsimony networks showing the phylogenetic relationships between *COI* haplotypes of several cyst nematode species: A. *Heterodera glycines*; B: *H. trifolii*, *H. betae*, *H. galeopsidis*; B: *H. mediterranea*. Pie (circle) sizes are proportional to the number of samples with a particular haplotype. New sequences are indicated in boldface.

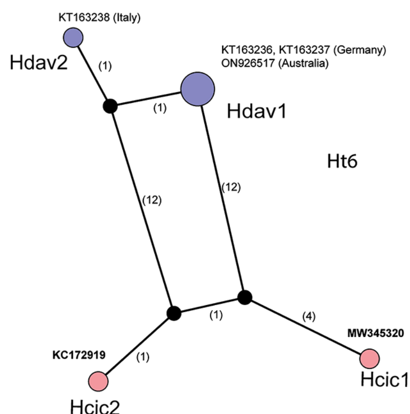


Fig. 7. Statistical parsimony network showing the phylogenetic relationships between *COI* haplotypes of *Heterodera daverti* and *H. ciceri*. Pie (circle) sizes are proportional to the number of samples with a particular haplotype. New sequences are indicated in boldface.

sequences of the partial *hsp90* gene were obtained in this study. The alignment length was 337 bp. Phylogenetic relationships within the *Schachtii* group species are given in Figure 8. This gene fragment was able to differentiate all studied species from each other.

Phylogeographical analysis and molecular clock

The ancestral ranges to each node in a tree were evaluated accounting for phylogenetic uncertainty in DIVA optimisation. The most likely ancestral areas were mapped on the BI majority consensus tree and are presented in Figure 3. The ancestral area for the clade with *H. glycines* + *H. medicaginis* + *H. sonchophila* + *Heterodera* sp.1 + *H. schachtii* + *Heterodera* sp.2 that was suggested with highest probability encompassed the Iberic Peninsula, the Canary Islands and Morocco. This world region also could be considered likely as ancestral for the *Schachtii* group. North America and Japan were suggested as ancestral areas for *H. glycines* and Western Asia for *H. trifolii*. The tree topology retrieved from BEAST software contradicted a tree yielded by MrBayes in the position of *H. ciceri* and *H. daverti*. Estimated node ages for some main clades are given in Figure 3. The earliest divergence within the *Schachtii* group was estimated at 0.96 Mya (million years ago). It further split into clades with *H. glycines* at 0.13 Mya, *H. schachtii* 0.13 Mya and *H. mediterraneae* 0.60 Mya (Fig. 3).

Discussion

Species delimiting and DNA barcoding

The results of the present SP analysis of the ITS rRNA gene sequences showed that this marker has some limitations in species delimiting and diagnostics of some *Schachtii* group species. The SP analysis differentiated *H. galeopsidis*, *H. sonchophila*, *H. cicero* and *H. mediterranea* from each other. This analysis showed that some sequences of *H. glycines* and *H. medicaginis* belonged to the same haplotype. Some sequences of *H. betae*, *H. daverti*, *H. schachtii* and *H. trifolii* could also not be sufficiently differentiated from each other. Thus, the ITS rRNA gene has a rather high level of heterogeneity that complicates species delimitation. The *COI* gene sequences differentiated the studied species *H. schachtii*, *H. glycines*, *H. cajani*, *H. medicaginis* and *H. sonchophila* from each other; however, our study revealed that partial *COI* gene sequences were identical for *H. trifolii*, *H. betae* and *H. galeopsidis*.

Heterodera betae

The yellow beet cyst nematode was discovered in Dutch beet fields in 1975 and was first considered as a biotype of the clover cyst nematode, *H. trifolii* (Maas & Heijbroek, 1982) before being described as a new species by Wouts *et al*. (2001). This nematode has 35-36 chromosomes and reproduces by mitotic parthenogenesis (Steele & Whitehand, 1984). It parasitises many plants in the families Caryophylaceae, Cruciferae, Chenopodiaceae, Leguminosae and Polygonaceae (Subbotin *et al*., 2010). PCR-ITS-RFLP with the restriction enzymes *Mva*I and *Scr*FI generated a unique profile for this species (Wouts *et al*., 2001; Amiri *et al*., 2002; Gracianne *et al*., 2014). Madani *et al*. (2007) revealed that the ITS rRNA gene sequences of *H. betae* clustered with those of *H. schachtii*. Sekimoto *et al*. (2017) was the first to show that the *COI* sequence of *H. betae* was similar to that of *H. trifolii* and our study confirmed this observation.

