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

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Summary – The *Humuli* group of the genus *Heterodera* contains species that parasitise dicotyledons and are characterised by a lemon-shaped cyst having a bifenestrate vulval cone (ambifenestrate for *H. fici*), long vulval slit and weak underbridge. Presently, the *Humuli* group includes seven species: *H. amaranthusiae*, *H. fici*, *H. humuli*, *H. litoralis*, *H. ripae*, *H. turcomanica* and *H. vallicola*. In this study we provided comprehensive phylogenetic analyses of *COI* and ITS rRNA gene sequences of species from the *Humuli* group using Bayesian inference, maximum likelihood, and maximum and statistical parsimony. All seven valid species from the *Humuli* group, one putatively new species belonging to this group and the willow cyst nematode, *H. salixophila*, sharing a common ancestor with the *Humuli* group, were analysed. Some 84 *COI* and 5 ITS rRNA new gene sequences from 37 nematode populations collected from 12 countries were obtained in this study. Our results confirmed that the *COI* gene is a powerful DNA barcoding marker for identification of populations and species from the *Humuli* group. Based on the results of phylogeographical analysis and age estimation of clades with a molecular clock approach, it was hypothesised that some species of the *Humuli* group primarily originated and diversified in Western and Middle Asian regions during the Pleistocene and Holocene periods and then dispersed from this region across the world. Two secondary diversification centres of the *Humuli* group were likely located in East and Southeast Asia, Russian Far East, and Oceania.

Keywords – *COI* gene, haplotypes, *Heterodera fici*, *Heterodera humuli*, *Heterodera ripae*, *Heterodera salixophila*, hop cyst nematode, ITS rRNA gene, molecular clock, speciation.

Cyst-forming nematodes (Heteroderidae) are highly derived and considered as agriculturally important plant parasites. The majority of these nematodes belong to the genus *Heterodera* Schmidt, 1871, containing 87 valid species. By using morphological and molecular characteristics, this genus has been divided into nine species groups: *Afenestrata*, *Avenae*, *Bifenestra*, *Cardiolata*, *Cyperi*, *Goettingiana*, *Humuli*, *Sacchari* and *Schachtii*. Four *Heterodera* species (*H. salixophila* Kirjanova, 1969, *H. skohensis* Kaushal, Sharma & Singh, 2000, *H. spinicaudata* Wouts, Shoemaker, Sturhan & Burrows, 1995 and *H. zeae* Koshy, Swarup & Sethi, 1971) are presently not assigned to any of these groups (Subbotin *et al*., 2010, 2021; Handoo & Subbotin, 2018). The *Humuli* group contains species that parasitise dicotyledons and are characterised by a lemon-shaped cyst having a bifenestrate cone (ambifenestrate for *H. fici* Kirjanova, 1954), long vulval slit, weak underbridge and presence or absence of bullae. Presently, the *Humuli* group includes seven species, namely *H. amaranthusiae* Jiang, Hu, Li, Bian, Huang, Gu, Liu, Huang, Kong, Liu, Peng & Peng, 2021, *H. fici*, *H. humuli* Filipjev, 1934, *H. litoralis* Wouts & Sturhan, 1996,

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H. ripae Subbotin, Sturhan, Rumpenhorst & Moens, 2003, *H. turcomanica* Kirjanova & Shagalina, 1965 and *H. vallicola* Eroshenko, Subbotin & Kazachenko, 2001. Only two species from this group are considered as nematode agricultural pests: the hop cyst nematode, *H. humuli* on hop plants and the fig cyst nematode, *H. fici* on fig trees (Subbotin *et al*., 2010).

Subbotin *et al*. (2001) showed that the willow cyst nematode, *H. salixophila*, shared a common ancestor with the *Humuli* group. These relationships were further supported by additional phylogenetic analysis (Handoo & Subbotin, 2018; Rezaee Danesh *et al*., 2020). The corn cyst nematode, *H. zeae* Koshy, Swarup & Sethi, 1971, also clustered with *H. salixophila* and the *Humuli* group species (Handoo & Subbotin, 2018). *Heterodera zeae* and *H. salixophila* are related, but do not belong to the *Humuli* group.

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

Materials and methods

Nematode species and populations

Species and populations from different hosts, localities and countries used in this study are listed in Table 1. A total of 37 nematode populations collected in 12 countries were analysed in this study. Six valid species from the *Humuli* group and one putatively new species belonging to the *Humuli* group were obtained. Samples of *H. salixophila* collected in three locations were also included in this study. Cysts were extracted from soil samples using standard flotation and sieving techniques. Species delimiting of the studied populations was accomplished by integrating results of morphological and morphometric studies, phylogenetic and sequence analysis, as well as analysis of nematode host-plant specificity and geographic distribution of studied samples (Subbotin *et al*., 2010).

Distribution maps

Several published sources and original data were used to create distribution maps for *H. humuli* (López & Romero, 1989; López-Robles, 1995; Tanha Maafi *et al*., 2003; Madani *et al*., 2004; Sturhan, 2006; Subbotin *et al*., 2010; Fatemy *et al*., 2017; Akyazi *et al*., 2019; Yao *et al*., 2021), *H. ripae* (Subbotin *et al*., 1997; Madani *et al*., 2004; Sturhan & Lišková, 2004; Andersson & Manduric, 2006; Sturhan, 2006; López-Robles *et al*., 2011; Wang *et al*., 2012; Yao *et al*., 2021), *H. fici* (Romero *et al*., 1973; Yuksel, 1981; Maqbool *et al*., 1987; Krnjaic *et al*., 1997; Madani *et al*., 2004; Abrantes *et al*., 2008; Subbotin *et al*., 2010; Fanelli *et al*., 2019) and *H. salixophila* (Sturhan & Lišková, 2004; Sturhan, 2006; Subbotin *et al*., 2010; Rezaee Danesh *et al*., 2020).

