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Summary – Cyst nematodes of the genus *Heterodera* are obligatory sedentary endoparasites of great economic importance throughout the world. The *Goettingiana* group of this genus consisted of 17 species parasitising dicotyledons and are characterised by a lemon-shaped cyst having an ambifenestrate cone, long vulval slit, weak underbridge and presence or absence of bullae. In this study, we provided comprehensive phylogenetic analyses of 164 *COI* and 108 ITS rRNA gene sequences of several species of the *Goettingiana* group, including *H. carotae, H. circeae, H. cruciferae, H. goettingiana, H. microulae, H. persica* and *H. urticae* and several unidentified species using Bayesian inference, maximum likelihood, and maximum and statistical parsimony. 126 new *COI* and 46 new ITS rRNA gene sequences from 45 nematode populations representing six valid and two unidentified species collected in 13 countries were obtained in this study. *Heterodera scutellariae* syn. n is considered as a synonym of *H. circeae* based on similar molecular and morphological characters. The carrot cyst nematode *H. carotae* is reported in France on a wild carrot species growing in natural ecosystems and in California from an agricultural field for the first time. The nettle cyst nematode in Spain and France is also reported for the first time. Our study showed that the ITS rRNA gene

sequence can be used for discrimination of some species from the *Goettingiana* group; however, it did not allow differentiating *H. carotae, H. cruciferae, H. urticae* belonging to the *H. cruciferae* species complex from each other. The *COI* gene sequences clearly distinguished all studied species of the *Goettingiana* group from each other and can be recommended as a DNA barcoding marker for this group. It has been hypothesised that the majority of the *Goettingiana* group species originated and diversified in regions located in Western and Eastern Asia and Central and Western Europe during the Pleistocene and then dispersed from these regions across the world.

Keywords – *COI* gene, haplotypes, *Heterodera carotae, Heterodera circeae, Heterodera cruciferae, Heterodera goettingiana, Heterodera urticae,* molecular clock, speciation.

Cyst forming nematodes of the genus *Heterodera* Schmidt, 1871 are obligatory sedentary endoparasites of great economic importance throughout the world. 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 *Goettingiana* group contains species that parasitise dicotyledons and are characterised by a lemon-shaped cyst having an ambifenestrate cone, long vulval slit, weak underbridge and presence or absence of bullae. Species of this group are differentiated from each other in morphology and morphometrics of the second-stage juveniles and cyst structures.

The *Goettingiana* group consisted of seventeen species: *H. amygdali* Kirjanova & Ivanova, 1975, *H. bergeniae* Maqbool & Shahina, 1988, *H. carotae* Jones, 1950, *H. circeae* Subbotin & Sturhan, 2004, *H. cruciferae* Franklin, 1945, *H. glycyrrhizae* Narbaev, 1987, *H. goettingiana* Liebscher, 1892, *H. johanseni* (Sharma, Kaushal, Singh, Pande, Pokharel & Upreti, 2001) Sturhan, 2002, *H. kirjanovae* Narbaev, 1988, *H. menthae* Kirjanova & Narbaev, 1977, *H. microulae* Li, Li, Ni, Peng, Liu, Luo & Xu, 2020, *H. persica* Tanha Maafi, Sturhan, Subbotin & Moens, 2006, *H. plantaginis* Narbaev & Sidikov, 1987, *H. scutellariae* Subbotin & Sturhan, 2004, *H. turangae* Narbaev, 1988, *H. urticae* Cooper, 1955 and *H. uzbekistanica* Narbaev, 1980. Only three species from this group are considered as nematode of agricultural or economic importance in different countries: the carrot cyst nematode *H. carotae*, the cabbage cyst nematode *H. cruciferae* and the pea cyst nematode *H. goettingiana* (Sykes & Winfield, 1966; Mugniery & Bossis, 1988; Greco *et al.*, 1991; Subbotin *et al.*, 2010; Sasanelli *et al.*, 2013; Montarry *et al.*, 2024).

Accurate diagnosis of pests is the first step in selecting appropriate control measures. New diagnostic tools must be also validated against the widest genetic diversity, including, if possible, populations outside the cultivated compartment, sampled from wild host plants related to commercial cultivars. Indeed, these wild populations of pests may act as reservoir of genetic diversity and initiate local crop epidemics (Gracianne *et al*, 2016).

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 were used to discriminate species including some representatives of the *Goettingina* group. Szalanski *et al.* (1997) and Sabo *et al.* (2001) were the first to provide ITS rRNA gene sequences for *H. goettingiana* and *H. carotae*, respectively. Using the ITS rRNA gene sequences, Subbotin *et al.* (2001) reconstructed phylogenetic relationships within the genus *Heterodera*, including the *Goettingiana* group containing *H. goettingiana, H. carotae, H. urticae* and *H. cruciferae*. Phylogenetic analyses resolved some of relationships within the *Heterodera* groups and placed the *Goettingiana* group at the basal position of the genus phylogeny. Although DNA sequences of some species of this group collected from different world regions were also obtained and analysed by several other authors (Madani *et al.*, 2004, 2018; Subbotin & Sturhan 2004; Chizhov *et al.* 2009; Sasanelli

et al. 2013; Vovlas *et al.* 2017; Yu *et al.* 2017; Escobar-Avila *et al.* 2018), comprehensive phylogenetic and sequence analysis of these nematodes have been not conducted.

The main goals of this study were to: i) analyse phylogenetic relationships within selected species of the *Goettingiana* group species using sequences of ITS rRNA and partial *COI* genes; ii) provide intra-specific diversity knowledge on the recognised species of the *Goettingiana* group using partial *COI* gene sequences and iii) propose and test hypotheses of the origin and distribution of the *Goettingiana* 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. A total of 45 nematode populations of seven nominal species and two unidentified species collected in 13 countries were analysed. There are: *H. carotae, H. circeae, H. cruciferae, H. goettingiana, H. urticae, H. scutellariae* syn. n., *H. persica* and unidentified: *Heterodera* sp. 6 and *Heterodera* sp. 7. Samples were taken with a shovel from the upper 30 cm of soil. Cysts were extracted from soil samples using flotation and sieving techniques (Coolen, 1979) in the CDFA Nematology lab and the IAS-CSIC lab. Species identification was made using morphological and molecular methods. 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 reconstruct distribution maps for *H. carotae, H. cruciferae, H. goettingiana* and *H. urticae* (Stone & Course, 1974; Mathews, 1975; Stone & Rowe, 1976; Krall, 1977; Anon, 2003; Subbotin *et al*., 2010; Sasanelli *et al.*, 2013; Jabbari *et al.* 2015; Mehalaine *et al.*, 2020; Toktay *et al.*, 2022).

DNA EXTRACTION, PCR AND SEQUENCING

For molecular analyses, nematode DNA was extracted from single cysts using proteinase K. Crushed cyst with second-stage juveniles and eggs or crushed juvenile and males were transferred to an Eppendorf tube containing 18 μl distilled water, 2 μl 10 x PCR buffer and 2 μl proteinase K (600 μg/ml) (Promega, USA). The tubes were incubated at 65 °C (1 h) and then at 95 °C (15 min). PCR and sequencing were completed in two laboratories: CDFA, USA and IAS-CSIC, Spain. All protocols were described by Subbotin *et al.* (2018) and Castillo *et al*. (2003), respectively. Several primers for amplification of ITS rRNA and partial *COI* gene were used in the present study (Table 2). Sequencing was performed at Azenta

(CA, USA) and the Stab Vida sequencing facilities (Caparica, Portugal). New sequences were deposited in the GenBank database under accession numbers: MW361365, PV138305-PV138349 (ITS rRNA gene) and PV132996-PV133010, MW363061-MW363090, PV134933-PV134974, PV134896-PV134932, PV138973, PV138974 (*COI* gene) as indicated in Table 1 and phylogenetic networks and trees.

REAL-TIME PCR IDENTIFICATION OF *HETERODERA CAROTAE*

Cyst nematode samples of *H. carotae, H. cruciferae, H. circeae* and *H. urticae* type B were submitted for additional testing using Real-time TaqMan PCR with *H. carotae* specific primers (Hca-83-F, Hca-194-R) and probe (Hca-128P) developed by Fouville *et al.* (2024) (Table 2). Real-time PCR assay was performed using the SensiFast Probe Lo-ROX Kit (Bioline, USA). The reaction was performed in a total volume of 20 µl containing 10 µl 2X SensiFast Probe Lo-ROX, 1 µl of each primer (10 µM) (table 2), 0.4 µl of Taqman probe (10 µM), 5.6 µl nuclease-free water, and 2 µl of DNA and carried out in Applied Biosystem QuantStudio 7 Flex Real-Time PCR system. Real-time PCR temperature profile was with an initial denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 10 sec, annealing/extension at 60 °C for 60 sec. PCR amplicons were also run on agarose gels for band verification.

