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

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Summary – Among the recognised species groups of *Heterodera*, the *Avenae* group is one of the largest with a total of 12 species. Ten of them, *H. arenaria*, *H. aucklandica*, *H. australis*, *H. avenae*, *H. filipjevi*, *H. mani*, *H. pratensis*, *H. riparia*, *H. sturhani* and *H. ustinovi*, are morphologically closely related and represent the *H. avenae* species complex, and the other two, *H. hordecalis* and *H. latipons*, are morphologically more distinct from this complex. In this study we provide comprehensive phylogenetic analyses of several hundred *COI* and ITS rRNA gene sequences from the *Avenae* group using Bayesian inference, maximum likelihood and statistical parsimony. Some 220 *COI* and 11 ITS rRNA new gene sequences from 147 nematode populations collected in 26 countries were obtained in this study. Our study showed that the *COI* gene is a powerful DNA barcoding marker for identification of populations and species from the *Avenae* group. A putatively new cyst nematode species related to *H. latipons* was revealed from the analysis of *COI* and ITS rRNA gene datasets. *COI* gene sequences allow distinguishing *H. arenaria*, *H. australis* and *H. sturhani* from each other and other species. Problems of species delimiting of these species are discussed. The results of the analysis showed that *COI* haplotypes corresponded to certain pathotypes of the cereal cyst nematodes. It is recommended that information on *COI* haplotypes of studied populations be included in research with these nematodes. Based on the results of phylogeographical analysis and age estimation of clades with a molecular clock approach, it was hypothesised that several species of the *Avenae* group primarily originated and diversified in the Irano-Anatolian hotspot during the Pleistocene and Holocene periods and then dispersed from this region across the world. Different geographic barriers, centres and times of origin might explain current known distribution patterns for species of the *Avenae* group. Possible pathways, including a long distance trans-Atlantic dispersal, and secondary centres of diversification are proposed and discussed.

Keywords – cereal cyst nematodes, *COI* gene, haplotypes, *Heterodera australis*, *Heterodera avenae*, *Heterodera sturhani*, Irano-Anatolian hotspot, ITS rRNA, molecular clock, trans-Atlantic dispersal.

Cyst nematodes of the genus *Heterodera* Schmidt, 1871 are serious damaging parasites that limit production of many crops. Using morphological and molecular characteristics, the species of this genus have been divided into nine groups (Subbotin *et al*., 2010; Handoo & Subbotin, 2018). The *Avenae* group is one of the largest and contains species that parasitise only monocotyledons and are characterised by a lemon-shaped cyst having a bifenestrate cone, short vulval slit and presence of bullae. Presently, the *Avenae* group contains 12 species, namely *H. latipons* Franklin, 1969 and *H. hordecalis* Andersson, 1975, plus ten species of the *H. avenae* complex: *H. are-*

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naria Cooper, 1955, *H. avenae* Wollenweber, 1924, *H. aucklandica* Wouts & Sturhan, 1995, *H. australis* Subbotin, Sturhan, Rumpenhorst & Moens, 2002, *H. filipjevi* (Madzhidov, 1981) Stelter, 1984, *H. mani* Mathews, 1971, *H. pratensis* Gäbler, Sturhan, Subbotin & Rumpenhorst, 2000, *H. riparia* (Kazachenko, 1993) Subbotin, Sturhan, Rumpenhorst & Moens, 2003, *H. sturhani* Subbotin, 2015, and *H. ustinovi* Kirjanova, 1969. Five species from this group are considered as nematode pests and are collectively known as cereal cyst nematodes (CCN) (Smiley *et al*., 2017). Four CCN species: the European CCN *H. avenae*, the Filipjev CCN *H. filipjevi*, the Sturhan CCN *H. sturhani* and the Australian CCN *H. australis*, are major, economically important, nematode pests in cereal-growing areas, whereas the other six species of the *H. avenae* complex are found parasitising various grass species of less economic relevance to agriculture. *Heterodera avenae* and *H. filipjevi* have been found in many countries of Europe, Asia and Northern America, where they occur often in mixed populations, whereas *H. australis* has a limited distribution and has only been reported to date in Australia while *H. sturhani* has only been found in China. The Mediterranean CCN, *H. latipons*, is also considered a damaging nematode parasite of cereals in the Mediterranean region. The barley cyst nematode, *H. hordecalis*, is reported mainly from grasslands, forests and coastal areas, and also from cereal fields (Philis, 1997, 1999; Subbotin *et al*., 2010; Smiley *et al*., 2017).

One of the main and effective measures to control CCN is the use of resistant cultivars of cereals. However, different CCN species show different pathogenicity and virulence, thus complicating their management. A reliable diagnosis of cereal nematode populations in the field is imperative for the recommendation of resistant cultivar use in cereal nematode-infested agricultural operations.

Traditional diagnoses of the cyst nematode species belonging to the *Avenae* group are based on morphological and morphometric characteristics of cysts and second-stage juveniles (J2) and require careful and time-consuming study of many specimens from a sample for a correct identification. One of the main problems in using morphology for identification is the significant overlap of morphological and morphometric characters between species, making their separation difficult and unreliable. During the last decade, the introduction of molecular methods has mitigated this problem. The availability of ITS rRNA gene sequences for almost all species of the *Avenae* group made possible the discrimination of the species because the fragments of this gene showed sufficient nucleotide differences to facilitate separation (Ferris *et al*., 1994, 1999; Subbotin *et al*., 2001, 2003). Using the biochemical and molecular approach, several new species were also described. Presently, developed molecular diagnostic tools, such as PCR-RFLP (Bekal *et al*., 1997; Subbotin *et al*., 1999, 2003; Gäbler *et al*., 2000; Rivoal *et al*., 2003; Tanha Maafi *et al*., 2003; Abidou *et al*., 2005a; Ou *et al*., 2008; Fu *et al*., 2011), conventional PCR (Qi *et al*., 2012; Peng *et al*., 2013; Toumi *et al*., 2013a, b; Yan *et al*., 2013) and Real Time PCR (Toumi *et al*., 2015b) with specific primers, enable the differentiation of agriculturally important species both from their siblings and from less economically important species. In many of these techniques, the ITS rRNA gene was used as a marker for specific diagnostics. Several molecular tools, together with direct sequencing of the ITS rRNA gene, have been used in many regional surveys to identify the CCN species.