DNA extraction, PCR and sequencing

DNA extraction, PCR and sequencing were performed as described by Subbotin *et al*. (2018). Two 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 for amplification of the partial *COI* gene and *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 as described by Subbotin *et al*. (2018). The amplified fragments were directly sequenced by Genewiz using the primer pairs for PCR. New sequences were deposited in the GenBank database under accession numbers: MT804359-MT804363 (ITS rRNA gene), MT808338-MT808393, MT813456, MT911890-MT911898, MW279109-MW279122 and ON007076-ON007079 (*COI* gene) as indicated in Table 1, Phylogenetic trees and networks.

Phylogenetic, sequence and phylogeographic analysis

Alignments with the ITS rRNA and *COI* gene sequences were created using ClustalX 1.83 (Chenna *et al*., 2003) with default parameters. New sequences were aligned with corresponding published gene sequences (Eroshenko *et al*., 2001; Subbotin *et al*., 2001, Tanha Maafi *et al*., 2003, Madani *et al*., 2004; Toumi *et al*., 2013; Fatemy *et al*., 2017; Sun *et al*., 2017; Fanelli *et al*., 2019; Rezaee Danesh *et al*., 2020; Darling *et al*., 2021; Jiang *et al*., 2021 and others). Several alignments were created: *i*) ITS rRNA gene alignment containing only reference sequences of the *Humuli* group species and *H. salixophila*; *ii*) ITS rRNA gene alignment containing all sequences of *H. fici*, *H. humuli*, *H. ripae* and

Table 1. Species and populations of cyst nematodes from the *Humuli* group and *Heterodera salixophila* used in the present study.

Species	Location	Host	Sample code	*COI* haplotype	*COI* GenBank accession number	ITS rRNA GenBank accession number	Source and/or reference
H. fici	Iran, Kordestan, Sanandaj	*Ficus elastica*	Zah29	Hfic1	MT808373-MT808375	AF498385	Z. Tanha Maafi; Tanha Maafi *et al.* (2003, 2004)
H. fici	USA, California, Fresno County, Parlier	*F. carica*	CD2067	Hfic1	MT808376	MT804359	S.A. Subbotin
H. fici	Abkhazia, Sukhumi	*F. carica*	CD2963	Hfic2	MT808377	AF274409	Subbotin *et al.* (2001)
H. fici	Greece, Arta	*F. carica*	CD3322	Hfic1, Hfic3	MT911895-MT911898	–	N. Vovlas
H. humuli	Belgium	*Humulis lupulus*	CD2877a, b, c	Hhum1	MT808370, MW279114	–	S.A. Subbotin
H. humuli	Belgium	*H. lupulus*	CD3363a, b, c	Hhum1	MW279115-MW279117	–	S.A. Subbotin
H. humuli	Russia, Chuvash Republic, Tsivilsk	*H. lupulus*	CD2880a, b, CD3368a, b	Hhum1, Hhum2	MW279109, MW279120-MW279122	–	S.A. Subbotin
H. humuli	Russia, Moscow, Jauza river	*Urtica dioica*	37a, c; CDCD3144b-1, CD3144b-4	Hhum1, Hhum2	MT808366, MT808371, ON007076, ON007077	–	S.A. Subbotin
H. humuli	USA, Ohio	*H. lupulus*	CD1303	Hhum2	MT808368	MT804361	S.A. Subbotin
H. humuli	Germany, Münster	*H. lupulus*	CD2885a, b, c	Hhum1, Hhum2	MT808369, MW279118, MW279119	–	D. Sturhan
H. humuli	Germany	*U. dioica*	344	Hhum2	MT808367	–	D. Sturhan
H. humuli	Iran, Mazandaran province, near Amol	*U. dioica*	Zah22	Hhum3, Hhum4	MT808358-MT808365	AF498384	Z. Tanha Maafi; Tanha Maafi *et al.* (2003)
H. humuli	Kyrgyzstan, Kemin district	Unknown	CD2125	Hhum5	MT808372	MT804360	S.A. Subbotin
H. litoralis	New Zealand, Auckland, Glen Innes, the Hahuna Torea Nature Reserve	*Sarcocornia quinqueflora*	111a, CD3257a, b, c	Hlit	MT808378-MT808381	AF274410	W. Wouts; Subbotin *et al.* (2001)
H. ripae	Belgium, West Flanders, Het Zwin, sample 1	*U. dioica*	35a	Hrip1	MT808340	–	S.A. Subbotin
H. ripae	Belgium, West Flanders, Het Zwin, sample 2	*U. dioica*	CD2879	Hrip2	MT808352, MT808353	–	S.A. Subbotin
H. ripae	Belgium	*U. dioica*	CD3370	Hrip1	MW279112	–	S.A. Subbotin
H. ripae	Germany, sample 1	*U. dioica*	58	Hrip1	MT808338	AF274407	D. Sturhan; Subbotin *et al.* (2001)
H. ripae	Germany, sample 2	*U. dioica*	CD2890	Hrip1	MT808342	–	D. Sturhan

Table 1. (Continued.)