The yellow beet cyst nematode was reported in several European countries: Belgium, The Netherlands, Sweden, Germany, Switzerland, Spain, Portugal, France, Italy (Subbotin *et al*., 2010; Gracianne *et al*., 2014) and Morocco (Amiri *et al*., 2002). Based on habitat use analysis, Gracianne *et al*. (2014) showed that *H. betae* was preferentially located in warm habitats of southern Europe and demonstrated that *H. schachtii* and *H. betae* are widely distributed along the Atlantic coastline, with

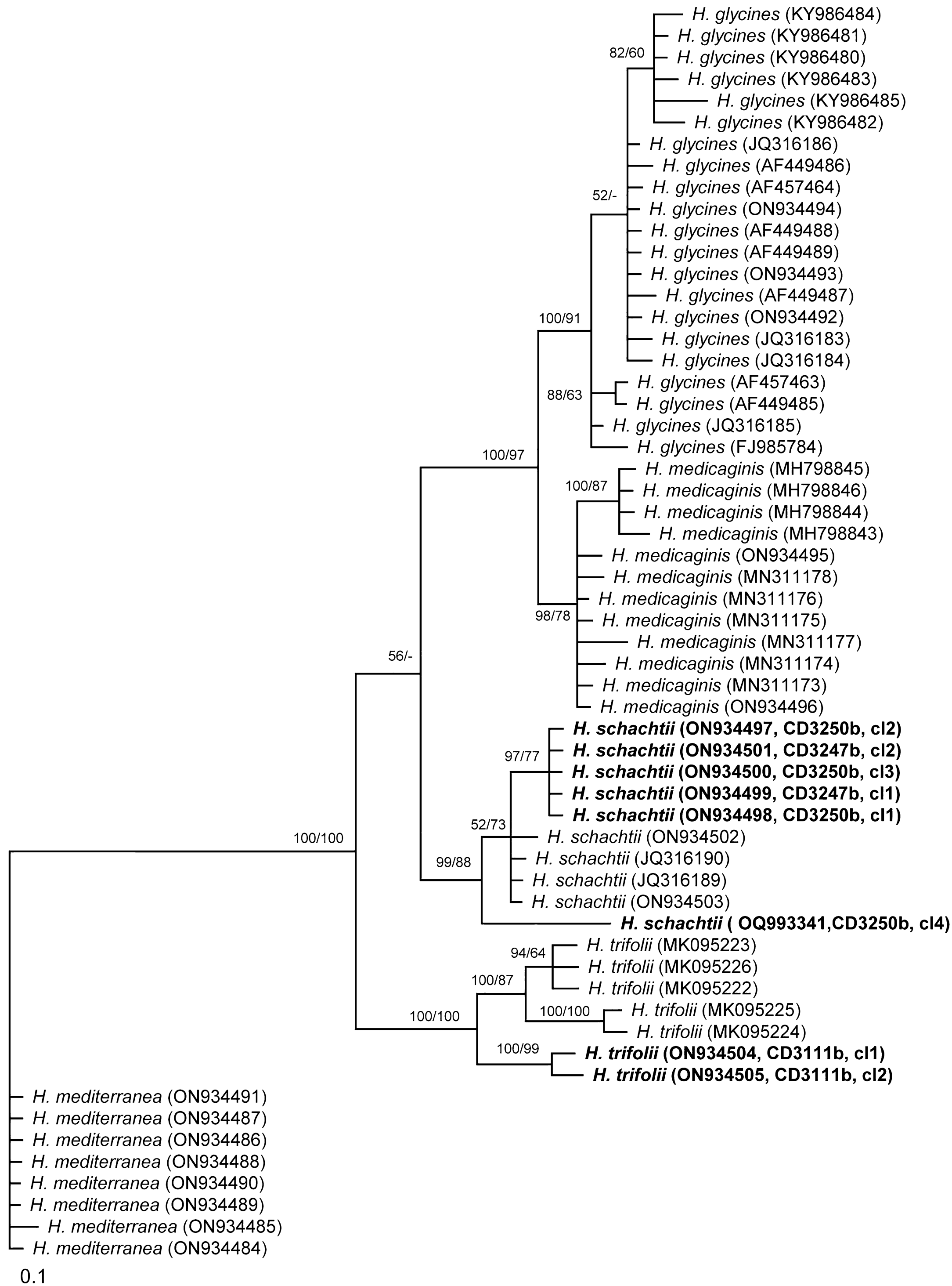


Fig. 8. Phylogenetic relationships between some species from the *Schachtii* group as inferred from Bayesian analysis of partial *hsp90* protein coding gene sequences under the GTR + I + G model. Posterior probability (BI) and bootstrap (ML) values are given for appropriate clades. New sequences are indicated in boldface.

a large area of overlapped distribution on a wild beet relative, the sea beet, *Beta vulgaris* ssp. *maritima*.

Mwamula *et al*. (2018) reported a cyst nematode identified as *H. trifolii* infecting Chinese cabbage, *Brassica rapa chinensis* L., a high-value economic highland crop in Korea. These authors found high similarity in morphology, morphometrics, *COI* and ITS rRNA gene sequences between the Korean population and those of *H. trifolii*. However, our comparison of published second-stage juvenile morphometrics showed that the Korean population likely belongs to *H. betae* and, thus, it is the first report of *H. betae* in Asia. It has been known that cabbage is a good host for *H. betae* (Subbotin *et al*., 2010).

Heterodera cajani

The pigeon pea cyst nematode is an important nematode pest of pigeon pea, *Cajanus cajan*, and found in all major growing regions of this crop in India and also reported in Egypt, Myanmar and Pakistan (Subbotin *et al*., 2010). *Heterodera cajani* was first molecularly characterised by PCR-ITS-RFLP (Subbotin *et al*., 2000). Sequences of ITS rRNA (Subbotin *et al*., 2001), β-tubulin (Sabo & Ferris, 2004), the D2-D3 expansion segments of 28S rRNA (Subbotin *et al*., 2006) and *COII* (Riepsamen *et al*., 2011) genes were published for the Indian populations of this species. The sequences of the ITS rRNA and *COI* gene placed this species together with *H. dunensis* at the basal position of the *Schachtii* group.