Recently, the mitochondrial *COI* gene has also been applied for the identification of several CCN species. It has been shown that mtDNA genes evolve much more quickly than the ITS rRNA gene. Furthermore, a high variability has been also shown in the mtDNA *COI* gene, a variability that can be employed as a powerful DNA barcoding marker. This gene fragment has already been used for discrimination of the majority of species of Longidoridae (Palomares-Rius *et al*., 2017), Criconematidae (Powers *et al*., 2014), Heteroderidae (Subbotin *et al*., 2017), *Meloidogyne* (Kiewnick *et al*., 2014), *Pratylenchus* (Troccoli *et al*., 2016; Janssen *et al*., 2017), *Hemicriconemoides* (Van den Berg *et al*., 2015), and others. The usefulness of *COI* gene sequences for identification of some species from the *Avenae* group has been shown by Toumi *et al*. (2013b) and Subbotin (2015), but only a limited number of samples were analysed in those studies. Mitochondrial DNA analysis has also proved to be a powerful tool for assessing intraspecific genetic patterns and phylogeography in plant-parasitic nematodes. This approach has not been explored for the representatives of the *Avenae* group of the genus *Heterodera*.

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

Materials and methods

Nematode species and populations

Species and populations from different hosts, localities and continents used in this study are listed in Table 1. A total of 147 nematode populations collected in 26 countries was analysed in this study. Of the 12 valid species belonging to the *Avenae* group, 11 were studied, the exception being *H. riparia* due to a lack of DNA material. 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

Species distribution maps for representatives of the *Avenae* group were made using personal communications from colleagues listed in the Acknowledgements and from published sources (Meagher, 1977; Tikhonova, 1978; Abidou *et al*., 2005b; Holgado *et al*., 2006; Tanha Maafi *et al*., 2007; Peng *et al*., 2009; Pankaj *et al*., 2011; Dawabah *et al*., 2012; Mokrini e*t al*., 2012, 2017; Pridannikov *et al*., 2015; Toumi *et al*., 2015a; Li *et al*., 2016; Tirchi *et al*., 2016; Cui *et al*., 2017, and others).

DNA extraction, PCR and sequencing

DNA was extracted from 50 or more J2 and embryonated eggs released from single cysts. One or several cysts were used from each sample. Cysts were soaked for 10-20 min in double distilled water in a tube. One cyst was put into 20 μl ddH_2O on a glass slide, punctured by a needle under a dissecting microscope, and released J2 and eggs were cut by an eye scalpel or a stainless steel dental needle. Cut nematodes in water suspension were transferred into a 0.2 ml Eppendorf tube, 3 μl proteinase K (600 μg ml^{-1}) (Promega) and 2 μl 10$\times$ PCR buffer (*Taq* PCR Core Kit, Qiagen) were added to each tube. The tubes were incubated at 65°C (1 h) and 95°C (15 min) consecutively. After incubation, the tubes were centrifuged and kept at $-$20°C until use.

Two gene fragments were amplified and sequenced for this study. For the ITS rRNA gene study, 2 μl of extracted DNA was transferred to 0.2 ml Eppendorf tube containing 2.5 μl 10$\times$ PCR buffer, 5 μl Q solution, 0.5 μl dNTPs mixture (*Taq* PCR Core Kit, Qiagen), 0.15 μl of each primer (1.0 μg μl^{-1}) (synthesised by Integrated DNA Technologies), 0.1 μl *Taq* Polymerase, and 12.6 μl distilled water. The forward primer TW81 (5′-GTT TCC GTA GGT GAA CCT GC-3′) and the reverse primer AB28 (5′-ATA TGC TTA AGT TCA GCG GGT-3′) as described by Joyce *et al*. (1994) were used in PCR. The PCR amplification profile consisted of 4 min at 94°C, 35 cycles of 1 min at 94°C, 1 min 30 s at 55°C, and 2 min at 72°C, followed by a final step of 10 min at 72°C. For the partial *COI* gene study, 3 μl of extracted DNA was transferred to a 0.2 ml Eppendorf tube containing: 10 μl DreamTaq Green PCR Master Mix (2$\times$) (Thermo Fisher Scientific), 10 μl water and 0.15 μl of each primer (1.0 μg μl^{-1}). The forward primer Het-coxiF (5′-TAG TTG ATC GTA ATT TTA ATG G-3′) and the reverse primer Het-coxiR (5′-CCT AAA ACA TAA TGA AAA TGW GC-3′) as described by Subbotin (2015) were used in PCR. The PCR amplification profile consisted of 4 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 45°C, and 1 min 30 s at 72°C, with a final extension at 72°C for 10 min. Two μl of the PCR product was run on a 1% TAE-buffered agarose gel (100 V, 40 min). PCR products were purified using QIAquick PCR Purification Kit (Qiagen) and directly sequenced. Sequencing was made in Quintara Biosciences. New sequences were deposited in the GenBank under accession numbers: MG522927-MG523136, MG523139-MG523146 (*COI* gene) and MG523147-MG523157 (ITS rRNA gene) as indicated in Table 1, and phylogenetic trees and networks. Sequences of *H. goldeni* Handoo & Ibrahim, 2002 samples obtained from Iran are given under accession numbers MG523137 and MG523138.

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, 2003; Tanha Maafi *et al*., 2003; Madani *et al*., 2004; Ou *et al*., 2008; Fu *et al*., 2011; İmren *et al*., 2012, 2015, 2017; Liu *et al*., 2012; Toumi *et al*., 2013b; Wang *et al*., 2013; Baklawa *et al*., 2015; Subbotin, 2015; Tirchi *et al*., 2016; Cui *et al*., 2017; Mokrini *et al*., 2017, and others). Several alignments were created: *i*) ITS rRNA gene alignment containing only reference sequences of each *Avenae* group species; *ii*) ITS rRNA