Species	Location	Host	Sample code	*COI* haplotype	*COI* GenBank accession number	ITS rRNA GenBank accession number	Source and/or reference
H. ripae	Belgium, Luxemburg province, sample 1	*U. dioica*	29	Hrip1	MT808345	–	S.A. Subbotin
H. ripae	Belgium, Luxemburg province, sample 2	*U. dioica*	CD3226	Hrip1	MT808346	–	S.A. Subbotin
H. ripae	Belgium, Luxemburg province, sample 3	*U. dioica*	CD3371a, b	Hrip1	MW279110, MW279111	–	S.A. Subbotin
H. ripae	UK, St. Albans	*U. dioica*	CD2882a, b	Hrip1, Hrip6	MT808339, MT808347	–	S.A. Subbotin
H. ripae	Russia, Jaroslavl region	*U. dioica*	CD2883a, b	Hrip1	MT808341, MT808344	–	S.A. Subbotin
H. ripae	Russia, Moscow, Jauza river	*U. dioica*	CD3144b-2, CD3144b-3	Hrip1	ON007078, ON007079	–	S.A. Subbotin
H. ripae	Russia, Moscow region, Mytishchinsky District	*U. dioica*	CD2887a, b	Hrip1	MT808343, MW279114	–	S.A. Subbotin
H. ripae	Greece, Epirus	*U. dioica*	CD2873a, b	Hrip3, Hrip4	MT808350, MT808351	AY347927	N. Vovlas; Madani *et al.* (2004)
H. ripae	Germany, sample 3	*U. dioica*	CD1502	Hrip5	MT808354, MT808355	–	D. Sturhan
H. ripae	Russia, Primorsky territory, Ussurijskii natural reserve	*U. laetevirens*	423a	Hrip7	MT808356	AF393840	A.S. Eroshenko; Eroshenko *et al.* (2001)
H. ripae	Slovakia	*U. dioica*	CD2878a, b	Hrip4	MT808348, MT808349	–	D. Sturhan
H. salixophila	Germany	*Salix* sp.	CD2884	Hsal1, Hsal2, Hsal3	MT808384, MT808388-MT808390	–	D. Sturhan
H. salixophila	Ukraine, Kherson	*S. album*	CD2888a, c CD3258a, b, c	Hsal2	MT808383, MT808385-MT808387, MW279113	AF274405, MT804362, MT804363	S.A. Subbotin; Subbotin *et al.* (2001)
H. salixophila	Russia, Moscow	*Salix* sp.	CD3321	Hsal4	MT911890-MT911894	–	V.N. Chizhov
H. salixophila	Belgium, Nieuwpoort	*Salix* sp.	CD3259	Hsal5	MT808391-MT808393	AF274406	S.A. Subbotin; Subbotin *et al.* (2001)
H. turcomanica	Iran, Ardabil, Meshkin shahr	Unknown	Zah42	Ht1	MT813456	AF498386	Z. Tanha Maafi; Tanha Maafi *et al.* (2003, 2004)
H. vallicola	Russia, Primorsky territory, Mikhailovsky district	*Ulmus japonica*	CD2876a	Hv1	MT808357	AF393841	A.S. Eroshenko; Eroshenko *et al.* (2001)
Heterodera sp.1	Australia, Victoria	Unknown	603a	Hsp1	MT808382	–	I. Riley

H. vallicola; *iii*) ITS rRNA gene alignment of *H. salixophila*; *iv*) *COI* gene alignment containing only reference haplotype sequences of the *Humuli* group and *H. salixophila*; and *v*) *COI* gene sequence alignments containing sequences of *H. fici*, *H. humuli*, *H. ripae* or *H. salixophila*. The ITS rRNA gene sequence alignments were manually edited using GeneDoc 2.5.0 (Nicholas *et al*., 1997). 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 and *COI* gene sequence alignments were analysed with maximum likelihood (ML) and maximum parsimony (MP) using PAUP* (Swofford, 2003) 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 network estimation using statistical parsimony (SP) as implemented in POPART software (http://popart.otago.ac.nz) (Bandelt *et al*., 1999). The estimation of divergence times with BEAST 2.4.5 (Bouckaert *et al*., 2014) were performed as described by Subbotin *et al*. (2018). The tree prior a lognormal relaxed clock with uncorrelated rates was 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 one generation per year was considered for *Humuli* group species (Subbotin *et al*., 2010).

Statistical Dispersal-Vicariance Analysis (S-DIVA) as implemented in RASP 4.2 (Yu *et al*., 2015) was used to evaluate the alternative ancestral ranges at each node in the trees. The distribution range of the *Humuli* group species was divided into nine geographical regions (A-I). The S-DIVA tested using a simulated dataset of 100 randomly sampled trees from 18 000 BI trees and a condensed tree. Most likely ancestral regions for each node were mapped on the 50% majority rule consensus BI tree inferred from the analysis of the *COI* sequence alignment.

Results

Phylogenetic and sequence analysis with ITS rRNA gene

Two approaches were applied to analyse the ITS rRNA gene sequences and of the *Humuli* group species. The first approach included BI, ML and MP analyses of ITS rRNA gene alignment, containing only reference sequences of each *Humuli* group species, and the second approach included SP analysis of the alignment of all available in the Genbank and new ITS rRNA gene sequences for selected species.

Humuli group and Heterodera salixophila

Phylogenetic relationships within seven valid species of the *Humuli* group species as inferred from MP, BI and ML analyses of the ITS rRNA gene reference sequences are given in Figure 1. *Heterodera ripae*, *H. vallicola*, *H. humuli* and *H. amaranthusiae* formed a strongly supported clade. *Heterodera litoralis* and *H. fici* clustered together in all trees with moderate or weak supports. *Heterodera turcomanica* had a basal position, and relationships of this species with other representatives of the *Humuli* group were not well resolved.