Heterodera ciceri

The chickpea cyst nematode was described from *Cicer arietinum* L. from Syria and presently reported also from Lebanon, Jordan and Turkey. This species parasitises wild annual *Cicer* spp., but not sugar beet and clovers (Subbotin *et al*., 2010). In the SP network, newly obtained and published ITS rRNA gene sequences of *H. ciceri* clustered together and also with some sequences of *H. schachtii* from Sanliurfa, Turkey, obtained by Cui *et al*. (2016). These authors already noted close relationships of sequences of *H. schachtii* from Sanliurfa, Turkey, with those from Australia (EF611123) and Morocco (EF611118), which can be also observed in the SP network with the fourth group of the ITS sequences for *H. schachtii*. Close relationships of sequences between Syrian *H. ciceri* and the Sanliurfa *H. schachtii* might be explained by hybridisation events between these species with possible disruption of concerted evolution homogenising rRNA gene copies in the last species. In the *COI* gene tree, *Heterodera ciceri* clustered with the other amphimictic species *H. daverti*.

Heterodera daverti

The Davert clover cyst nematode, *Heterodera daverti*, parasitising white clover was described from Germany. This species was detected in several countries in Europe (Germany, France, Italy, The Netherlands, UK), Africa (Egypt, Tunisia) (Subbotin *et al*., 2010) and recently in Australia (Jain *et al*., 2022). The specimens collected from the type locality and Italy were molecularly characterised by Vovlas *et al*. (2015) and those from Australia by Jain *et al*. (2022). All *COI* gene sequences are unique for this species.

Heterodera dunensis

Heterodera dunensis was described from Gran Canaria (Singh *et al*., 2020) and parasitises *Tetraena fontanesii*, a halophyte succulent plant growing in pure sand on the dunes and on rocky soils along the coast in Macaronesia and northwest Africa. The sequences of the *COI* gene occupy a basal position in the phylogenetic *COI* gene tree of the *Schachtii* group like that of *H. cajani*.