Table 1. Species and populations of cyst nematodes from the *Avenae* group 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
Heterodera arenaria	The Netherlands	Marram grass	550a, 862b	Har1	MG522943, MG522944	–	G. Karssen; Subbotin *et al.* (2003)
H. arenaria	UK, Lincolnshire	Marram grass	863	Har1	MG522942	AF274396	J. Rowe; Subbotin *et al.* (1999, 2001, 2003)
H. arenaria	Italy, Terranova	Marram grass	CD1440	Har2	MG522939-MG522941	MG523155	N. Vovlas
H. aucklandica	UK, St Albans	Grasses	CD2391	Hauc1	MG523088, MG523089	AY148380	Subbotin *et al.* (2003)
H. aucklandica	New Zealand, Auckland	Grasses	326, CD2397	Hauc1, Hauc2	MG523085-MG523087	AF274398	W. Wouts; Subbotin *et al.* (2001)
H. australis	Australia, Yorke Peninsula	Cereals	CD1485	Haus1	KU147202, MG523101	AY148392, AY148395, AY148396	Subbotin *et al.* (2003); Subbotin (2015)
H. australis	Australia	Cereals	520a	Haus1	MG523103	–	Subbotin *et al.* (2002, 2003)
H. australis	Australia, South Australia	Cereals	607	Haus1	MG523102	AY148394	Subbotin *et al.* (2002, 2003)
H. australis	Australia, Victoria	Cereals	CD1284	Haus1	MG523100	AY148393	Subbotin *et al.* (2002, 2003)
H. avenae	France, Argentan	Cereals	CD1466	HavA1	MG522937	AF274395	R. Rivoal; Subbotin *et al.* (2003)
H. avenae	USA, Oregon, Union County	Cereals	CD1285	HavA1	MG522927-MG522930	–	R. Smiley
H. avenae	Germany, Bavaria, Gravenreuth	Cereals	523b	HavA1	MG522938	–	D. Sturhan; Subbotin *et al.* (2003)
H. avenae	USA, Oregon	Cereals	CA117	–	–	MG523156	R. Smiley
H. avenae	USA, Oregon, Cove	Cereals	CD1519	HavA1	MG522936	–	R. Smiley
H. avenae	France, Villasavary	Cereals	88b; CD2399	HavA1, HavA3	MG522931-MG522933, MG522950	AY148372-AY148374	R. Rivoal; Subbotin *et al.* (2003)
H. avenae	Germany, Bavaria, Rinkam	Cereals	CD1478	HavA2	MG522953	–	D. Sturhan; Subbotin *et al.* (1999)
H. avenae	Serbia	Cereals	CD1556	HavA2	MG522956	–	V. Oro
H. avenae	Slovakia	Cereals	CD1464	HavA2	MG522954	–	D. Sturhan
H. avenae	Unknown	Cereals	517a	HavA2	MG522955	–	H.J. Rumpenhorst
H. avenae	Switzerland	Cereals	CD1465	HavA2	MG522957	–	J. Rowe
H. avenae	Morocco, Chaouia, Settat	Cereals	CD2335	HavA2, HavA5	MG522958-MG522960	–	F. Mokrini
H. avenae	Norway	Cereals	CD2336	HavA3	MG522948	–	R. Holgado
H. avenae	France, St Georges du Bois	Cereals	CD1461	HavA3	KU147188	–	Subbotin (2015)
H. avenae	USA, Idaho, St. Anthony	Cereals	CD1521	HavA3	MG522949	–	R. Smiley
H. avenae	Germany, Lower Saxony, Taaken	Cereals	533b	HavA3	MG522951	AY148353	D. Sturhan; Subbotin *et al.* (1999, 2003)
H. avenae	Norway	Cereals	CD2337	HavA3	MG522952	–	R. Holgado
H. avenae	Spain, Santa Olalla	Cereals	CD1443, CD2395	HavA3, HavA4	MG522945-MG522947	AY148354-AY148356	M. Romero; Subbotin *et al.* (2003)
H. avenae	Morocco	Cereals	527a, b	HavA5, HavA6	MG522961, MG522962	AY148367-AY148369	S. Amuri; Subbotin *et al.* (2003)
H. avenae	USA, Oregon, Island City	Cereals	CD1520	HavA7	MG522934	–	R. Smiley
H. avenae	USA, Washington, Palouse	Cereals	CD1522	HavA8	MG522935	–	R. Smiley
H. avenae	Turkey, Adana, Sarıçam, Dutluca	Wheat	CD2004	HavB1	MG522993, MG522994	–	I.H. Elekçioğlu
H. avenae	Turkey	Cereals	CD1488	HavB1	MG522991, MG522992	–	D. Sturhan
H. avenae	Turkey	Cereals	343	HavB1	MG522990	–	D. Sturhan
H. avenae	Turkey, Adana, Sarıçam, Çabutlu	Wheat	CD2005	HavB1	MG522995	–	I.H. Elekçioğlu
H. avenae	Turkey, Adana, Sarıcam, Buca tepesi	Wheat	CD2044	HavB1	MG522989	–	I.H. Elekçioğlu
H. avenae	Tunisia, Siliana	Cereals	CD1456, CD1504	HavB1	MG522997, MG522998	–	L. Waeyenberge
H. avenae	India	Cereals	503a	HavB2	MG522977	AY148362	H.J. Rumpenhorst; Subbotin *et al.* (2003)
H. avenae	Syria, Al-Hasakah	Wheat	–	HavB2	KC172909	–	Toumi *et al.* (2013b)

Table 1. (Continued.)