A total of 24 ITS rRNA gene sequences of the *Humuli* group species were analysed. The ITS2 region of the

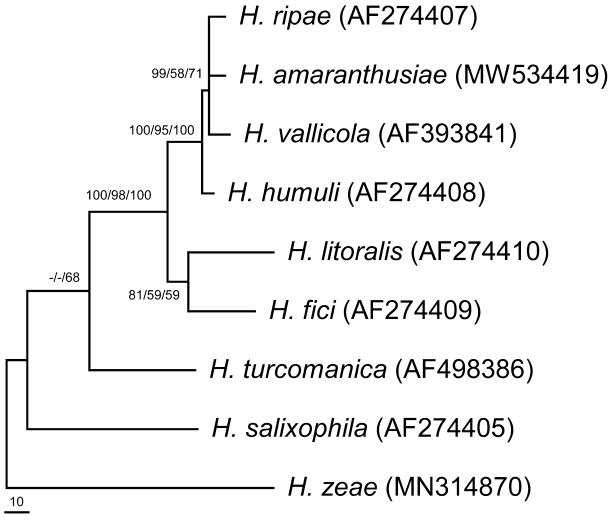

Fig. 1. Phylogenetic relationships between *Heterodera* species from the *Humuli* group as inferred from maximum parsimony (MP) analysis of the ITS rRNA gene sequences. Posterior probability and bootstrap support values for BI, ML and MP analysis are given for appropriate clades, respectively. Values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

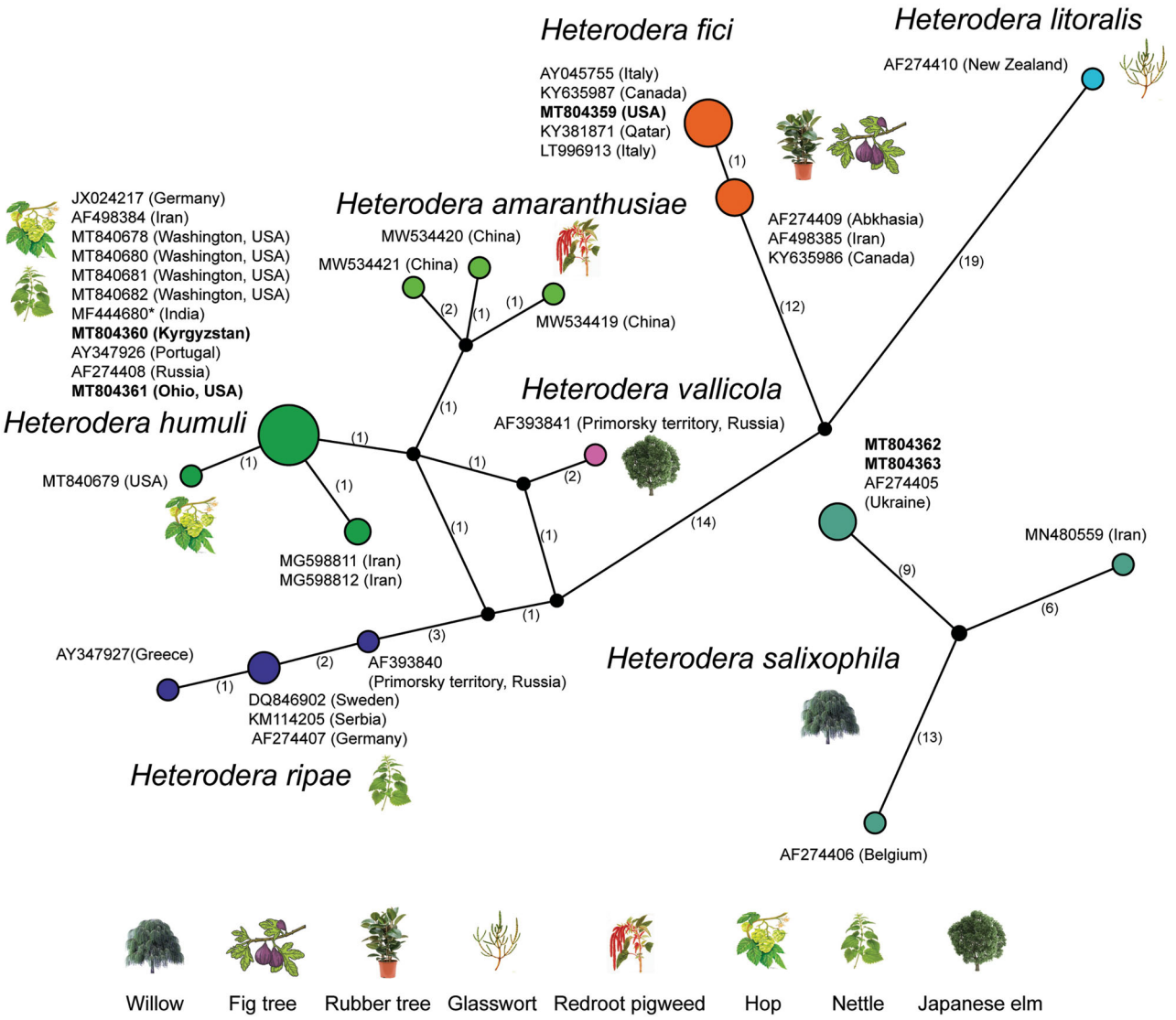

Fig. 2. Statistical parsimony networks showing the phylogenetic relationships between ITS rRNA gene sequences of some *Heterodera* species from the *Humuli* group and *H. salixophila*. The sequences of each species are marked by different colours. Pies (circles) represent sequences of each species with 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 in bold font. * Identified as *H. skohensis* in GenBank.

sequence under the accession number MF444680 and the first 20 and more nucleotides of the sequences under numbers MG598811 and DQ846902 were excluded from the analysis because of the presence of possible reading mistakes. The sequence under accession number MF444680 identified as *H. skohensis* is considered here as *H. humuli*. SP network showing the phylogenetic relationships between ITS rRNA gene sequences of some *Heterodera* species from the *Humuli* group is given in Figure 2. Maximal sequence variation for this group was 7.3%.