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 (Subbotin *et al.*, 2001; Tanha Maafi *et al.* 2003; Madani *et al.*, 2004, 2018; Subbotin & Sturhan, 2004; Chizhov *et al.*, 2009; Sasanelli *et al.*, 2013; Toumi *et al.*, 2013; Vovlas *et al.*, 2017; Yu *et al.*, 2017; Escobar-Avila *et al.*, 2018). Several alignments were created: *i*) ITS rRNA gene sequence alignment containing reference sequences of ten species of the *Goettingiana* group; *ii*) ITS rRNA gene sequence alignment containing new and published sequences of the *Goettingiana* group, *iii*) *COI* gene sequence alignment containing reference haplotype sequences of valid and undescribed species of the *Goettingiana* group; *iv*) *COI* gene alignments containing sequences of four species: *H. carotae, H. circeae, H. cruciferae, H. goettingiana, H. urticae* and *Heterodera* sp.6.

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* 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 *COI* haplotypes were identified based on the SP results. 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 *Goettingiana* group species (Subbotin *et al.,* 2010).

COI gene polymorphism was estimated for some species. 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 9 geographical regions (A-I), 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.

Results

Real-time PCR identification of *Heterodera carotae*

Real-time TaqMan PCR tests with species-specific primers for detection of the carrot cyst nematode showed strong amplification signals (Ct=24-32) and distinct bands on gels for all *H. carotae* samples only. No signals and no bands were obtained from the samples of other non-target species: *H. cruciferae, H. circeae* and *H. urticae* type B. The sample (CD2404) previously identified as *H. cruciferae* by Escobar-Avila *et al.* (2018) from California showed a strong signal amplification (Ct=24-26) in three replicates, where each replicate was obtained from a single cyst. This sample was assigned here as *H. carotae.*

Phylogenetic and sequence analysis with ITS rRNA gene

Phylogenetic relationships within seven valid and three undescribed species of the *Goettingiana* group species as inferred from MP, BI and ML analyses of the ITS rRNA gene reference sequences are given in Figure 1. The ITS rRNA gene sequence alignment containing reference sequences of these ten species of the *Goettingiana* group was 1053 bp in length. Phylogenetic analyses revealed the presence of several clades (groups) within the *Goettingiana* group: *i*) *H. carotae, H. cruciferae, H. urticae, Heterodera* sp. 6 and *Heterodera* sp. 7; *ii*) *H. circeae, H. microulae, H. persica* and *H. goettingiana; iii*) *Heterodera* sp.A. Relationships between these clades were poorly resolved.

A total of 108 ITS rRNA gene sequences of seven nominal species and three undescribed species of the *Goettingiana* group were analysed in this study. Forty-six new ITS rRNA gene sequences were obtained in the present study. The ITS rRNA gene sequence alignment containing new and published sequences of the *Goettingiana* group was 982 bp in length and included 24 sequences of *H. carotae*, 2 sequences of *H. circeae* (=*H. scutellariae* syn. n.), 22 sequences of *H. cruciferae*, 38 sequences of *H. goettingiana*, 7 sequences of *H. microulae*, 8 sequences of *H. urticae*, 2 sequences of *Heterodera* sp. A. and one sequence for each of the following three species: *H. persica*, *Heterodera* sp. 6 and *Heterodera* sp. 7. Maximal sequence variation for the ITS rRNA gene between species of the *Goettingiana* group reached 8.7%, for *H. goettingiana* – 1.7% and *H. microulae* – 0.7%. The SP network showing the phylogenetic relationships among ITS rRNA gene sequences of species listed above are given in Figure 2. The ITS rRNA gene sequences of *H. goettingiana* belonged to the central Hg1 haplotype and two peripheral haplotypes, whereas sequences of the *H. cruciferae* species complex represented the central Hs1 and 17 peripheral haplotypes (Fig. 2).

PHYLOGENETIC AND SEQUENCE ANALYSIS WITH *COI* GENE

A total of 126 new *COI* gene sequences were obtained in this study. The *COI* gene sequence alignment containing reference haplotype sequences of valid and undescribed species of the *Goettingiana* group was 499 bp in length. Phylogenetic relationships within the *Goettingiana* group species containing 41 reference haplotype sequences from this group and two sequences of outgroup species as inferred from BI and ML analyses are given in Figure 3. Relationships between the clades were poorly resolved. Most of the described and undescribed species appeared as monophyletic groups, except for samples identified as *H. urticae* which split into two separate lineages. The parameters of *COI* gene polymorphism for some species are given in Table 3. Values of Tajima's D-test obtained for several species indicated that *COI* gene evolved neutrally.

Heterodera carotae

A total of 48 sequences of *H. carotae* from 16 populations were analysed. The *COI* gene sequence alignment was 499 bp in length. Eleven *COI* haplotypes were revealed. The geographical distribution of *H. carotae* is illustrated in Figure 4A and the haplotype phylogenetic network is given in Figure 4B. The Hca1 (14 sequences), Hca2 (10 sequences), and Hca3 (8 sequences) haplotype were widely distributed. The haplotype Hca4 was found in Canada, Ontario, the haplotypes Hca5, Hca8, Hca9 and Hca10 in Italy, Hca6 in South Africa, Hca7 in France and Hca11 in France and Denmark. Cysts with Hca1 and Hca3 haplotypes were found from carrot (*Daucus carota sativus*) and wild carrot (*D. carota gummifer*) plants. Maximal intraspecific sequence diversity was 2.2%.

Heterodera cruciferae

A total of 35 sequences of *H. cruciferae* from 8 populations were analysed. The *COI* gene sequence alignment was 472 bp in length. Only two *COI* haplotypes Hcr1 and Hcr2 were revealed. The geographical distribution of *H. cruciferae* is illustrated in Figure 5A and the haplotype phylogenetic network is given in Figure 5B. Maximal intraspecific sequence diversity was 0.6%.

Heterodera circeae

Four sequences of *H. circeae* and two sequences of *H. scutellariae* syn. n., which is considered here as a synonym of *H. circeae*, were included in this study. The *COI* gene sequence alignment was 424 bp in length. Three *COI* haplotypes were revealed. The haplotype phylogenetic network is given in Figure 5C. Maximal intraspecific sequence diversity was 0.5%.

Heterodera goettingiana

A total of 51 sequences of *H. goettingiana* from 12 populations were analysed. The *COI* gene sequence alignment was 441 bp in length. Ten *COI* haplotypes were revealed. The geographical distribution of *H. goettingiana* is illustrated in Figure 6A and the haplotype phylogenetic network is given in Figure 6B. The haplotypes Hgo1, Hgo2, Hgo3 and Hgo5 were found in Italy, Hgo4 in Germany, Hgo6, Hgo9, Hgo10 in Spain, Hgo7 in France and Hgo8 in Iran. Cysts belonging to the Hgo1 haplotype were found from pea (*Pisum sativum*), broad bean (*Vicia faba*) and lucerne (*Medicago lupulina*), whereas the Hgo8 haplotype was collected from white clover (*Trifolium repens*). Maximal intraspecific sequence diversity reached 11.4%.

Heterodera urticae

Eighteen new sequences identified as *H. urticae* from 3 populations were obtained in this study. The sequences collected in Spain and Morocco were assigned to *H. urticae* type A, whereas sequences from a sample found in France were classified as *H. urticae* type B. The geographical distribution of *H. urticae* is illustrated in Figure 7A. A total of 16 sequences of *H. urticae* type A were analysed. The *COI* gene sequence alignment was 393 bp in length. The *COI* haplotype phylogenetic network for type A is given in Figure 7B. Maximal intraspecific sequence diversity for *H. urticae* type A was 1.3%. Three sequences of *H. urticae* type B were identical. Sequence differences between A and B types were 10.5-11.0%.

Heterodera persica

Two sequences from one sample belonging to two *COI* haplotypes were obtained. Intraspecific sequence diversity was 2.5%.

Heterodera sp. 6 and *Heterodera* sp. 7

Five sequences belonging to five *COI* haplotypes were revealed for *Heterodera* sp. 6. The *COI* gene sequence alignment was 424 bp in length. The haplotype phylogenetic network for this undescribed species is given in Figure 7C. Intraspecific sequence diversity was 1.9%. Two sequences of *Heterodera* sp. 7 were assigned to two *COI* haplotypes, which were different in 1 bp (0.2%).

PHYLOGEOGRAPHICAL ANALYSIS AND MOLECULAR CLOCK

The ancestral areas were reconstructed by S-DIVA and ancestral areas were mapped on the BI majority consensus tree (Fig. 3). The most probable ancestral area occupied by the ancestor for the *Goettingina* group encompassed Western Asia, Central and Western Europe and Eastern Asia (China). The ancestral area for the clade with *H. goettingiana* + *H. carotae* + *H. cruciferae* + *H. urticae* + *Heterodera* sp.B. + *Heterodera* sp.6 + *Heterodera* sp.7 was suggested with highest probability encompassing Western Asia. Central, Western and Southern Europe, and Western Asia were suggested as ancestral areas for *H. carotae* and *H. cruciferae*. Estimated node ages for some main clades are given in Figure 3. The earliest divergence within the *Goettingiana* group was estimated at 0.65 Mya (million years ago). It further split into clades with *H. goettingiana* at 0.38 Mya, *H. carotae* + *H. cruciferae* - 0.24 Mya and *H. persica* + *H. microulae* - 0.20 Mya (Fig. 3).