Heterodera galeopsidis

The hemp nettle cyst nematode, *H. galeopsidis* Goffart, 1936, was described from *Galeopsis tetrahit* L. from Germany. Triantaphyllou & Hirschmann (1978) noticed that *H. galeopsidis* probably represents a tetraform, distinct from all *H. trifolii* populations by its 32 chromosomes. A comparative study of chromosomal forms of *H. trifolii* and *H. galeopsidis* has demonstrated that each chromosomal form can be distinguished morphologically when observed in critical comparisons by light and scanning electron microscopy (Hirschmann & Triantaphyllou, 1979). However, Mass *et al*. (1982) considered this species as a synonym of *H. trifolii*, Siddiqi (2000) and Wouts *et al*. (2001) agreed with this proposal, whereas Baldwin & Mundo-Ocampo (1991) and Subbotin *et al*. (2010) considered it as a valid species. Two ITS rRNA gene sequences from *H. galeopsidis* from Germany were obtained and analysed in this study. The SP analysis showed that these sequences clustered separately from other *Schachtii* group species while placed with the sequence of a cyst nematode identified as *Heterodera* sp. 3 from Iran by Tanha Maafi *et al*. (2003). This Iranian cyst nematode sample is considered here as representative of *H. galeopsidis*. The partial *COI* gene sequence of

this sample is identical to the sequences of widely distributed Ht1/Hb1 haplotype of *H. trifolii* and *H. betae*. Thus, only the ITS rRNA gene sequence allows differentiating *H. galeopsidis* from other species and confirmed a separate species status.

Heterodera glycines

The soybean cyst nematode occurs in most of the soybean-producing countries of the world, including Argentina, Brazil, Canada, China, Russia, the USA and Iran. Soybean is the major economically important host of *H. glycines*, although this nematode has a wide host range consisting mainly on legumes and weeds from at least 22 plant families (*e.g.*, Boraginaceae, Capparaceae, Caryophyllaceae, Chenopodiaceae, Brassicaceae, Lamiaceae, Fabaceae, Scrophulariaceae and Solanaceae) (Haque & Khan, 2021). The ITS rRNA and *COI* genes of *H. glycines* were intensively sequenced by many researchers (Szalanski *et al*., 1997; Zheng *et al*., 2000; Subbotin *et al*., 2001; Tanha Maafi *et al*., 2003; Sekimoto *et al*., 2017; Ko *et al*., 2019; Powers *et al*., 2019). Although the ITS rRNA gene sequences do not clearly differentiate this species from some *H. medicaginis* populations, the *COI* and *hsp90* gene sequences allow a clear separation of this species from all others. In this study, the *COI* tree shows a population structure consisting of isolated populations from Japan, South Korea, USA and the Russian Far East containing unique haplotypes.

Heterodera medicaginis

The lucerne cyst nematode was reported from several regions of Ukraine, several regions of the Southern Federal District of Russia, Kazakhstan and Uzbekistan and is known as a parasite of species of *Medicago* (Subbotin *et al*., 2010). Recently, this nematode was collected from alfalfa fields in Kansas, Montana (Powers *et al*., 2019) and Utah (Handoo *et al*., 2020) in the USA. Powers *et al*. (2019) and the present results showed that the ITS rRNA sequences provide unclear distinction between *H. medicaginis* and *H. glycines* due to the sequence heterogeneity within both species; however, the *hsp90* gene sequence clearly separates the two species. Although *COI* gene sequences differentiated *H. medicaginis* from *H. glycines*, there were only a few nucleotide differences between the lucerne cyst nematode and *H. sonchophila* and a putatively new *Heterodera* sp.1 from the grassleaf orache, *Atriplex littoralis*, found on a North Sea beach in Belgium.

Heterodera mediterranea

The Mediterranean olive cyst nematode parasitises only three woody host plant species, *Pistacia lentiscus* and *P. vera* and *Olea europaea*, which belong to the families Anacardiaceae and Oleaceae, respectively (Vovlas & Inserra, 1981). Presently, this species is found in Italy (Vovlas *et al*., 1981), Spain (Castillo *et al*., 1999) and Tunisia (Guesmi-Mzoughi *et al*., 2018). Sequences of the ITS-rRNA gene for *H. mediterranea* have been published by Sabo *et al*. (2001) and Madani *et al*. (2004). In this study, high *COI* gene sequence diversity was found for this species in Southern Spain and the population from Cádiz, Tarifa, was substantially different from others in both studied genes. *COI* gene sequences are unique for this species.