A total of five ITS rRNA sequences of *H. salixophila* were analysed. SP network showing the phylogenetic relationships between ITS rRNA gene sequences of some populations of *H. salixophila* is given in Figure 2. Maximal intraspecific variation for this species was 2.3%.

PHYLOGENETIC AND SEQUENCE ANALYSIS WITH *COI* GENE

Two approaches were applied to analyse the *COI* gene sequences of the *Humuli* group species. The first approach included BI and ML analyses of the *COI* gene alignment, containing only reference haplotype sequences of the *Humuli* group species, whilst the second approach included SP analyses of alignments of *COI* sequences for each species.

Heterodera humuli group

A total of 65 new *COI* gene sequences were obtained in this study for the *H. humuli* group species. The *COI* gene alignment was 424 bp. Most nematode populations contained only one *COI* gene haplotype, whereas two haplotypes were found in seven populations. Phylogenetic relationships within the *Humuli* group species and *H. salixophila*, containing 25 reference haplotype sequences as inferred from BI and ML analyses, are given in Figure 3. *Heterodera turcomanica* occupied the basal position in the BI and ML trees.

Heterodera humuli

A total of 26 new *COI* gene sequences were obtained in this study and analysed. Five haplotypes were revealed:

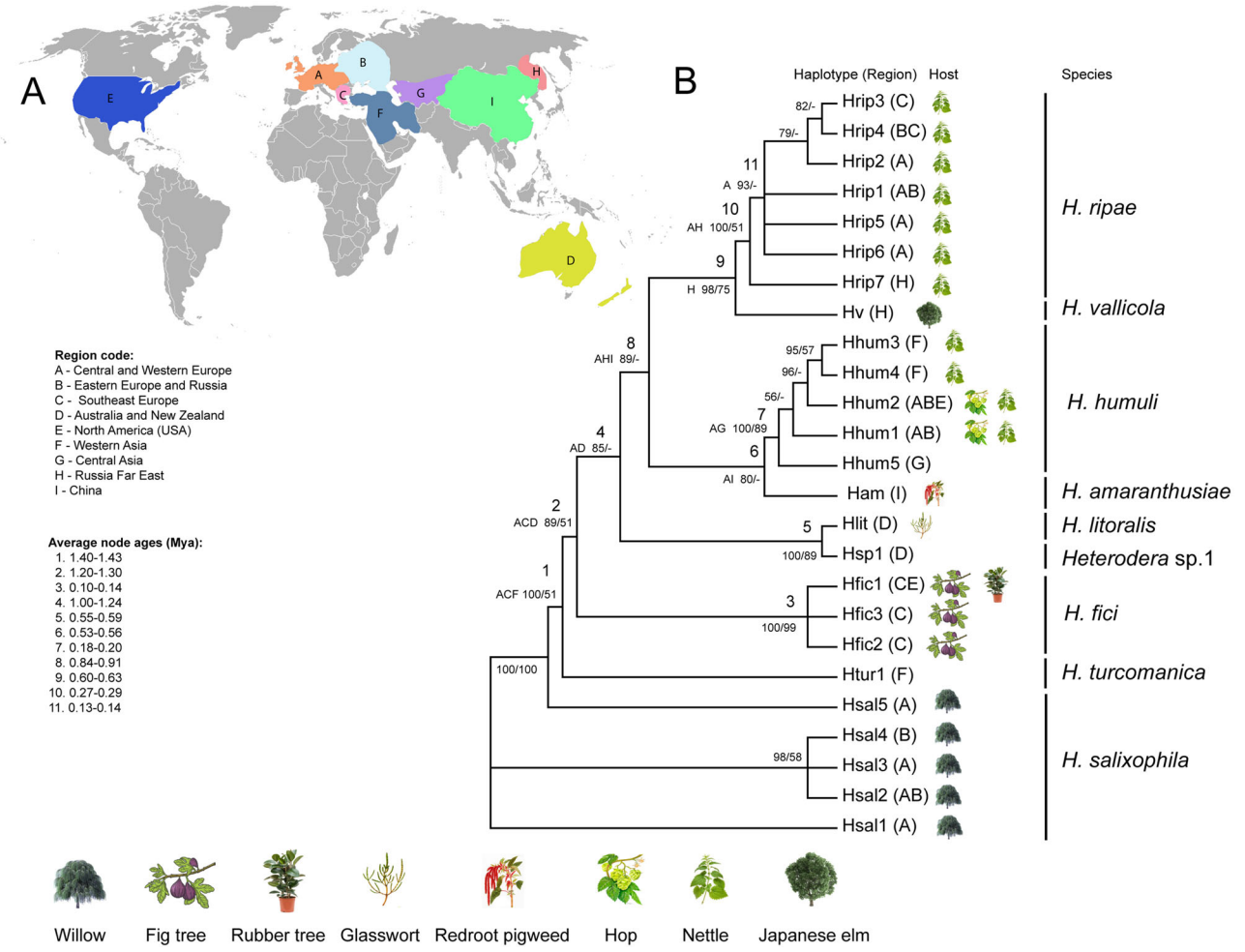

Fig. 3. Phylogenetic relationships between *COI* haplotypes of the *Humuli* group species as inferred from Bayesian analysis with mapping of regions and plant-hosts and indication of node ages. A: World map with region codes; B: Phylogenetic tree. Codes with most probable ancestral regions, posterior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