Species	Location	Host	Sample code	*COI* haplotype	*COI* GenBank accession number	ITS rRNA GenBank accession number	Source and/or reference
H. avenae	Turkey, Mardin, Midyat, Idil Road	Barley	CD2006	HavB2	MG522971, MG522972	–	I.H. Elekçioğlu
H. avenae	Turkey, Diyarbakir, Ergani, Koyunalan	Wheat	CD2002	HavB2	MG522981, MG522982	–	I.H. Elekçioğlu
H. avenae	Syria, El-Hassake, Tel Aswad	Barley	CD1568	HavB2	MG522976	–	F. Toumi (Toumi *et al*., 2015a)
H. avenae	Syria, El-Hassake, Khazna	Barley	CD1570	HavB2	MG522973	–	F. Toumi (Toumi *et al*., 2015a)
H. avenae	Turkey, Diyarbakir, Centrum, Bağpınar	Wheat	CD2007	HavB2	MG522965, MG522966	–	I.H. Elekçioğlu
H. avenae	Turkey, Sanlıurfa, Birecik, Kocaali	Barley	CD1999	HavB2	MG522963	–	I.H. Elekçioğlu
H. avenae	Syria, El-Hassake, Am Roos	Barley	CD1569	HavB2	MG522978	–	F. Toumi (Toumi *et al*., 2015a)
H. avenae	Turkey, Sanlıurfa Centrum, Kabahaydar	Wheat	CD2003	HavB2	MG522974, MG522975	–	I.H. Elekçioğlu
H. avenae	Egypt, Al Tasa	Cereals	CD1982	HavB2	MG522979	–	M. Baklawa
H. avenae	Egypt, Abou Sultan	Cereals	CD1981	HavB2	MG522983	–	M. Baklawa
H. avenae	Israel, Ha-hoola	Cereals	529b	HavB2	MG522980	AY148366	M. Mor; Subbotin *et al.* (2003)
H. avenae	Egypt, El Salihiyya Al Jadidah	Cereals	CD1980	HavB2	MG522967	–	M. Baklawa
H. avenae	Turkey, Sanlıurfa, Suruc, Çalışkanlar	Barley	CD2001	HavB2	MG522968	–	I.H. Elekçioğlu
H. avenae	Syria, El-Hassake, Sibat	Wheat	CD1566	HavB2	MG522964	–	F. Toumi (Toumi *et al*., 2015a)
H. avenae	Israel, Niz Oz	Cereals	CD1515	HavB2, HavB6	MG522969, MG522970	AY148365	D. Orion; Subbotin *et al.* (2003)
H. avenae	Turkey, Hatay	Wheat	CD1598	HavB3	MG523011	–	F. Toumi
H. avenae	Tunisia, Kairouan	Cereals	CD1505	HavB3	MG523005, MG523006	–	L. Waeyenberge
H. avenae	Turkey, Osmaniye Centrum, Günyazı	Wheat	CD2000	HavB3	MG523009	–	I.H. Elekçioğlu
H. avenae	Turkey, Osmaniye Centrum, Dervişler	Wheat	CD1996	HavB3	MG523007, MG523008	–	I.H. Elekçioğlu
H. avenae	Turkey, Osmaniye Mustafabeyli, Imran	Wheat	CD1995	HavB3	MG523003, MG523004	–	I.H. Elekçioğlu
H. avenae	Turkey, Adana, Sarıçam, Torunlar	Wheat	CD2042	HavB3	MG523002	–	I.H. Elekçioğlu
H. avenae	Turkey, Kilis	Wheat	CD1597	HavB3	MG523010	–	F. Toumi
H. avenae	Turkey, Hatay, Reyhanlı-Müşrüfe	Wheat	CD2048	HavB3	MG523001	–	I.H. Elekçioğlu
H. avenae	Tunisia, Zaghouan	Cereals	CD1455	HavB4	MG523000	–	L. Waeyenberge
H. avenae	Turkey, Osmaniye Centrum, Cevdetiye	Wheat	CD2041	HavB5	MG522999	–	I.H. Elekçioğlu
H. avenae	Israel, Bet Dagan	Cereals	477	HavB6	MG522996	AY148363	D. Orion; Subbotin *et al.* (2003)
H. avenae	Saudi Arabia	Cereals	504a	HavB7	MG522988	AY148361	H.J. Rumpenhorst; Subbotin *et al.* (2003)
H. avenae	Iran, Ilam, Mehran-Reza Abad	Wheat	Zah33	HavB7	MG522984-MG522987	AF498378	Z. Tanha Maafi; Tanha Maafi *et al.* (2003)
H. avenae	Syria, Deir Al-Zor	Wheat	–	HavB8	KC172908	–	Toumi *et al.* (2013b)
H. filipjevi	Serbia	Cereals	CD1557b	HfA1	MG523053		V. Oro; Oro *et al.* (2012)
H. filipjevi	Russia, Saratov	Oat	561	HfA1	MG523050	AF274399	Subbotin *et al.* (2001)
H. filipjevi	Russia, Nizhnii Novgorod region, Gorodets	Oat	535b	HfA1	MG523049	–	L. Nasonova; Subbotin *et al.* (1999)
H. filipjevi	USA, Oregon, Imbler	Cereals	CD1517, CD1518	HfA1	MG523051, MG523052	–	R. Smiley
H. filipjevi	Russia, Leningrad region, Pushkin	False wheat	563b	HfA1	MG523048	–	Subbotin *et al.* (2003)
H. filipjevi	Tadzhikistan, Dushanbe	Wheat	525a, CD2393	HfA2, HfA3	MG523033-MG523035	AY148402	Subbotin *et al.* (2003)
H. filipjevi	Russia, Bashkortostan, Baymak	Wheat	536, CD2400	HfA2-HfA4	MG523030-MG523032	–	Subbotin *et al.* (2003)
H. filipjevi	Ukraine, Chabany	Cereals	CD1477	HfA3, HfA4	MG523036-MG523038	–	S.A. Subbotin
H. filipjevi	USA, Washington	Oat	CD1876, CD1878	HfA4	MG523039, MG523040	–	T.C. Paulitz
H. filipjevi	USA, Montana	Cereals	CD1989a	HfA4	MG523042	KP878490	A. Skantar; Dyer *et al.* (2015)

Table 1. (Continued.)