Heterodera schachtii

The sugar beet cyst nematode is considered as a major limiting factor in sugar beet production worldwide and has been recognised and listed as a quarantine nematode in several countries. This nematode parasitises over 200 plant species (Subbotin *et al*., 2010). The sequence analysis revealed a rather high level of interspecific variation of the ITS rRNA gene up to 2.5%, which is similar to that reported by Madani *et al*. (2007) and Oro & Tabakovic (2020). Although the ITS rRNA gene sequences cannot clearly separate *H. schachtii* from some species of the *Schachtii* group, PCR-ITS-RFLP showed that the restriction enzyme *Mva*I produced a rather complex and unique RFLP profile containing fragments with different intensities but similar for all studied populations. This profile can be used for identification of *H. schachtii*. This observation confirms the complexity and heterogeneity of ITS for this species (Amiri *et al*., 2002; Tanha Maafi *et al*., 2003; Madani *et al*., 2004, 2007).

Our results revealed that ITS rRNA gene sequences of *H. schachtii* were distributed among four groups. Most ITS rRNA gene sequences obtained from populations from Europe, North America, Africa and Asia belonged to group 1, group 2 included only sequences of European populations, and group 3 included sequences of populations from Europe, Iran and Morocco. Group 4 contained sequences from Turkey, Australia and The Netherlands and was not well defined because it clustered with sequences of *H. ciceri*, *H. trifolii* and *H. betae*. The ITS rRNA gene sequences of *H. schachtii* obtained from the Sanliurfa populations from Turkey belonging to the fourth group were similar to those of *H. ciceri*. Species

identification of the Sanliurfa populations was confirmed by morphological and morphometrical studies and PCR-*H. schachtii* specific primer assay as described by Cui *et al*. (2016).

The present study revealed that *COI* gene sequences are also unique for this species and can be used as a DNA diagnostic barcode for the sugar beet cyst nematode. The SP analysis revealed seven *COI* haplotypes for this species.

It has been shown that AFLP results revealed a clear divergence between *H. schachtii* populations from Western Europe, Australia and Africa (Madani *et al*., 2007). Phylogeographic study using 15 microsatellite markers of *H. schachtii* sampled from four continents revealed the existence of cryptic lineages within this species, with the Korean populations comprising one group and the populations from Europe, Australia, North America and western Asia comprising another group (Kim *et al*., 2016). The SP network analysis of the ITS rRNA gene did not show separation of Western Europe, Australia, Africa or South Korean populations. The *COI* gene sequences did not also differentiate these groups, but instead distinguished southern Spanish populations from all other *H. schachtii* populations. Southern Spanish populations of *H. schachtii* have some biological peculiarities. Fournet *et al*. (2018) showed the existence of two distinct groups of the sugar beet cyst nematode populations: one including southern Spain and Morocco populations for which a low temperature (–3°C) strongly reduced hatching rate, and a second one including all other populations where exposure at low temperatures had only a limited impact on subsequent hatching. The survey of *Heterodera* species parasitising the sea beet, *Beta vulgaris* ssp. *maritima*, which is widely distributed all along the European Atlantic and Mediterranean coastlines, revealed that *H. schachtii* seemed to be relatively rare in southern Spain, whereas it was more frequent on the northern coast of Spain, in France, in The Netherlands and Denmark, showing that *H. schachtii* occurred preferentially in northern Europe and therefore in cooler habitats (Gracianne *et al*., 2014). It cannot be excluded that our studied samples from southern Spain belong to an isolated group of populations of *H. schachtii* occurring in natural habitats and having different biology.

Heterodera sonchophila

The sow thistle cyst nematode, *Heterodera sonchophila*, was described from *Sonchus oleraceus* L. from Estonia and also found in Latvia, Poland and Bashkiria, Russia (Subbotin *et al*., 2010). In the present study, the first molecular characterisation of this species is given using ITS rRNA and *COI* genes. This species is related to *H. medicaginis* and an unidentified *Heterodera* sp. 1. parasitising *A. littoralis* from Belgium.