Discussion

The results of the present analysis of the ITS rRNA gene sequences showed that this marker can be used for discrimination of some species within the *Goettingiana* group. The SP analysis of this DNA fragment clearly differentiated *H. circeae, H. microulae, H. persica,* and *H. goettingiana* from each other and from the *H. cruciferae* species complex. However, three species *H. carotae, H. cruciferae, H. urticae* from the *H. cruciferae* species complex cannot be distinguished from each other using ITS rRNA gene sequences. The *COI* gene sequences clearly differentiated most of the studied species of the *Goettingiana* group from each other and can be recommended as a DNA barcoding marker for this group. The studied *COI* gene region yielded phylogenetic resolution on more recent temporal and finer geographical scales than could be achieved with the ITS rRNA gene. However, this *COI* gene region does not allow estimating deeper phylogenetic relationships between species.

HETERODERA CAROTAE

So far, the carrot cyst nematode was only found from cultivated and wild carrots belonging to several subspecies of *Daucus carota* and *D. pulcherrimus* (family Apiaceae) and on other plants from the genus *Torilis*, including *T. leptophylla* under controlled experiments in the laboratory, greenhouse or in the field (Jones, 1950; Mugniéry & Bossis, 1988; Montarry *et al.*, 2024). However, there was no reason for assuming that *H. carotae* is present only in the field and this study demonstrates for the first time the existence of populations in natural ecosystem of the north-west of France close to the seashore on the wild carrot *D. carota* subsp. *gummifer*. Presently, *H. carotae* is found in most carrot-growing regions worldwide including several European countries, USA (Michigan and California), Canada (Ontario), Mexico, South Africa and Chile (Mathews, 1975; Mugniery & Bossis, 1988; Berney & Bird, 1992; Subbotin *et al.*, 2010; Yu *et al.*, 2017; Escobar-Avila *et al.*, 2018; Gautier *et al.*, 2019; Shubane *et al.*, 2021; Wram *et al.*, 2022; Montarry *et al.*, 2024).

In this study, sixteen populations of *H. carotae* from seven countries were analysed. Although eleven *COI* haplotypes were identified from these samples, the maximal *COI* sequence diversity was estimated only at 2.2%. This is consistent with the results of Esquibet *et al*. (2020) who used the microsatellite markers to explore the genetic structure of *H. carotae* populations in a French carrot-growing area located in west Normandy and highlighted a strong gene flow among populations. Such a pattern appears to be common for cyst nematode populations occurring in cultivated areas, where low genetic differentiation suggests a strong connection between populations likely due to soil transport associated with planting, harvesting and soil management practices carried out by processing factories (Gracianne *et al*. 2016).

Three of these sixteen populations were sampled on the wild relative *D. carota* Gummifer and belonged to two of the three most common haplotypes Hca1 and Hca 3. Sharing similar *COI* haplotypes between populations from cultivated and wild carrots may indicate gene flow between these two compartments. Wild *Daucus* species are known to be widespread in the temperate parts of the Northern Hemisphere and most commonly in the Mediterranean region. Although our sampling effort for wild *Daucus* was limited to the northwestern France, it could be hypothesised that the carrot cyst nematode shared a long evolutive history with these wild carrots and only more recently transferred to cultivated carrots by places. These initial corresponding populations may then have spread across Europe and beyond through human crop trade and selection. For instance, Gracianne *et al*. (2014) showed that *Heterodera betae* which was already described as a crop species was commonly distributed all along the European sea shore on the wild sea beet *Beta maritima*. The hypothesis of a Mediterranean origin of *H. carotae*, closely tied to the evolutionary history of its host plant was also well supported by our phylogenetic analysis and molecular clock estimates which suggest the Western Asia, Western and Southern Europe as ancestral areas for *H. carotae*.

Heterodera carotae is morphologically similar to *H. cruciferae* and *H. urticae*, which are different from each other and the carrot cyst nematodes in minor morphometric characters of second-stage juveniles and

males. *Heterodera carotae*, *H. cruciferae, H. urticae* and four unidentified species *Heterodera* spp. 6, 7, A and B are recently diverged species and considered here as representatives of the *H. cruciferae* species complex. The ITS rRNA gene sequence analysis showed very high molecular similarity between these species (Subbotin *et al*., 2001; Madani *et al*., 2004). Recently, using a genome region containing microsatellites, Fouville *et al*. (2024) have developed two primer sets targeting specifically *H. carotae* and *H. cruciferae* and validated them through real-time PCR protocols. Species-specific real-time PCR assay for *H. carotae* has been used to verify identification of some samples from our collection and this test revealed that a sample from California previously identified as *H. cruciferae* by Escobar-Avila *et al*. (2018) indeed belonged to *H. carotae*. It confirmed a notice made by Huston *et al.* (2022) who suggested this sample might be misidentified. Thus, it becomes the first report of the carrot cyst nematode in California.

HETERODERA CIRCEAE

Two species of cyst-forming nematodes: the enchanter's nightshade cyst nematode *H. circeae* from *Circaea lutetiana* (family Onagraceae) and the skullcap cyst nematode *H. scutellariae* from *Scutellaria galericulata* (family Lamiaceae) were described in woodlands in Germany by Subbotin and Sturhan (2004). The ITS rRNA gene sequences of *H. circeae* and *H. scutellariae* were very similar and differed from each other by only three nucleotides situated in the ITS1. These species were distinguished from each other in shape of the stylet knobs, which was rounded in *H. scutellariae* and slightly concave anteriorly in *H. circeae* (Subbotin & Sturhan, 2004). The analysis of sequences of the ITS rRNA and *COI* genes and the analysis of intraspecific variation of stylet knob shapes for some cyst nematode species showed that these characters could be considered in a range of intraspecific variations for this nematode group. Thus, these species should be suggested as co-specific, and *H. scutellariae* syn. n. is considered here as a synonym of *H. circeae.*

HETERODERA CRUCIFERAE

Heterodera cruciferae was first reported from the United Kingdom by Franklin (1945). The cabbage cyst nematode infects many species of *Brassica* (family Brassicaceae), including common agricultural crops such as cabbage, Brussels sprouts, cauliflower, broccoli and radish. The Brassicaceae is a large plant family with more than 330 genera and 3,700 species and shows a worldwide distribution, although most of the taxa are found in temperate regions of the Northern Hemisphere (Lysak & Koch, 2011). Presently, *H. cruciferae* was found in many European counties including Armenia, and also in Azerbaijan, Iran, Pakistan, Turkey, Libya, USA and Australia (Stone & Rowe, 1976; Subbotin *et al*., 2010). The cabbage cyst nematode could be considered as one of the globally distributed species from the *Goettingiana* group. Findings of two similar *COI* haplotypes only in all samples collected from different world regions might

indicate a recent dispersal of this species, which most likely occurred after the last glacial maximum and continued during the Holocene.

HETERODERA GOETTINGIANA

The pea cyst nematode was isolated and described from pea (*Pisum sativum*) and vetch (*Vicia sativa*) growing in fields of the Agricultural Institute at Göttingen, Germany. *Heterodera goettingiana* parasitises many leguminous plants (family Fabaceae) and two species of the genus *Sonchus: S oleraceus* and *S. asper* (family Asteraceae) (Vovlas, 2005; Subbotin *et al.*, 2010; Vovlas *et al.*, 2017). This nematode is reported from a number of European counties and also from Israel, Japan, Jordan, Turkey, Algeria, USA and Iran (Thorne, 1961; Stone & Course, 1974; Di Vito & Greco, 1986; Handoo *et al.*, 1994; Tanha Maafi *et al.*, 2003). In the USA, this species has limited distribution in Washington state or reported from greenhouses in other states (Thorne, 1961; Handoo *et al.*, 1994). Reports of *H. goettingiana* from China require confirmation. Some findings of cyst nematodes initially identified as *H. goettingiana* in this country indeed belong to *H. microulae* or an unidentified *Heterodera* sp. A.