Species	Location	Host	Sample code	*COI* haplotype	*COI* GenBank accession number	ITS rRNA GenBank accession number	Source and/or reference
H. filipjevi	Germany, Gimbte, near Münster	Cereals	CD1442	HfA4	MG523044	AY148400	H.J. Rumpenhorst; Subbotin *et al.* (2003)
H. filipjevi	Sweden	Cereals	CD1445b	HfA4	MG523041	–	J. Rowe
H. filipjevi	Russia, Nizhnii Novgorod region, Vad	Oat	CD1475	HfA4	MG523043	AY148401	L. Nasonova; Subbotin *et al.* (2003)
H. filipjevi	Spain, Torralba de Calatrava	Cereals	560a; CD2392	HfA5	MG523077, MG523078	AY148399	M. Romero; Subbotin *et al.* (2003)
H. filipjevi	Serbia	Cereals	CD1584	HfA5	MG523081	–	F. Toumi
H. filipjevi	China, Henan Province, Xiayi	Cereals	CD2359	HfA5, HfA8	MG523079, MG523080	–	H.-M. Li
H. filipjevi	UK, Akenham	Cereals	526, CD2401	HfA6, HfA7	MG523045-MG523047	AY148403	Subbotin *et al.* (2003)
H. filipjevi	Turkey	Cereals	CD1479	HfA9	MG523082	–	D. Sturhan
H. filipjevi	Turkey, Eskisehir	Cereals	42B	HfA10	MG523084	–	F. Toumi (Toumi *et al.*, 2013a, b)
H. filipjevi	Greece, Crete	Unknown	CD2039	HfA10	MG523083	–	Z. Handoo
H. filipjevi	Turkey, Ankara	Wheat	CD1567	HfA11	MG523071	–	F. Toumi
H. filipjevi	Turkey, Central Anatolia	Oat	CD1476	HfA11	MG523072	–	H.J. Rumpenhorst
H. filipjevi	Turkey, Ankara	Cereals	Fat23	HfA11	MG523074, MG523075	–	F. Toumi (Toumi *et al.*, 2013a, b)
H. filipjevi	Turkey, K. Maras, Afşin, Elbistan Road	Cereals	CD1998	HfA11, HfA12	MG523068, MG523069	–	I.H. Elekçioğlu
H. filipjevi	Turkey, K.Maras, Elbistan, Büyük Yapalak-II	Wheat	CD1993	HfA11, HfA12	MG523066, MG523067	–	I.H. Elekçioğlu
H. filipjevi	Turkey, K. Maras, Elbistan, Büyük Yapalak-I	Wheat	CD1991	HfA11, HfA12	MG523064, MG523065	–	I.H. Elekçioğlu
H. filipjevi	Turkey, K. Maras, Ekinözü, Centrum	Wheat	CD1994	HfA11, HfA12	MG523062, MG523063	–	I.H. Elekçioğlu
H. filipjevi	Turkey, Adana Pozantı Road	Wheat	CD2040	HfA12	MG523070	–	I.H. Elekçioğlu
H. filipjevi	Turkey, Afşin, Termik Santral	Wheat	CD1990	HfA12	MG523058, MG523059	–	I.H. Elekçioğlu
H. filipjevi	Turkey, K. Maras, Afşin, Centrum	Wheat	CD1997	HfA12	MG523060, MG523061	–	I.H. Elekçioğlu
H. filipjevi	Turkey	Cereals	528b	HfA13	MG523076	–	H.J. Rumpenhorst; Subbotin *et al.* (2003)
H. filipjevi	Syria, El-Hassake, Tel-Ailol	Barley	CD1571	HfA14	MG523057	–	F. Toumi (Toumi *et al.*, 2015a)
H. filipjevi	Syria, El-Hassake, Jriba	Wheat	CD1572	HfA15	MG523056	–	F. Toumi (Toumi *et al.*, 2015a)
H. filipjevi	Syria, El-Hassake, Khanka	Barley	CD1617	HfA16	MG523054	–	F. Toumi (Toumi *et al.*, 2015a)
H. filipjevi	Syria, El-Hassake, Jolebsan	Cereals	Fat125	HfA16	MG523073	–	F. Toumi (Toumi *et al.*, 2013a, b)
H. filipjevi	Syria, El-Hassake, Jriba	Wheat	CD1573	HfA16	MG523055	–	F. Toumi (Toumi *et al.*, 2015a)
H. filipjevi	Iran, Eastern Azerbaijan, Marand	Wheat	Zah34	HfB1, HfB2	MG523012-MG523014	–	Z. Tanha Maafi; Tanha Maafi *et al.* (2003)
H. filipjevi	Iran, Kerman, Bardsir	Wheat	Zah44	HfB3	MG523026	–	Z. Tanha Maafi; Tanha Maafi *et al.* (2003)
H. filipjevi	Iran, Yazd Marvast	Wheat	Zah36 CD1460	HfB3, HfB4	MG523023-MG523025, MG523027	–	Tanha Maafi *et al.* (2003)
H. filipjevi	Iran, Fars, Abaadeh, Tashk	Wheat	CD1439	HfB4	MG523029	–	Tanha Maafi *et al.* (2003)
H. filipjevi	Iran, Marvast	Cereals	505a	HfB4	MG523028	AY148404	H.J. Rumpenhorst; Subbotin *et al.* (2003)
H. filipjevi	Iran, Kurdistan, Qorveh	Wheat	Za8	HfB5	MG523022	–	Z. Tanha Maafi; Tanha Maafi *et al.* (2003)
H. filipjevi	Iran, Hamadan, Kabootar Ahang	Wheat	Zah35	HfB6	MG523015, MG523016	–	Z. Tanha Maafi; Tanha Maafi *et al.* (2003)

Table 1. (Continued.)

Species	Location	Host	Sample code	*COI* haplotype	*COI* GenBank accession number	ITS rRNA GenBank accession number	Source and/or reference
H. filipjevi	Iran, Esfahan	Wheat	Zah37	HfB6	MG523017, MG523018	AF498380	Z. Tanha Maafi; Tanha Maafi *et al.* (2003)
H. filipjevi	Iran, Aligoudarz	Wheat	Did15	HfB6	KC172910	JX024208	Toumi *et al.* (2013a, b)
H. filipjevi	Syria, Al-Hasakah	Wheat	Fat125	HfB6	KC172911	–	Toumi *et al.* (2013a, b)
H. filipjevi	Iran	Cereals	Fat15	HfB6	MG523021	–	F. Toumi
H. filipjevi	Iran, Estahan	Wheat	CD2365	HfB7	MG523020	–	Z. Tanha Maafi
H. filipjevi	Iran, Khuzestan, Ramshir	Wheat	CD2364	HfB8	MG523019	–	Z. Tanha Maafi
H. hordecalis	Israel, Reim	Cereals	CD1492c	HhA1	MG523146	–	M. Mor
H. hordecalis	Tunisia	Wheat	TunisB	HhA1	KC172912	JX024216	Toumi *et al.* (2013a, b)
H. hordecalis	Israel, Gelat	Cereals	CD1491c	HhA2	MG523145	MG523147	M. Mor
H. hordecalis	Sweden	Cereals	CD1486	HhB1	MG523142	MG523150	J. Rowe
H. hordecalis	Slovakia	Grasses	CD2396 (E1568)	HhB2	MG523140	MG523148	D. Sturhan
H. hordecalis	Slovakia	Grasses	511	HhB3	MG523141	–	D. Sturhan
H. hordecalis	Germany	Grasses	CD1484	HhB4	MG523143, MG523144	MG523151	D. Sturhan
H. hordecalis	Iran, Gilan, Bandar Anzali	Grasses	Zah23	HhB5	MG523139	–	Z. Tanha Maafi
H. hordecalis	Iran, Ardabil, Meshkin shahr	Wheat, grasses	Zah27	HhB5, HhB6	MG523135, MG523136	AF498381, MG523149, MG523152, MG523153	Z. Tanha Maafi; Tanha Maafi *et al.* (2003, 2007)
H. latipons	Syria, El-Hassake, Aamer	Wheat	CD1590	HlA1	MG523118	–	F. Mokrini (Toumi *et al.*, 2013a, b; 2015a)
H. latipons	Turkey, Kilis, Merkez, Yığmatepe	Barley	CD2047	HlA1	MG523116	–	I.H. Elekçioğlu
H. latipons	Syria, El-Hassake, Damkhia El-Sakira	Wheat	CD1609	HlA1	MG523115	–	F. Toumi (Toumi *et al.*, 2015a)
H. latipons	Turkey, Elbistan, Göksun road	Cereals	CD1992	HlA1	MG523117	–	I.H. Elekçioğlu
H. latipons	Iran, Zanjan, Abhar	Cereals	Zah6	HlA2	MG523123	–	Z. Tanha Maafi; Tanha Maafi *et al.* (2003)
H. latipons	Iran, Western Azerbaijan, Salmas	Cereals	Zah5, Zah9	HlA2, HlA3	MG523121, MG523122	AF498382	Z. Tanha Maafi; Tanha Maafi *et al.* (2003)
H. latipons	Iran, Zanjan	Wheat	CD2366	HlA3	MG523120	–	Z. Tanha Maafi;
H. latipons	Syria, Deir Al-Zor	Wheat	–	HlA4	KC172913	–	Toumi *et al.* (2013b)
H. latipons	Turkey, Kilis	Wheat	CD1595	HlA5	MG523119	–	F. Toumi
H. latipons	Syria, Aleppo, Atien	Barley	CD1604	HlA6	MG523127	–	F. Toumi
H. latipons	Syria, Aleppo, Akhtarin	Barley	CD1606	HlA6	MG523128	–	F. Toumi
H. latipons	Syria, Breda	Cereals	Breda	HlA7	MG523124	AF274402	U. Scholz; Subbotin *et al.* (2001)
H. latipons	Israel, Gelat	Cereals	Gelat, CD1491b	HlA8, HlA9	MG523123, MG523126	–	M. Mor
H. latipons	Russia, Rostov region	Grasses	484c	HlB1	MG523129	AY347925	V.N. Chizhov; Madani *et al.* (2004)