Heterodera trifolii

The clover cyst nematode is considered a pest of several agricultural crops and pasture plants. This species is cosmopolitan, and its host plant list includes many species of Fabaceae and other families. Triantaphyllou & Hirschmann (1978) suggested that *H. trifolii*, which probably represents the basic stock of this parthenogenetic complex, shows extensive diversification with regard to chromosome numbers. It has been suggested that the clover cyst nematode, *H. trifolii*, appeared to be a conglomerate of independently evolving mitotic parthenogenetic populations (Triantaphyllou & Hirschmann, 1978; Wouts & Sturhan, 1978) comprising polyploid and aneuploid forms with 24-35 chromosomes and host races with more or less wide host ranges (Mulvey & Anderson, 1974; Maas *et al*., 1982). With a basic chromosome number of n = 9 for the genus *Heterodera* (Triantaphyllou, 1975), there appeared to be two chromosomal forms within *H. trifolii*: one triploid with 26-28 and another tetraploid with 33-35 chromosomes. In this study we identified this species from *Trifolium repens*, *Plantago* sp., *Rumex* sp. and *Polygonum* sp. having worldwide distributed *COI* haplotype Ht1, which is identical to those in *H. betae* and *H. galeopsidis*. A low level of sequence diversity of these haplotypes might suggest a recent origin for this species.

About the validity of *Heterodera agrostis*

Heterodera agrostis was described from roots of colonial bent grass on Sakhalin Island, Russia, by Kazachenko (1993). It is the only species among the *Schachtii* group that parasitises monocotyledons. The species differs from *H. trifolii* by shorter body and tail length of the second-stage juveniles. Several cysts identified and provided by A. Eroshenko as *H. agrostis* from Sakhalin Island (sample 422) were used for this study. The *COI* gene sequence of this sample was identical to those of the haplotype Ht1/Hb1/Hgal1 and identified here (Table 1) as a representative of *H. trifolii*. Thus, additional morphological and molecular study of *H. agrostis* is still required to verify the validity of this species.

Origin and dispersal of the *Schachtii* group species

In this paper we attempted to analyse and understand distribution patterns of some *Schachtii* group species. Because the ITS rRNA gene sequences contain limited information for phylogeographic analysis, only the *COI* gene marker was used; however, it should be considered that a single marker may not accurately reflect species history. It has been already shown for the *Avenae* group (Subbotin *et al*., 2018) and the genus *Globodera* (Subbotin *et al*., 2020) that some species from these groups originated and diversified in several biodiversity hotspots. Our present study showed that the centre of origin and diversification of some *Schachtii* group species are in biodiversity hotspots located in mountainous regions. Mountains played a key role as refugia for biota during the Neogene and Quaternary global cooling (Fjeldså & Lovett, 1997; Médail & Diadema, 2009; López-Pujol *et al*., 2011). Mountainous and inter-mountain regions could play a critical role for the long-term survival of host plants. These regions had relatively eco-climatic stability along climatic cycles due to continued moisture availability and varied topography providing many sheltered habitats from cold winds and a wide diversity of microhabitats (Fjeldså & Lovett, 1997; Tzedakis *et al*., 2002). Populations of plants and the associated pests occurring in such isolated areas are characterised by high levels of genetic diversity, whereas populations established in other regions show relatively low genetic variation.

In our study we applied the molecular clock hypothesis to estimate divergence time between species and populations in the *Schachtii* group. Our estimation of haplotype diversities may indicate that species of this group originated during the Pleistocene (2.58 million to 11 700 years ago). Splitting of *H. glycines* and *H. schachtii* was estimated at 0.33 million years ago, whereas Radice *et al*. (1988) suggested that these two species diverged between 7 and 15 million years ago. Because many controversies have been focused on the most common use of the clock assumptions, such as the dating of divergence times, our results using estimation divergence dates should be viewed with caution (Subbotin *et al*., 2018).