Vovlas *et al.* (2017) showed that the ITS rRNA and *COI* gene sequences clearly discriminated *H. goettingiana* from other species of the *Goettingiana* group. Intraspecific variation for the ITS rRNA gene sequences was found very low, but up to 5.0% for *COI* gene sequences (Volvas *et al.*, 2017). Our study has revealed deep and extensive lineage diversity of *H. goettingiana.* Maximal *COI* intraspecific sequence diversity of this species was the highest (11.4%) among the species of the *Goettingiana* group, whereas the ITS rRNA gene sequence variation reached 1.7%. Intraspecific *COI* gene sequence variation found in our study for *H. goettingiana* is in a range of known intraspecific variations of this gene for other cyst nematodes reported in centers of their origin and diversification. For example, maximal intraspecific *COI* gene sequence diversity for *H. filipjevi* was estimated at 10.6% in the Irano-Anatolian region, Iran (Subbotin *et al.*, 2018), *G. pallida* - 20.7% and *G. rostochiensis* - 14.2% in Andes, Peru, and Bolivia (Subbotin *et al.*, 2020) and *H. mediterranea* - 8.1% in Iberian Peninsula, Spain (Subbotin *et al.*, 2024).

The analysis of highly concordant *COI* gene sequences, with a conservative rate calibration, indicated Pleistocene origin and indicated that this species is the oldest one from this group. The most plausible explanation for the patterns emerging from the *COI* analysis is that this species survives in different locations through periods of climatic changes. Drastic climate oscillations during past glacial and interglacial periods have facilitated genetic diversification for this species.

HETERODERA URTICAE

The nettle cyst nematode was morphologically described by Cooper (1955) from common nettle and then redescribed by Mathews (1970) based on preserved type specimens and additional specimens collected

from Northern Ireland. This species can be distinguished from the closely related *H. carotae* and *H. cruciferae* by minor morphometric differences of the second-stage juveniles. Only two species of the genus *Urtica* are known as hosts for *H. urticae*. In addition to Northern Ireland, this species is also widely distributed in UK, Belgium, Germany and Slovakia (Subbotin *et al.*, 2010). The nettle cyst nematode is reported in Spain and France for the first time in this study. However, we identified two molecular types of *H. urticae*. The first type A was closely related to *H. carotae* and *H. cruciferae* but another type B was more distantly related and represented by a single haplotype (HurB1) according to the *COI* gene phylogeny. This last haplotype was only found in a population isolated in the Southwest of France from *Urtica* sp. plants. Significant differences in *COI* gene sequences between these types may indicate on present of two sibling species under this specific name. Morphological and molecular analysis of additional samples of this species from the type locality and different European regions should be done to confirm this hypothesis.

HETERODERA PERSICA

The species was described by Tanha Maafi *et al.* (2006) and was isolated from soil and roots of Persian hogweed, *Heracleum persicum* (family Apiaceae) in a pastureland near a river located in the Alborz Mountains north of Tehran, Iran. There is no other information on distribution of this species. Two *COI* gene sequences obtained from one sample showed some differences.

UNIDENTIFIED SPECIES *HETERODERA*

Phylogenetic and sequences analysis of the ITS rRNA and *COI* gene sequences suggested the presence of four putative species: *Heterodera* spp. 6 and 7, *Heterodera* A and B within the *Goettingiana* group, which belongs to the *H. cruciferae* species complex. Two first species (*Heterodera* spp. 6 and 7) were found in Iran and previously molecularly characterised using the ITS rRNA gene by Tanha Maafi *et al*. (2003). The *COI* gene sequences obtained in this study confirmed their separated species status. The cyst nematode originally identified by Ou *et al*. (unpublished; the GenBank records: EU623623 and EU623624) as *H. goettingiana* in China, Qinghai province, Huangzhong county, Xinzhuang village from an unknow host is assigned here as *Heterodera* sp. A. This putative species showed differences in the ITS rRNA gene sequences from those of *H. goettingiana* and other representatives of the *Goettingiana* group. The cyst nematodes originally identified by Escobar-Avila *et al.* (2018) (GenBank record: MG563235) as *H. carotae* from a carrot culture from Italy is assigned here as putative species *Heterodera* sp. B. This sample required further molecular and morphological study.

BIOGEOGRAPHY OF THE *GOETTINGIANA* GROUP SPECIES

Several of our phylogeographic studies that focused on the cyst nematodes have proposed different centers of origin and diversification for these nematodes. These included the Irano-Anatolian region for the *Avenae* group of *Heterodera*, the Western Cape in South Africa, the Andes in South America, North and South Island mountains in New Zealand for *Globodera*, Western and Middle Asian regions for the *Humuli* group of *Heterodera* and the Mediterranean Basin and Sino-Japanese floristic region for the *Schachtii* group of *Heterodera*. The cyst nematode distribution patterns may also indicate other centers of origin and diversification in mountains of Central Africa for some *Heterodera* species and the Sierra Madre Mountain region in Mexico for *Cactodera* (Subbotin *et al.*, 2018, 2020, 2022, 2024).

Present phylogeographical analysis gave a high level of uncertainty for origin of the *Goettingiana* group from Central and Western Europe to Western and Eastern Asia. Only a limited number of samples were used in this study, and they do not reflect the entire natural distribution of the species from this group. Eleven species (*H. amygdali*, *H. bergeniae*, *H. cruciferae*, *H. glycyrrhizae*, *H. goettingiana*, *H. kirjanovae*, *H. menthae*, *H. persica*, *H. plantaginis*, *H. turangae* and *H. uzbekistanica*) of the presently valid 16 species that comprise the *Goettingiana* group have been reported or described from the Central Asian region, including the countries of Iran and Pakistan. Only three of them (*H. cruciferae*, *H. goettingiana* and *H. persica*) were molecularly characterized. Further cyst nematode surveys should be conducted in different world regions to obtain new datasets to identify centers of diversification of this species group.

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

Species	Location	Host	Sample code	*COI* haplotype	GenBank accession number		Source and/or reference
					COI gene	ITS rRNA gene	
Heterodera carotae	Italy, Barletta-Andria-Trani province, Barletta	*Daucus carota*	CJ96-CJ99	Hca8, Hca9	PV134924-PV134927	PV138340, PV138347	P. Castillo
H. carotae	Portugal, Alfândega da Fé, Braganza	*D. carota*	CM63, CM64	-	-	PV138342, PV138343	P. Castillo
H. carotae	France, Pays de la Loire, Sarthe, Montfort le Génois	*D. carota sativus*	CD4245, Hca 72-01	Hca2	PV134917, PV134919	PV147185	S. Fournet
H. carotae	France, Provence Alpes Côte d'Azur, Vaucluse, Carpentras	*D. carota sativus*	CD4251, Hca 84-01	Hca7	PV134915, PV134916	PV138344, PV138345	S. Fournet
H. carotae	France, Bretagne, Finistère, Plouguerneau	*D. carota gummifer*	CD4242, Fr-15-02	Hca1	PV134905, PV134909, PV134910	PV138346	S. Fournet
H. carotae	France, Bretagne, Finistère, Plouescat	*D. carota sativus*	CD4249, Hca 29-02	Hac1	PV134907, PV134911	PV138348	S. Fournet
H. carotae	Switzerland, Valais, Fully	*D. carota sativus*	CD4247, Hca Ch-01	Hca1	PV134906, PV134908	PV138349	S. Fournet
H. carotae	France, Bretagne-Finistère, Saint Hernot	*D. carota gummifer*	CD4241, Fr-12-04	Hca3	PV134897, PV134899, PV134902	-	S. Fournet
H. carotae	France, Auvergne Rhône Alpe, Ain Feillens	*D. carota sativus*	CD4248, Hca 01-01	Hca3	PV134898, PV134900	-	S. Fournet
H. carotae	France, Occitanie, Gard, Lédenon	*D. carota sativus*	CD4250, Hca 30-01	Hca3	PV134901	-	S. Fournet
H. carotae	France, Provence Alpes Côte d'Azur, Bouches du Rhône, Lambesc	*D. carota sativus*	CD4252, Hca 13-03	Hca11	PV134904, PV134913	-	S. Fournet