Table 1. (Continued.)

Species	Location	Host	Sample code	*COI* haplotype	*COI* GenBank accession number	ITS rRNA GenBank accession number	Source and/or reference
H. mani	USA, Washington, Olympic National Park	Grasses	CD956	Hm1	MG523095, MG523097	–	S.A. Subbotin
H. mani	Germany, Bavaria	Grasses	519	Hm1	MG523098	AY148378	H.J. Rumpenhorst; Subbotin *et al.* (2003)
H. mani	USA, California, San Luis Obispo County	Grasses	CD1237	Hm1	KU147203	MG523157	S.A. Subbotin; Subbotin (2015)
H. mani	Germany	Grasses	521a	Hm1	MG523096	–	H.J. Rumpenhorst; Subbotin *et al.* (2003)
H. mani	USA, California, Monterey County	Grasses	CD1252	Hm2	MG523099		S.A. Subbotin
H. pratensis	Germany	Grasses	CD2398	Hp1	MG523104-MG523107	–	D. Sturhan
H. pratensis	The Netherlands, near Rotterdam	Grasses	559	Hp1	KU147189	AY148388	Subbotin *et al.* (2003); Subbotin (2015)
H. pratensis	Germany, Missunde, near Schleswig	Grasses	537a	Hp1	KU147194	–	Subbotin (2015)
H. pratensis	Germany	Grasses	555	Hp1	KU147192	–	Subbotin (2015)
H. pratensis	Belgium, Zwin	Grasses	Zwi78	Hp1	KU147191	–	Subbotin (2015)
H. pratensis	Germany	Grasses	601	Hp1	KU147193	–	Subbotin (2015)
H. pratensis	Germany	Grasses	507a	Hp1	KU147190	–	Subbotin (2015)
H. pratensis	Russia, Leningrad region, Putilovo	False wheat	565, 595	Hp2	KU147200, KU147201	AY148351	Subbotin *et al.* (2002); Subbotin (2015)
H. pratensis	Iran, Gilan, Bandar Anzali	Grasses	Zah46	Hp3, Hp4	MG523110-MG523112	AF498379	Z. Tanha Maafi; Tanha Maafi *et al.* (2003)
H. pratensis	Iran, Tehran, Gachsar	Grasses	Zah41	Hp5	MG523108	–	Tanha Maafi *et al.* (2003, 2007)
H. pratensis	Iran, Ardabil, Meshkin shahr	Grasses	Zah30	Hp5	MG523109	–	Tanha Maafi *et al.* (2003, 2007)
H. sturhani	China, Jiangsu Province, Peixian	Cereals	CD2360	Hs1	MG523113, MG523114	–	H.-M. Li
H. sturhani	China, Beijing, Tongzhou district	Cereals	514a	Hs1	KU147195	AY148382	Subbotin *et al.* (2003); Subbotin (2015)
H. sturhani	China, Beijing, Fangshan district	Cereals	513b	Hs1	KU147196	AY148381	Subbotin *et al.* (2003); Subbotin (2015)
H. sturhani	China, Shanxi province, Wenxi	Cereals	CD1555	Hs1	KU147197, KU147198	–	Subbotin (2015)
H. sturhani	China, Beijing, Pinggu district	Cereals	515a	Hs2	KU147199	–	Subbotin (2015)
H. ustinovi	USA, Arizona	Grasses	DF8	Hus1	MG523090	–	M. McClure
H. ustinovi	USA, Arizona	Grasses	DF1	Hus1	MG523091	–	M. McClure
H. ustinovi	Belgium	Grasses	281, 282, 286	Hus1, Hus2	MG523092-MG523094	AY148407	Subbotin *et al.* (1999, 2003)
Heterodera sp.	Syria, El-Hassake, Tel Khaled	Wheat	CD1625	HspA1	MG523134	–	F. Toumi (Toumi *et al.*, 2013a, b; 2015a)
Heterodera sp.	Turkey, Kadintor	Cereals	524b, 556	HspB2-HspB5	MG523130-MG523133	MG523154	D. Sturhan

gene alignment containing sequences of species from the *H. avenae* complex; *iii*) ITS rRNA gene alignment of *H. hordecalis* sequences; *iv*) ITS rRNA gene alignment of *H. latipons* and *Heterodera* sp. sequences; *v*) *COI* gene alignment containing sequences of all studied samples; *vi*) *COI* gene sequence alignment containing reference haplotype sequences for all *Avenae* group species; and *vii*) several *COI* gene alignments containing sequences of certain species or group of species. Sequence alignments were also 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* and Bayesian inference (BI) using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). The best fit models of DNA evolution were obtained using the program jModeltest.0.1.1 (Posada, 2008) with the Akaike Information Criterion. Bootstrap support (BS) values for ML and MP trees were calculated by a heuristic search from 100 and 1000 replicates, respectively. The BI analysis for each gene was initiated with a random starting tree and was run with four chains for 3.0×10^6 generations. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. After discarding burn-in samples and evaluating convergence the remaining samples were retained for further analysis. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities (PP) were given on appropriate clades. Trees were visualised with the TreeView 1.6.6 program (Page, 1996) and drawn with Adobe Illustrator v.10.