Based on analysis of species diversity for cyst nematodes, Krall & Krall (1978) suggested a rather wide area of origin for representatives of *Heterodera*, namely Mediterranean, Caucasus and some Middle Asian regions. Twelve from 18 *Schachtii* group species, *i.e.*, *H. cajani*, *H. betae*, *H. ciceri*, *H. daverti*, *H. dunensis*, *H. galeopsidis*, *H. glycines*, *H. mediterranea*, *H. medicaginis*, *H. menthae*, *H. schachtii* and *H. trifolii*, are presently reported from these regions. The results of the S-DIVA, species distribution and haplotype diversity analyses showed that the Western Mediterranean region, including the Iberian mountain ranges, is likely the primary centre of origin and diversification of the *Schachtii* group. This region is a part of the Mediterranean Basin hotspot, which is one of the largest and most important biodiversity hotspots in the world (Blondel *et al*., 2020). Several unique haplotypes of *H. schachtii*, *H. mediterranea* and unidentified and putative new *Heterodera* sp. are associated with the Baetic mountain ranges and adjacent valleys located in the southern and eastern Iberian Peninsula. This range represents a discontinuous chain of mountains of recent origin (8 Mya). This mountain area is one of major centers of species richness and endemism in the Mediterranean Basin (Lobo *et al*., 2001; Thompson, 2005; Médail & Diadema, 2009). This richness was induced by the interaction of environmental factors, including a fragmented orography and contrasting soil and ameliorated climatic conditions during the glacial-interglacial fluctuations of the Pleistocene due to the lower latitude, maritime influence and wide altitudinal range, which led to low extinction rates and increased diversification (Molina-Venegas *et al*., 2013).

Krall & Krall (1978) suggested that the sugar beet cyst nematode parasitises wild beet and cabbage species, which are primary distributed from the Mediterranean to the Asian steppes, and recently moved to cultivated crops. A recent study confirmed that *H. schachtii* parasitises the sea beet, *Beta vulgaris* ssp. *maritima*, a wild beet relative, which is widely distributed all along the European Atlantic and Mediterranean coastlines (Gracianne *et al*., 2014). However, it has been shown that *H. schachtii* occurs preferentially in northern Europe, therefore in cooler habitats. It cannot be excluded that this species originated in the Mediterranean Basin mountains and, after the end of the glaciation period, moved to northern regions. Alternatively, Oro & Tabakovic (2020) suggested that the area across the Dutch-Belgian coastal region is a possible place of origin for the European *H. schachtii* populations, which spread to other countries. Unique *COI* haplotypes found in this region from our study suggest that this region could serve as a secondary centre of diversification for *H. schachtii* and other species. A putative new *Heterodera* sp.1 species from *Atriplex littoralis* was also reported in this coastal region.

The soybean cyst nematode was described by Ichinohe (1952) from Hokkaido, Japan, and then was found in the

United States in 1954 (Winstead *et al*., 1955). Since that time, nematologists have differed in their opinion of the origin of *H. glycines* in the USA. Some of them believe that the nematode is indigenous to North America and is derived from an ancient ancestor once widespread in Asia and North America (Ferris *et al*., 1985). An alternate hypothesis is that *H. glycines* has a more recent history, having been imported in soil from Japan in the late 1800s and early 1900s to provide inoculum of *Bradyrhizobium japonicum* (Kirchner) Jordan for nodulation of soybean (Noel, 1992). The result of the S-DIVA, haplotype diversity and species distribution analyses showed that the possible centre of origin and diversification of *H. glycines* could be North America or Japan. However, other eastern Asian regions also could not be excluded. The territories of northeastern China, Korea, the Japanese archipelago and the Russian Far East belong to the Sino-Japanese Floristic Region, which extends from the eastern Himalayas to the Japanese archipelago through south and central China (Wu & Wu, 1996) and is considered one of the most diverse temperate floras in the world. This diversity was linked to climatic and physiographical complexity and historical environmental changes associated with the Pleistocene (< 2.6 Mya) climatic oscillations (Qian & Ricklefs, 2000). The main Korean mountain range, the Chubu mountain region in Japan, the Changbai mountains and the Daxinganling mountains in China can serve as glacial refugia for some host plants of *H. glycines* in northeastern Asia. Not only geographical isolation but also host plant might have an important influence on the level of genetic diversity. Using microsatellite markers, Wang *et al*. (2015) showed that *H. glycines* was an inbred species that was highly genetically differentiated and the host plant *Rehmannia glutinosa* had an important influence on the genetic differentiation of the populations in China.