H. carotae	France, Bretagne, Finistère, Guissény	*D. carota gummifer*	CD4240, Fr-14	Hca1	PV134912, PV134918	-	S. Fournet
H. carotae	Denmark, Sjaelland, Holbaek	*D. carota sativus*	CD4253, Hca Dk-2	Hca11	PV134914	-	S. Fournet
H. carotae	Chile	Unknown	CD4256	Hca1	PV134903	-	S.A. Subbotin
H. carotae	Italy, Bari	Unknown	CD3306	Hca10	MW363076, MW363077	-	V.N. Chizhov
H. carotae	USA, California, Santa Barbara County, Cuyama	Potato field	CD2404	Hca2	MW363078, MG563228, MG563230		S.A. Subbotin, Escobar-Avila *et al.* (2018)
H. circeae (=*H. scutellariae* syn. n.)	Germany, Everinghausen	*Scutellaria galericulata*	547	Hci3	MW363064, MW363065	AY368995	D. Sturhan, Subbotin *et al.* (2004)
H. circeae	Germany, Münster, Nienberge	*Circaea lutetiana*	548, 578	Hci2, Hci1	MW363061-MW363063, MW363066	AY368994	D. Sturhan, Subbotin *et al.* (2004)
H. cruciferae	UK	Unknown	208	Hcr2	MW363071	-	J. Rowe
H. cruciferae	USA	Unknown	CD1388	Hcr2	MW363075	-	S.A. Subbotin
H. cruciferae	Russia, Moscow region	*Brassica oleraceae*	CD3307	Hcr2, Hcr1	MW363069, MW363072, MW363073	GU126667, GU126668	V.N. Chizhov, Chizhov *et al.* (2009)
H. cruciferae	USA, California, Yuba County	Unknown	CD2865	Hcr2	MW363074	-	S.A. Subbotin
H. cruciferae	USA, California, Yuba County	Unknown	CD2866	Hcr2	MW363070	-	S.A. Subbotin
H. cruciferae	Italy, Taranto province, Castellaneta	*B. oleraceae*	CJ67, CJ69, CJ81-CJ83	Hcr1, Hcr2	PV134920-PV134922, PV134928, PV134929	PV138341	P. Castillo
H. cruciferae	Italy, Barletta-Andria-Trani province, Barletta	*B. oleraceae*	CK01	Hcr2	PV134923	PV138316	P. Castillo
H. cruciferae	USA	Unknown	CD2720	Hcr2	PV134896	-	S.A. Subbotin

H. goettingiana	Iran, Lorestan, Doroud, Akbarabad	*Trifolium repens*	Zah11	Hgo8	MW363086	AF498374	Z. Tahna Maafi, Tahna Maafi *et al.* (2003)
H. goettingiana	France, Antibes	Unknown	CD3123	Hgo7	MW363087, MW363088	MW361365	S.A. Subbotin
H. goettingiana	Germany	Unknown	567	Hgo4	MW363085	-	D. Sturhan
H. goettingiana	Spain	*Pisum sativum*	DB65-DB68	Hgo9, Hgo10	PV138973, PV138974	PV138305-PV138308	M. Córdoba-Sánchez
H. goettingiana	Italy, Bari province, Bari	*P. sativum*	CH24-CH31	Hgo1, Hgo3	PV134944-PV134949, PV134958, PV134959	PV138318, PV138319, PV138323	P. Veronico
H. goettingiana	Italy, Bari province, Monopoli	*Medicago lupulina*	CG74-CG76, CG78	Hgo1	PV134938-PV134941	PV138311, PV138329	N. Greco, Vovlas *et al.* (2017)
H. goettingiana	Italy, Bari province, Monopoli	*Vicia faba*	CJ33-1	Hgo1	PV134942	PV138322	N. Greco, Vovlas *et al.* (2017)
H. goettingiana	Italy, Bari province, Monopoli	*P. sativum*	CJ36, CJ37, CJ39-CJ41	Hgo1	PV134933-PV134937	PV138315, PV138325, PV138333	N. Greco, Vovlas *et al.* (2017)
H. goettingiana	Italy, Fasano, Brindisi province, Torre Canne	*P. sativum*	CJ50, CJ51, CJ53, CJ57, CJ58	Hgo1, Hgo2, Hg5	PV134954-PV134957, PV134943	PV138312-PV138314, V138327	J.E. Palomares-Rius
H. goettingiana	Spain, Sevilla province, Utrera	*P. sativum*	CJ86-CJ90	Hgo6	PV1349496-PV134953	PV138317, PV138330-V138332	J.E. Palomares-Rius
H. goettingiana	Italy, Bari province, Monopoli	*P. sativum*	CA21-CA35	Hgo2	PV134960-PV134974	PV138320, PV138324, V138326	P. Veronico
H. goettingiana	Italy, Taranto province, Castellaneta	*P. sativum*	CJ82, CJ83	-	-	PV138321, PV138328	P. Castillo

H. urticae type A	Spain, Córdoba province, Córdoba	*Urtica* sp.	CS37, CS39, CS42-CS46, CS52- CS61	HurA1, HurA2	PV132996-PV133010	PV138334-PV138339	J.E. Palomares-Rius
H. urticae type A (=*Heterodera* sp. 8)	Morocco	Unknown	427	HurA3	MW363084	AY347918	S. Amiri, Madani *et al.*, (2004)
H. urticae type B	France, Nouvelle Aquitaine, Pyrénées Atlantiques,Viodos	*Urtica* sp.	Fra 64559, CD4243	HurB1	PV134930-PV134932	-	S. Fournet
H. persica	Iran, Tehran, Dizin	*Heracleum persicum*	Zah12, Zah25	Hpe1, Hpe2	MW363067, MW363068	AF498377	Z. Tahna Maafi, Tahna Maafi *et al.* (2003)
Heterodera sp. 6	Iran, Mazandaran, Khorramabad	Unknown	Zah3	Hsp64, Hsp65, Hsp61	MW363079, MW363080, MW363081,	AF498375	Z. Tahna Maafi, Tahna Maafi *et al.* (2003)
Heterodera sp. 6	Iran, Mazandaran, Amol	Unknown	Zah28	Hsp62, Hsp63	MW363082, MW363083	-	Z. Tahna Maafi, Tahna Maafi *et al.* (2003)
Heterodera sp. 7	Iran, Western Azerbaijan, Urumieh	Unknown	Zah16	Hsp71, Hsp72	MW363089, MW363090	AF498376	Z. Tahna Maafi, Tahna Maafi *et al.* (2003)

Table 2. Primer and probe sets used in the present study.

Primer code	Primer and probe set (5' > 3')	Gene	Reference
TW81	GTT TCC GTA GGT GAA CCT GC	ITS rRNA gene	Joyce *et al.* (1994)
AB28	ATA TGC TTA AGT TCA GCG GGT		
Het-coxiF	TAG TTG ATC GTA ATT TTA ATG G	*COI* gene	Subbotin (2015)
Het-coxiR	CCT AAA ACA TAA TGA AAA TGW GC		
JB3	TTT TTT GGG CAT CCT GAG GTT TAT	*COI* gene	Bowles *et al.* (1992)
JB4	TAA AGA AAG AAC ATA ATG AAA ATG		
JB3	TTT TTT GGG CAT CCT GAG GTT TAT	*COI* gene	Bowles *et al.* (1992)
JB5	AGC ACC TAA ACT TAA AAC ATA ATG AAA ATG		Derycke *et al.* (2005)
Hca-83-F	CCA AAG TCC ATC CTC AGC AT	microsatellite	Fouville *et al.* (2024)
Hca-194-R	ATG TCC AAA AAT GCG TGT CC	loci	
Probe_Hca-128P	[FAM]* CGA ACC ACC TCC TGC AT CCC [BHQ-1]**		

* reporter; ** quencher.

Table 3. Polymorphism of the *COI* gene fragment for some cyst nematode species from the *Goettingiana* 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 carotae	48	11	14	0.845 ± 0.030	0.00770 ± 0.00089	-1.10117*
H. circeae	6	3	2	0.800 ± 0.122	0.00276 ± 0.00058	1.03194*
H. cruciferae	35	2	1	0.511 ± 0.025	0.00292 ± 0.00014	1.63955*
H. goettingiana	51	10	54	0.773 ± 0.041	0.05070 ± 0.00541	-0.54127*
H. urticae type A	16	3	4	0.492 ± 0.117	0.00540 ± 0.00123	0.61302*
Heterodera sp. 6	5	5	8	1.000 ± 0.126	0.01061 ± 0.00220	0.29610*

* - P > 0.10

Fig. 1. Phylogenetic relationships between *Heterodera* species from the *Goettingiana* group as inferred from Bayesian (BI) 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 network showing the phylogenetic relationships between ITS rRNA gene sequences of some *Heterodera* species from the *Goettingiana* 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. * - identified as *H. goettingiana* in the GenBank.

Fig. 3. Phylogenetic relationships between *COI* haplotypes of the *Goettingiana* 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 with probability, posterior probability values for BI analysis and bootstrap values for ML analysis more than 50% are given to appropriate clades. * - identified as *H. carotae* in the GenBank (MG563235) and by Escobar-Avila *et al.* (2018).

Fig. 4. A: Distribution map of *Heterodera carotae* 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. Small black circles represent missing haplotypes. New sequences are indicated in bold. * - identified as *H. pratensis* in the GenBank; ** - identified as *H. cruciferae* in the GenBank and by Escobar-Avila *et al.* (2018).

Fig. 5. A: Distribution map of *Heterodera cruciferae* with indication of the studied samples of *H. cruciferae* and *H circeae*; B: Statistical parsimony network showing the phylogenetic relationships between *COI* haplotypes of *H. cruciferae* (A) and *H. circeae* (C). Pie (circle) sizes are proportional to the number of

samples with a particular haplotype. New sequences are indicated in bold. * - identified as *Heterodera cf. urticae* in the GenBank.

Fig. 6. A: Distribution map of *Heterodera goettingiana* with indication of the studied samples for this species; 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. Small black circles represent missing haplotypes. New sequences are indicated in bold.