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).

Testing for Global Molecular Clock with the *COI* gene sequence alignment was performed with PAUP* under the null hypothesis – the phylogeny was rooted and the branch lengths were constrained such that all of the tips can be drawn at a single time plane. Under the alternative hypothesis, each branch was allowed to vary independently. The Hasegawa, Kishino & Yano (Hasegawa *et al*., 1985) (HKY85) model of DNA substitution with among-site rate variation with a gamma distribution was used.

Divergence times were inferred with BEAST 2.4.5 (Bouckaert *et al*., 2014) using the *COI* sequence alignment. 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 one generation per year was considered for all *Avenae* group species (Subbotin *et al*., 2010). The analyses were run for 1×10^8 generations and sampled every 1000 generations. Convergence was monitored with Tracer 1.6. TreeAnnotator (Bouckaert *et al*., 2014) and used to summarise the set of postburn-in trees (with the first 20% samples discarded as burn-in) and their parameters to produce a maximum clade credibility (MCC) chronogram, which showed the mean divergence time estimates with 95% high posterior density intervals.

Statistical Dispersal-Vicariance Analysis (S-DIVA) as implemented in RASP 4.0 (Yu *et al*., 2010, 2015) was used to evaluate the alternative ancestral ranges at each node in the trees. The presently known distribution range of the *Avenae* group species was divided into 15 geographical regions (A-O). The S-DIVA tested using a simulated dataset of 100 randomly sampled trees from 58 000 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 of the *Avenae* group species. The first approach included BI, ML and MP analyses of ITS rRNA gene alignment containing only reference sequences of each *Avenae* group species and the second approach included SP analyses of several alignments of ITS rRNA gene sequences for the *H. avenae* species complex, *H. hordecalis*, *H. latipons*, and *Heterodera* sp.

Avenae *group*

Phylogenetic relationships within 11 valid and one putative new species of the *Avenae* group species as inferred from BI, ML and MP analyses of the ITS rRNA gene sequences are given in Figure 1. The *H. avenae* complex species formed a moderate or highly supported

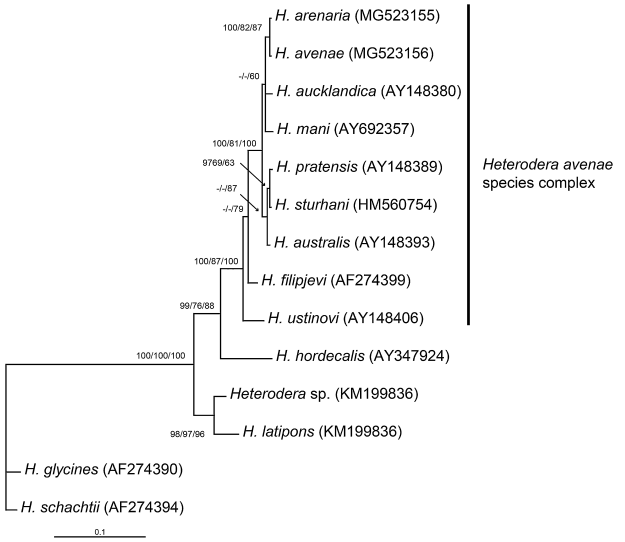


Fig. 1. Phylogenetic relationships between species from the *Avenae* group as inferred from Bayesian analysis of the ITS rRNA gene sequences. Posterior probability and bootstrap support values for BI, ML and MP analysis are given for appropriate clades, respectively. Values less than 50% are not indicated.

clade depending on applied method. *Heterodera latipons* and *Heterodera* sp. formed a basal clade in the tree. The ITS rRNA alignment was 763 bp in length.

Heterodera avenae *complex*

A total of 638 sequences was downloaded from GenBank. However, only 610 sequences were retained in the analysis because they met the length requirement and were nearly 738 bp in a length with both ITS1 and ITS2 regions, and also met the quality requirement as they were estimated as non-containing or containing a minimal number of sequence reading mistakes. Nucleotides suspiciously differing from homologous ones in the conservative 5′ and 3′ ends of sequence were considered as reading mistakes. After adding three new sequences, the final ITS1-5.8S-ITS2 alignment contained 613 sequences. The alignment was 758 bp in a length and included 114 sequences of *H. avenae*, two sequences of *H. arenaria*, three sequences of *H. aucklandica*, six sequences of *H. mani*, four sequences of *H. ustinovi*, 126 sequences of *H. filipjevi*, 329 sequences of *H. sturhani*, 11 sequences of *H. pratensis*, and 18 sequences identified as *H. australis*. Majority sequences of *H. avenae* and *H. arenaria* were represented by the haplotype Hav1 (93 sequences). Those of *H. filipjevi* contained one main haplotype Hf1 (77 sequences) and those of *H. sturhani* included three main haplotypes: Hst1 (148 sequences), Hst2 (31 sequences), and Hst3 (31 sequences) (Table 2).

A phylogenetic network for the ITS rRNA sequences, reconstructed using SP with POPART software, is given in Figure 2. This method allowed the discrimination of *H. avenae* + *H. arenaria*, *H. mani*, *H. australis*, *H. ustinovi*, *H. filipjevi*, and *H. sturhani* + *H. pratensis* from each other. *Heterodera sturhani* + *H. pratensis* differed from *H. avenae* + *H. arenaria* by at least four nucleotide changes and from sequences designated as *H. australis* by at least one change. *Heterodera avenae* + *H. arenaria* can be differentiated from *H. mani* by two changes and from *H. aucklandica* by four changes. The species-pair *H. avenae* and *H. arenaria* was not distinguishable based on the ITS rRNA gene sequences. Although all 11 sequences of *H. pratensis* were related to *H. sturhani*, nine sequences of *H. pratensis* represented unique haplotypes and only two sequences were identical to those of *H. sturhani*. Maximum sequence divergence for species of the *H. avenae* complex was 4.1%.

Heterodera hordecalis

The ITS rRNA gene sequence alignment was 839 bp and included 22 sequences including seven new ones. The phylogenetic network for the ITS rRNA gene sequences reconstructed using statistical parsimony is given in Figure 3A. Sequences of the Italian and German populations were related to those of Israeli, Tunisian and Algerian samples. Iranian sequence differed from those from Sweden, UK, Slovakia and Estonia by 3-6 changes, whereas from Israeli, Tunisian and Algerian samples in seven or more changes. Maximal intraspecific variation for *H. hordecalis* was 2.0%.