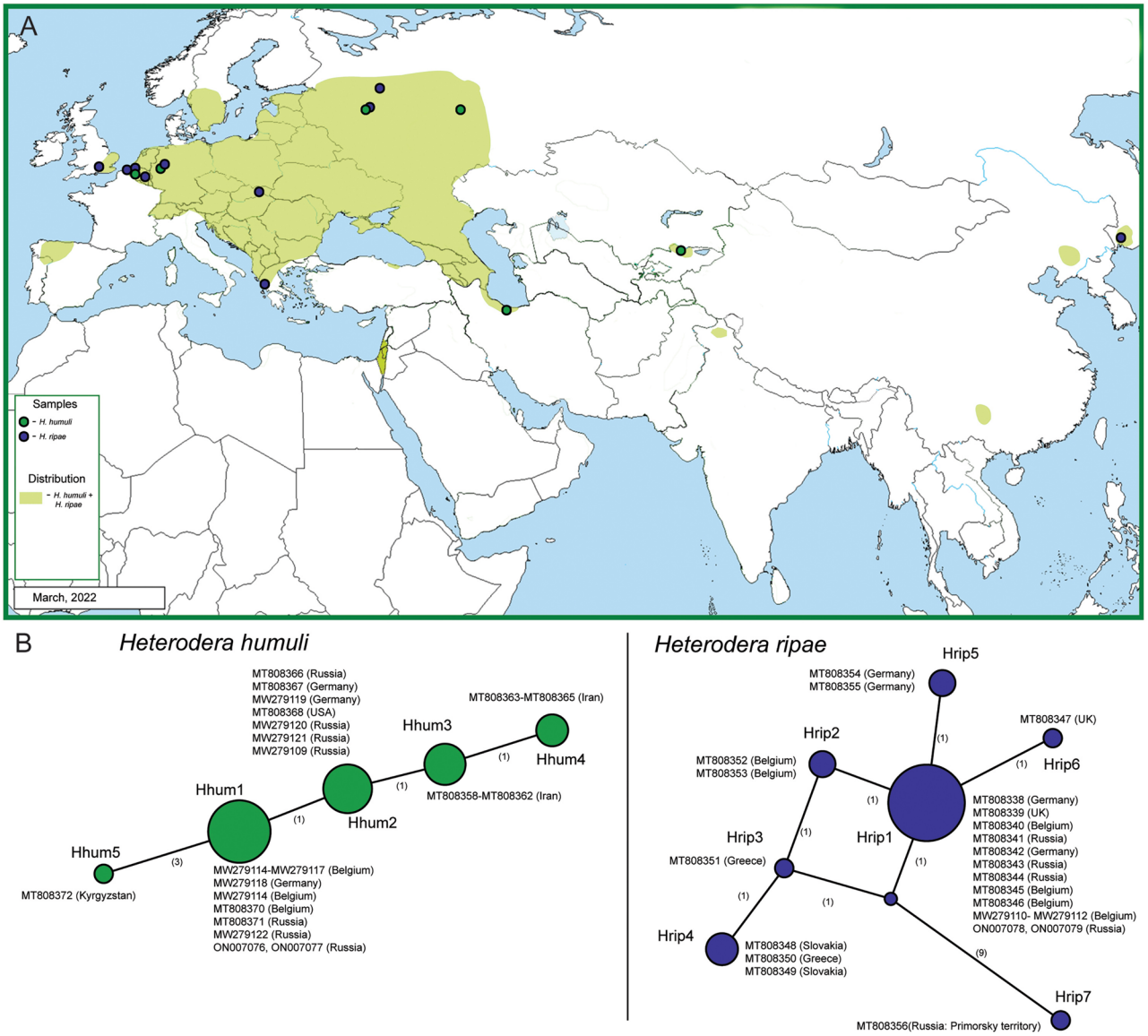

Fig. 4. Distribution maps of *Heterodera humuli* and *H. ripae* with indication of the studied samples; B: Statistical parsimony network showing the phylogenetic relationships between *COI* haplotypes of *H. humuli* and *H. ripae*. Pie chart sizes are proportional to the number of samples with a particular haplotype.

Hhum1 and Hhum2 from Belgium, Germany, Russia, and USA, Hhum3 and Hum4 from Iran, and Hhum5 from Kyrgyzstan (Fig. 4). Maximal intraspecific variation was 1.1%. Sequences of this species from Washington State, USA (MT840683 and MT8406860) were not included in the analysis due to shorter length. They were identical to those of Hhum1 and Hhum2 haplotype sequences.

Heterodera ripae

A total of 24 new *COI* gene sequences were obtained in this study and analysed. Seven haplotypes were revealed: Hrip1 from Belgium, Germany, Russia, UK, Hrip2 from Belgium, Hrip 3 from Greece, Hrip4 from Greece and Slovakia, Hrip5 from Germany, Hrip6 from the UK, and Hrip7 from Russian Far East (Fig. 4). Maximal intraspecific variation was 2.6%.