Fig. 7. A: Distribution map of *Heterodera urticae* type A with indication of the studied samples; B: Statistical parsimony network showing the phylogenetic relationships between *COI* haplotypes of *H. urticae* type A (B) and *Heterodera* sp. 6 (C). Pie (circle) sizes are proportional to the number of samples with a particular haplotype. Small black circles represent missing haplotypes. New sequences are indicated in bold.

Figure 1

Click here to access/download;Figure;Fig1_ITS-ref-final.jpg

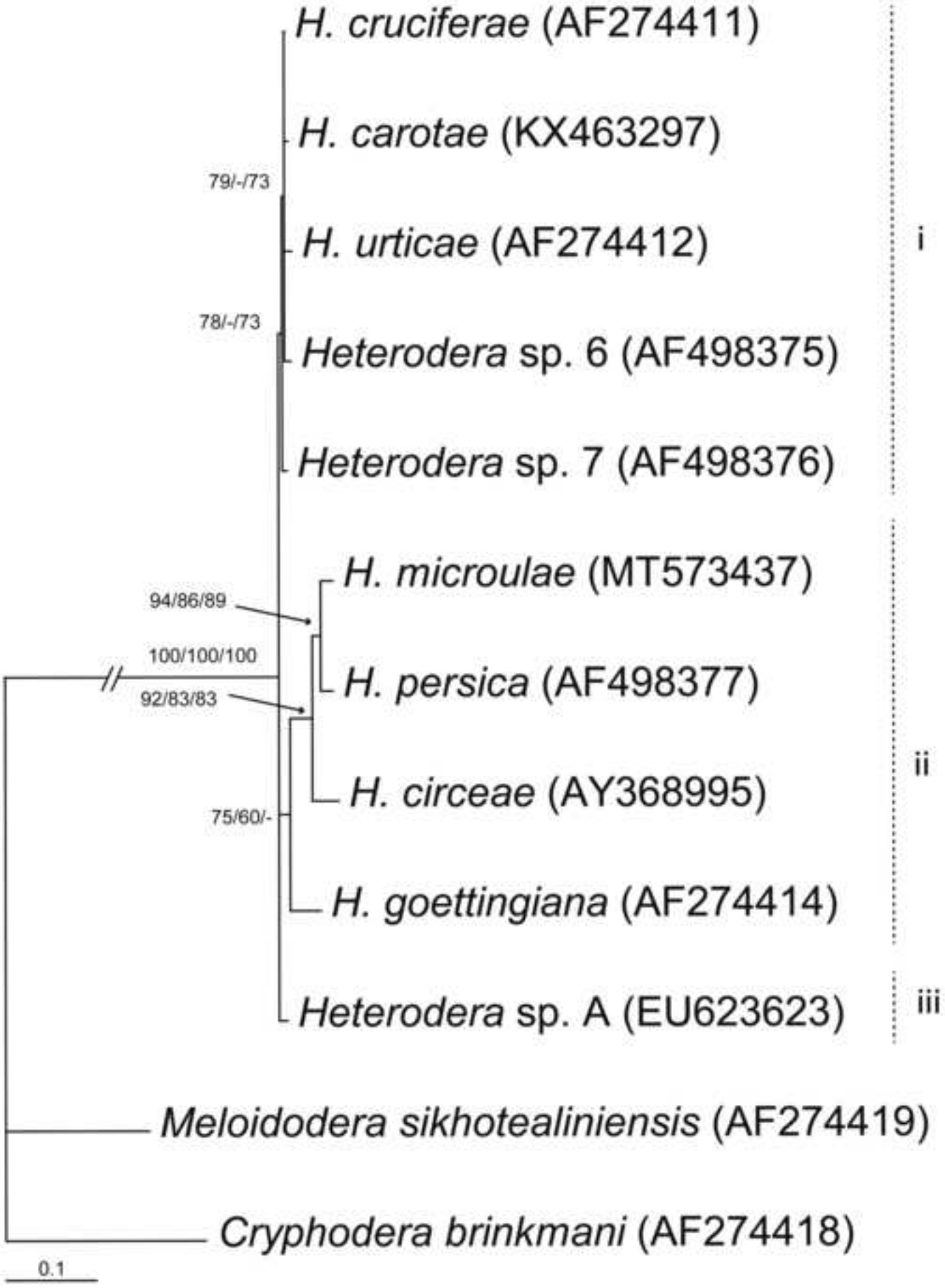

Figure 2

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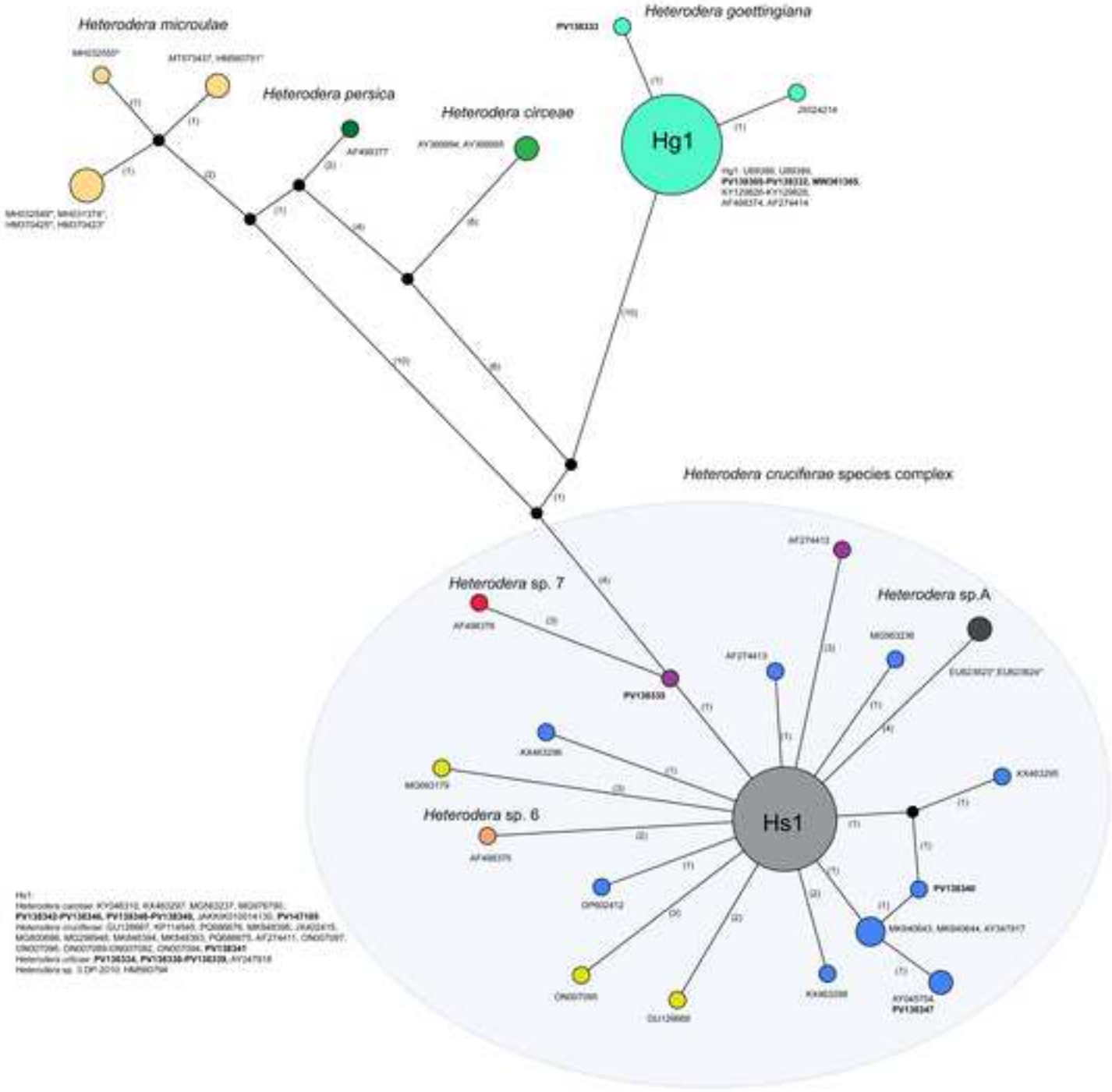