After analysis of esterase allozymes of soybean cyst nematode from China, Japan and the USA, Noel *et al*. (1998) concluded that more loci and alleles in populations from China and phylogenetic similarities among populations from Japan and the USA were consistent with a founder effect resulting from dissemination of progenitor *H. glycines* from China to Japan and subsequent introductions of founder populations from Japan to the United States. In our study, haplotype network and tree analysis did not indicate an ancestral haplotype. The presence of unique haplotypes in Japan, South Korea and the Russian Far East, and the haplotypes found in North and South Americas and Asia, including Japan, South Korea and China, suggested that the haplotypes might have survived during cold phases of the Pleistocene in some periglacial refugia, diversifying on the Eurasian continent before the last glacial period with further great spatial expansion. The difference in haplotypes might also indicate multi trans-Pacific dispersal events of *H. glycines* to North America before and after the last glacial period. We believe, that colonisation of North America by *H. glycines* did not occur as the result of human agricultural activity, but was due to earlier natural dispersal events. This nematode has a wide range of suitable host plants in North America and only relatively recently native nematode populations located in different regions inhabiting native plants dispersed to soybean fields. This scenario has already been described by Subbotin *et al*. (2018) for some *Avenae* group species, and it seems to be correct also for *H. medicaginis*.

Hybridisation and polyploidisation within the *Schachtii* group species

Hybridisation and polyploidy are widely considered important sources of novelty in organism evolution. Several studies demonstrate the usefulness of the ITS rRNA gene sequence in tracking hybridisation events and reticular evolution (Wan *et al*., 2014; Xu *et al*., 2017). Our sequence analyses indicated that such events might have occurred within the *Schachtii* group. For example, the SP results might indicate that some Turkish *H. schachtii* populations have recombined ITS rRNA gene sequences that appear to be a result of hybridisation of *H. schachtii* with *H. ciceri*. High similarity between some ITS rRNA gene sequences of *H. glycines* and *H. medicaginis* also suggest possible hybridisation events between these species. The presence of recombination patterns should be studied and tested for the *Schachtii* group species in more detail to exclude the effect of possible incomplete lineage sorting or gene duplication during evolution of this group.

The clover cyst nematode, *H. trifolii*, is considered a conglomerate of independently evolving mitotic parthenogenetic populations (Triantaphyllou & Hirschmann, 1978; Wouts & Sturhan, 1978). These parthenogenetic forms could be regarded as polyploid forms that have evolved from diploid amphimictic relatives of the *Schachtii* group species. Based on the close relationships between parthenogenetic *H. betae* and *H. trifolii* with amphimictic *H. schachtii* and *H. daverti*, two hypotheses for the origin of *H. betae* and *H. trifolii* could be suggested. The first one, formulated by Triantaphyllou & Hirschmann (1978) for the

H. trifolii species complex, states that forms having 35-36 chromosomes (tetraploid) and reproducing by mitotic parthenogenesis have evolved from autopolyploidisation of the diploid, amphimictic unknown species. According to the second hypothesis, this parthenogenetic species originated as a result of hybridisation of two diploid species (Madani *et al*., 2007), *i.e.*, allopolyploidisation. *Heterodera schachtii* or *H. daverti* could be suggested as one of the parental species, whereas the other parental species bearing Ht1/Hb1/Hgal *COI* haplotype is still unknown.

Concerted evolution may not be effective in polyploids, because they are likely to have rRNA genes on non-homologous chromosomes. It would be expected that the ITS rRNA gene may contain more polymorphism for polyploids than for diploids because of divergence of chromosomally distinct nucleolar organising regions (Soltis & Soltis, 2009; Osuna-Mascaró *et al*., 2022). Our ITS sequence data, however, do not show the extensive nucleotide site polymorphism present in *H. trifolii*, and in contrast show more ITS rRNA gene polymorphic sites in diploid *H. schachtii*.

Taxa of hybrid origin should be characterised by a distinctive combination of parental morphological, biological and molecular characters. With respect to molecular characters, a recently derived hybrid species should combine the alleles of its parents but show few, if any, unique alleles. From a morphological perspective, recent hybrids are predicted to be a mosaic of both parental and intermediate morphological characteristics (Soltis & Soltis, 2009), which can be observed for *H. betae* and *H. trifolii*.

The conclusions obtained in our study are based on analysis of three genetic markers and require further confirmation with other molecular and biological datasets. Other geographical regions inadequately sampled for cyst nematodes should be included in future studies. Future whole genome sequence datasets for the *Schachtii* group species will allow testing the hypotheses proposed herein and will give a more distinct pattern of evolutionary relationships between *Heterodera* species and their origin and phylogeography.

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