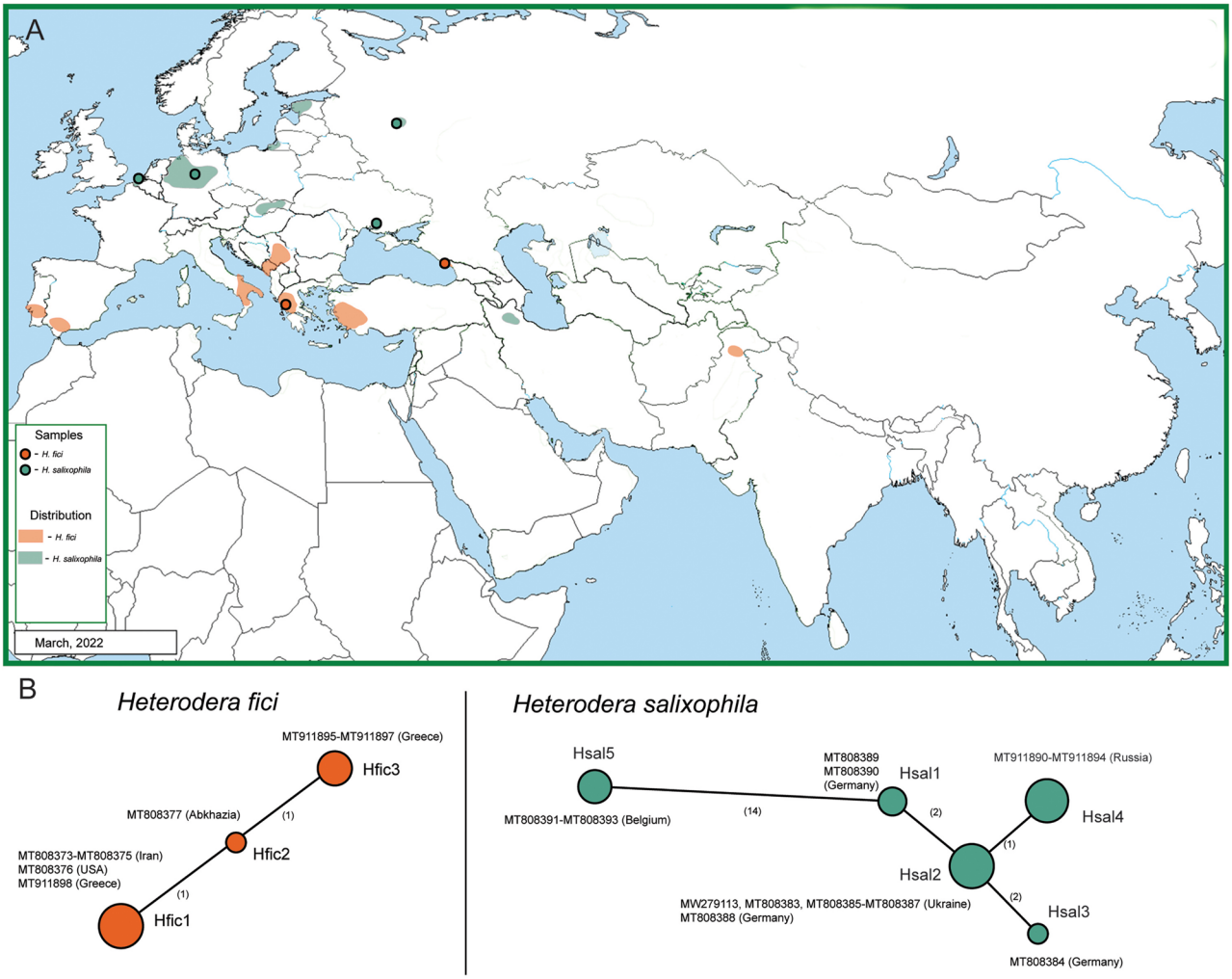

Fig. 5. Distribution maps of *Heterodera fici* and *H. salixophila* with indication of the studied samples; B: Statistical parsimony network showing the phylogenetic relationships between *COI* haplotypes of *H. fici* and *H. salixophila*. Pie chart sizes are proportional to the number of samples with a particular haplotype.

Heterodera fici

A total of nine new *COI* gene sequences were obtained in this study and analysed. Three haplotypes were revealed: Hfic1 from USA, Greece and Iran, Hfic2 from Abkhazia, and Hfic3 from Iran (Fig. 5). Maximal intraspecific variation was 0.5%.

Heterodera turcomanica, H. vallicola and Heterodera sp.1

These species were represented by one sequence for each.

Heterodera litoralis

Three similar sequences were obtained from this species.

Heterodera salixophila

A total of 17 new *COI* gene sequences were obtained in this study and analysed. Five haplotypes were revealed: Hsal1 and Hsal3 from Germany, Hsal2 from Germany and Ukraine, Hsal4 from Russia, and Hsal5 from Belgium (Fig. 5). Maximal intraspecific variation was 3.5%.

PHYLOGEOGRAPHICAL ANALYSIS AND MOLECULAR CLOCK

The ancestral ranges to each node in a tree were evaluated accounting for phylogenetic uncertainty and uncertainty in DIVA optimisation. Most likely one or several ancestral areas were mapped on the BI majority consensus tree (Fig. 3). Ancestral areas for the *Humuli* group clade were Europe and Western Asia. Central and Western Europe with Russian Far East and China are considered likely as ancestral for the clade with *H. ripae*, *H. vallicola*, *H. humuli* and *H. amaranthusiae*. The likelihood ratio test rejected the hypothesis of the Global Molecular Clock test at $P < 0.001$. The tree topology retrieved from BEAST software contradicted a tree yielded by MrBayes in the position of several clades. Estimated node ages for some main clades are given in Figure 3. The earliest divergence within the *Humuli* group was estimated at 1.40-1.43 Mya (million years ago). It further split into clades with *H. ripae* at 0.27-0.29 Mya; *H. humuli* at 0.18-0.20 Mya.

Discussion

The results of the present study confirmed that the ITS rRNA and *COI* gene markers are able to clearly differentiate all species within the *Humuli* group. The study showed that each species has unique *COI* sequences or DNA barcodes and these enable its identification and separation from all other species.