Figure 3

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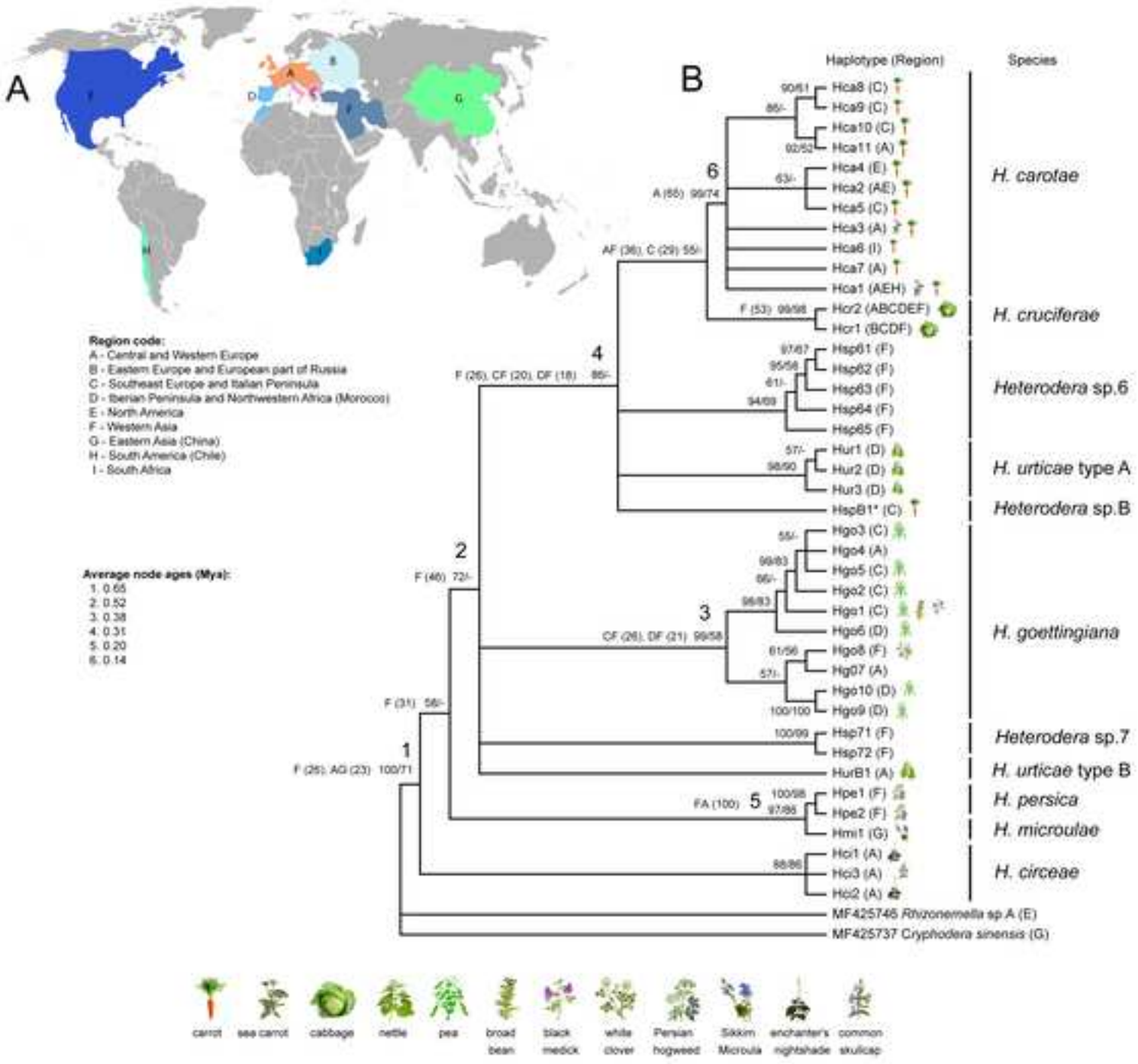

Figure 4

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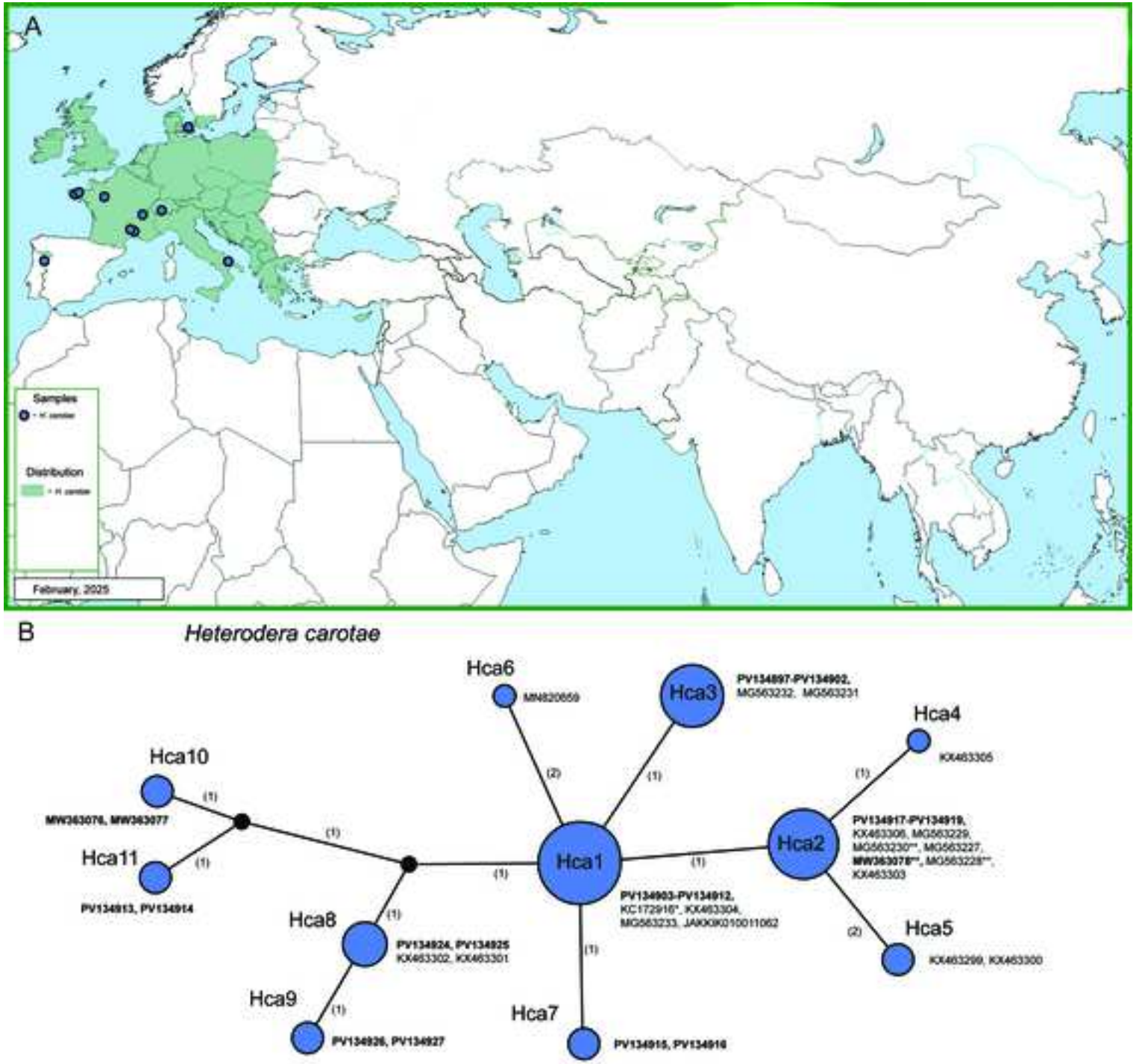