Phylogenetic relationships reconstructed using ITS rRNA and *COI* gene sequences for species of the *Humuli* group in this study are generally congruent to each other and similar to those published in previous works (Eroshenko *et al*., 2001; Subbotin *et al*., 2001; Tanha Maafi *et al*., 2003; Subbotin, 2010; Subbotin & Skantar, 2018; Rezaee Danesh *et al*., 2020). In this study we reconstructed phylogenetic relationships within the *Humuli* group using BI and ML analysis of *COI* gene sequences for the first time. Several clades had high or moderate statistical support and detected subclades correspond to separated groups in the SP haplotype network. Differences in the position of some species between phylogenetic trees were mainly due to weakly supported clades. According to our phylogenetic analyses, the *Humuli* group constituted from four distinct evolutionary lineages (subgroups): *i*) *H. humuli*, *H. ripae*, *H. vallicola* and *H. amaranthusiae*; *ii*) *H. litoralis* and *Heterodera* sp.1 from Oceania; *iii*) *H. fici*; and *iv*) *H. turcomanica* occupying a basal position within the *Humuli* group. In the ITS rRNA gene trees, the two species *H. salixophila* and *H. zeae* (Skantar *et al*., 2012; Subbotin & Skantar, 2018) clustered with the *Humuli* group with a high statistical support. These species do not belong to this group.

The hop cyst nematode, *H. humuli*, and *H. ripae* are the most widely distributed species in the *Humuli* group. *Heterodera ripae* was described as a sibling species of *H. humuli*, differentiating from it by several minor morphological characters, PCR-ITS-RFLP profiles and ITS-rRNA gene sequences (Subbotin *et al*., 1997, 2001). This species parasitises *Urtica* spp., and does not infect hop, whereas *H. humuli* parasitises both plants, nettle and hop. It is an interesting fact that on common nettle growing along a bank of the Jauza river in Moscow, Russia (Fig. 6), cysts of *Heterodera humuli* and *H. ripae* were found in 1:1 ratio. Although these species inhabit similar ecological niches and exhibit large overlap in geographical distribution, it seems that *H. humuli* colonises more regions. *Heterodera ripae* is not presently reported from America and Western and Central Asia, but this species is common in Europe (Subbotin *et al*., 1997), was also found in Primorsky territory, Russia (Eroshenko *et al*., 2001), and recently in southwest China together with *H. humuli* (Yao *et al*., 2021). The results of our study also showed that European and Asian populations of these two species have distinct *COI* haplotypes in the SP network. *COI* haplotypes of *H. humuli* from Iran are different from those from Europe and America and that from Kyrgyzstan. The *COI* haplotype of *H. ripae* from the Russian Far East is well separated from European *COI* haplotypes.

The fig cyst nematode, *H. fici*, was reported infesting common fig trees, *Ficus carica*, in several Mediterranean countries and southwest Asia. This species was also found on rubber tree, *F. elachista*, which is native to eastern parts of South Asia and southeast Asia. However, all these findings of *H. fici* were reported from potted rubber trees only (Subbotin *et al*., 2010).

Heterodera litoralis was described from native vegetation in South Island, New Zealand. The host of this species is beaded glasswort, *Sarcocornia quinqueflora* (Ung.-Sternb) A.J. Scott, a succulent species of the Chenopodiaceae which is abundant in New Zealand and is found along the coast near the high tide mark, in salt marshes, on coastal rock platforms and in rock crevices (Subbotin *et al*., 2010). The results of our *COI* gene sequence analy-

Fig. 6. Common nettle growing along a bank of the Jauza river in Moscow, Russia, and infected with *H. humuli* and *H. ripae*.

sis revealed that cysts provided by I. Riley from Victoria, Australia represent a new putative species, *Heterodera* sp.1, closely related to *H. litoralis*. The plant host of this species is unknown.

Heterodera turcomanica was reported from Turkmenistan and Iran only. In the ITS-rRNA (Tanha Maafi *et al*., 2003) and *COI* gene (present study) phylogenetic trees, this species occupied a basal position within the *Humuli* group. The plant host of this species is also unknown.

The willow cyst nematode, *H. salixophila*, was likely distributed from northwestern Europe to Iran on *Salix* spp. It has been shown that populations of *H. salixophila* can differ by PCR-ITS-RFLP with *Rsa*I restriction patterns. This enzyme restricted the ITS regions of the Belgian populations, but not that of the Ukrainian one (Subbotin *et al*., 2000). These populations were also different in the ITS rRNA gene sequences (Subbotin *et al*., 2001). The present study also confirmed a deep split of Belgian populations of this species collected along North Sea beaches from other European populations using *COI* gene sequences and makes intraspecific variations for both genes highest for *H. salixophila* than in any species of the *Humuli* group.

Krall & Krall (1978) suggested a rather wide area of origin for representatives of *Heterodera*, namely the Mediterranean, Caucasus and some Middle or Central Asian regions. It has been shown, based on the results of phylogeographical analysis and age estimation of clades with a molecular clock approach, that several species of the *Avenae* group of the genus *Heterodera* primarily originated and diversified in the Irano-Anatolian region during the Pleistocene and Holocene periods and then dispersed from this region across the world (Subbotin *et al*., 2018). Present phylogeographical analysis gave a high level of uncertainty for a Europe to Far East origin for the *Humuli* group. Evidently, the biogeographic datasets used in our study are collected with worldwide sampling

bias and do not reflect entire natural species distribution. Although discussion on centre of speciation is rather speculative, we can suggest a Western and Middle Asian origin of the *Humuli* group species that occurred during the Pleistocene and Holocene periods. In these regions, *H. humuli* and *H. turcomanica* are presently reported and the latter species occupies a basal position within the *Humuli* group in phylogenetic trees. Phylogeographical analysis also proposed two other secondary centres of diversification of the *Humuli* group, one in the Far East (*H. humuli*, *H. ripae*, *H. vallicola* and *H. amaranthusiae*) and another in Oceania (*H. litoralis* and *Heterodera* sp.1).

Nematology surveys conducted in different world regions would give more light on the time of origin and locations of centres and subcentres of diversification of this species group. Comprehensive sampling and experimental studies are required to provide robust evidence for or against the hypotheses raised in this study.

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