Figure 5

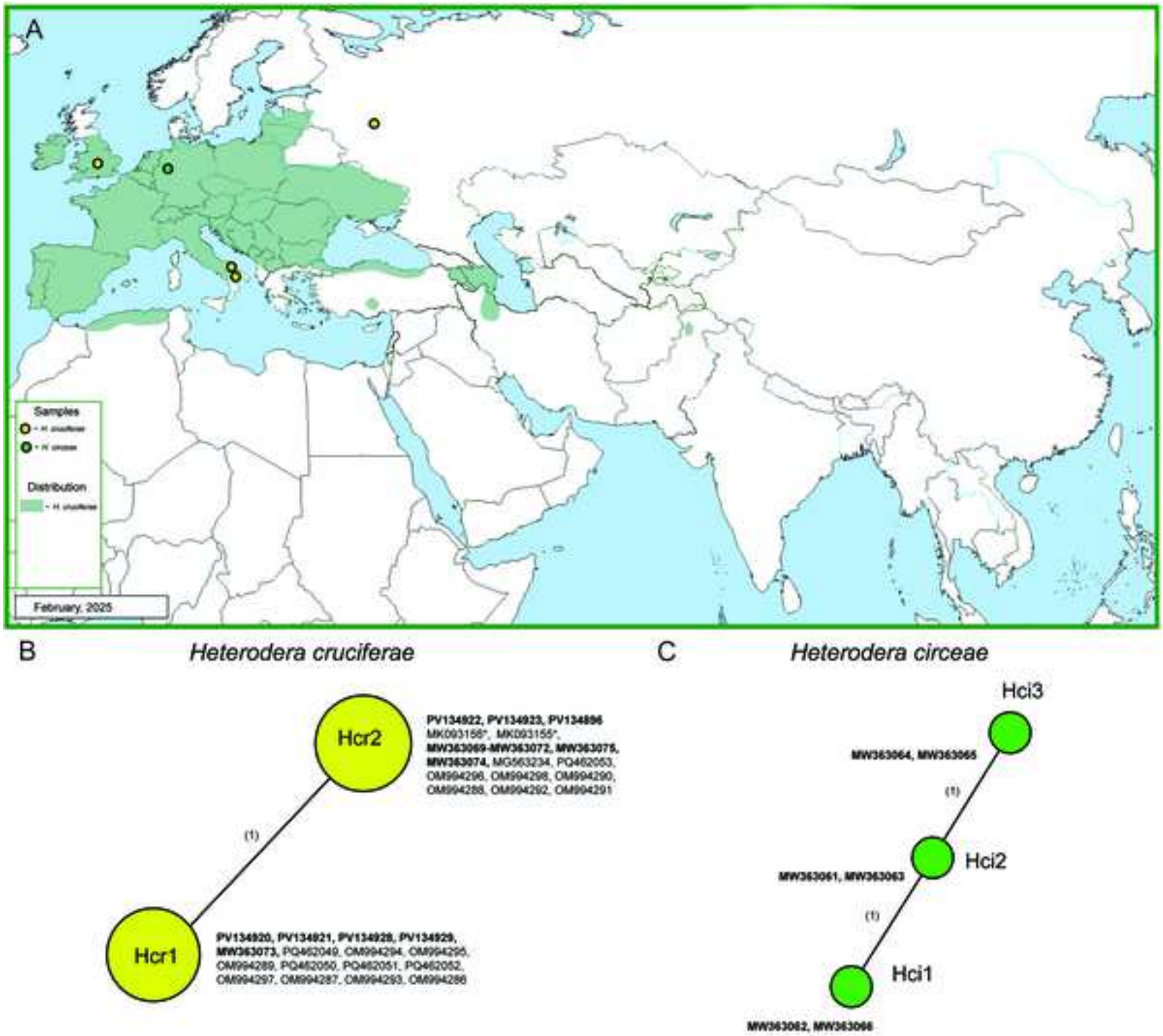

Figure 6

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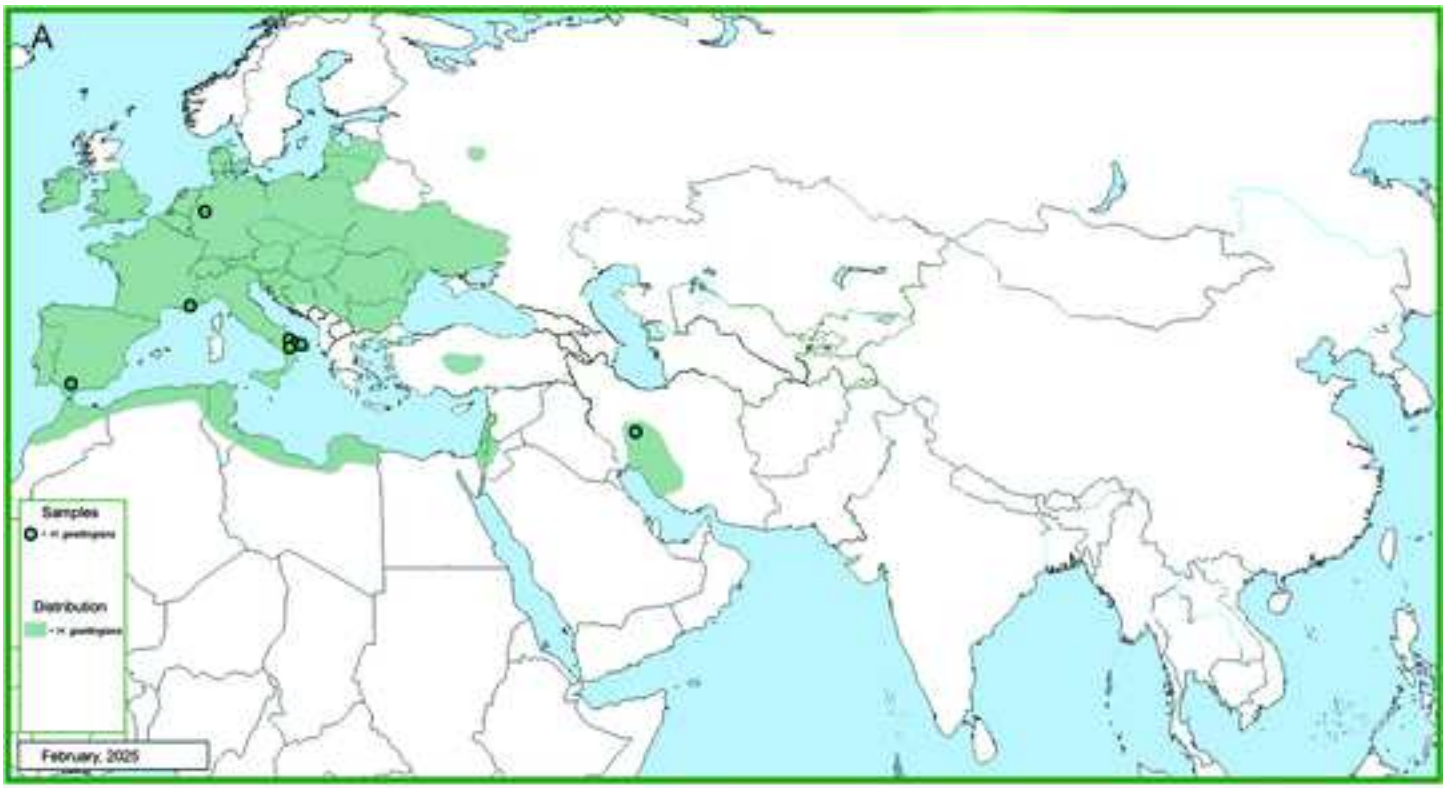

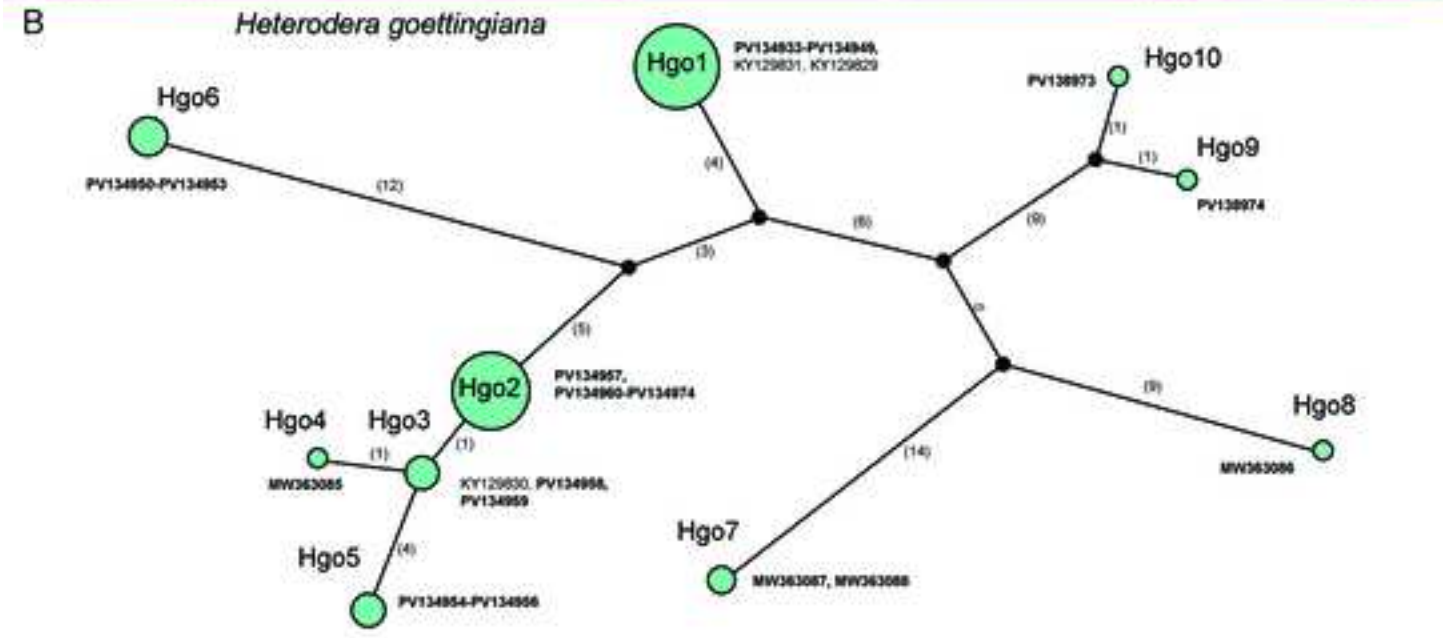

Figure 7

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