Heterodera latipons *and* Heterodera *sp.*

The ITS rRNA gene sequence alignment was 813 bp in a length and included 31 sequences of *H. latipons* and three sequences of an unknown, putatively new *Heterodera* species previously identified as *H. latipons*. Two from the last three sequences were from samples collected in two locations in Turkey (İmren *et al*., 2015) and one new sequence also came from a cyst sampled in this country (Table 1). Phylogenetic network for the ITS rRNA gene sequences reconstructed using SP is given in Figure 3B. Ten haplotypes were distinguished within *H. latipons*, five of which came from Turkey, and three haplotypes were from Syria. *Heterodera latipons* from Rostov, Russia, differed by 8-10 changes from other haplotypes. A putatively new *Heterodera* species differed by 21 or more changes from *H. latipons* haplo-

Table 2. GenBank accession numbers for several main ITS haplotypes of species from the *Heterodera avenae* complex.

Species, haplotype	GenBank accession numbers
Heterodera filipijevi, Hf1	KP708728, KP708753, KP708715, KP708732, HM027892, KY349106, KT314234, KR704302, KF225725, KP708739, GU079654, KF679395, KP708733, KX579919, KP708724, KP708738, JX024203, KP708725, KP708734, KP708731, KX579904, JX235959, KP708743, KP708730, KP708754, KX579909, KX579910, KX579916, KP708722, KP708721, KX579918, KX579915, KX579914, KX579905, KX579907, KX579921, KX579920, KX579912, KX579911, KX579917, KX579908, KX579913, KX579906, KX579903, KX579902, KX579901, KX579900, KX579899, KX574302, KX574299, KR704308, HQ380883, AY148399, AY148398, KU896215, AY148397, HQ380875, AY347922, KR704304, KR704303, KR704292, KR704290, KR704287, HM370418, HM208382, GU083595, HM147948, HM147947, HM147946, KR704291, JX024209, KU896216, AY148400, AY148403, AY148402, AY148401, AF274399
H. filipijevi, Hf2	KP708718, KP708723, KP708717, KP708716, KP708745, KP708749, KP708714, KP708746, KP708712
H. filipijevi, Hf3	KP708752, KP708741, KP708740, KP708742, KP708750, KM199842, KM199843, KM199841, KM199840
H. arenaria	MG523155, AF274396
H. avenae, Hav1	MG523156, KX574285, KX574291, KX574286, KX574294, KX574298, KX574297, KX574301, KX574305, KX574308, AY148371, KC736875, KC736860, KM199816, KF225723, JX024198, AY148363, KM199809, KM199837, KM199815, KM199817, KM199831, KM199821, AF274397, AF498378, JX024197, JX024191, AY148364, AY148365, AY148366, KM199828, KM199822, KM199829, KM199830, HM560770, KC736872, KM199806, HM560755, KF225719, KF225721, KF225722, JX024193, KC736871, KM199814, KM199839, KC736858, KX574288, KX574292, KX574295, KX574290, KX574296, KX574303, KX574300, KX574304, KX574310, KX574311, KM199808, KM199820, KM199818, KM199819, JX024199, KR704296, KR704298, KC736870, KF225720, KC736856, KC736869, KC736857, KC736874, KC736877, KC736859, KC736861, KC736862, KC736873, KC736876, JX024196, KC847094, KF225724, EF153843, AY148358, AY148353, AF274395, AY148357, AY148360, KC736879, KC736863, KC736878, KC736864, KC736866, KC736867, KC736868
H. pratensis	AY148384
H. sturhani, Hst1	HM560745, HM560751, HQ450292, HM560754, JF712594, HQ450308, HM560738, KF679380, HM560753, HM370409, KR704312, HQ450304, KJ921716, KC428096, JN657197, JF712591, JN657203, JF712609, HQ380891, HQ380873, HM560756, GU377241, KF679378, KJ921712, HQ450294, HQ450296, HQ456210, KF679385, JF712607, KF679379, KF679375, JN657198, KF679372, HM370415, KF679370, GU595426, KF679363, HM560742, JF712596, HQ450298, HM560749, HQ456215, KF679376, HM560773, KF679384, JF712599, KF679388, KF679390, HM370407, HQ697363, KU896210, KF679367, GU595418, JN831600, HM370428, HM370414, HM560776, HM560772, HM560750, KF679387, GU595424, HM560736, KF679382, HQ380868, GU377247, GU377243, KF679368, JN831608, HM560748, KF679397, HM560771, JF712611, HM560759, HM370419, JN657200, EU106175, HM027887, HQ380876, JF712590, JN657201, GU377236, HQ450303, AY148381, KU896211, HQ450289, JF712605, KF679377, JF712601, KF679389, HM370426, KF679365, EU623614, HM370410, KC428085, JF712598, KC428095, KF679381, KF679386, EU623625, KJ921724, HM027891, HM560758, GU377249, GU377253, KF679364, HM560735, GU595419, KF679392, GU377246, JF712589, GU377235, GU377248, KF679371, HM560739, KF679391, JN657199, KF679366, GU083590, KC428099, HQ456216, HQ456214, HM370408, KF679398, HQ456213, GU377251, KF679383, HM370416, HM560746, HM370417, KC428098, HM560774, GU377240, HM370411, KF679373, HM370424, KJ921722, HQ450293, HM560733, KF679393, JQ067679, KR704309, HM370406, KF679396, HM560765, KC428093, KC428097, HM370420, HQ450306
H. sturhani, Hst2	HM027890, HQ450307, HM560757, EU623630, HM370427, JF712610, JX481893, KF679394, HM560775, HM560743, HM560777, JX481890, JX481894, JX481895, JX481897, GU595421, HM560747, JX481899, HQ698921, GU595422, EU623629, JF712604, GU595423, GU595425, HQ456211, HQ456212, GU377242, GU377244, GU377239, HQ456209, HQ450309
H. sturhani, Hst3	EU623627, EU616684, EU616700, EU623626, EU616705, HM560768, HM370429, HM560752, EU623634, HM560766, HM560762, EU616683, HM560763, EU616685, EU623608, EU616686, EU616688, EU623613, EU623609, EU623610, EU623611, EU623615, EU623618, EU623621, EU616691, GU595420, EU616701, EU616702, EU616703, EU616707, EU623612

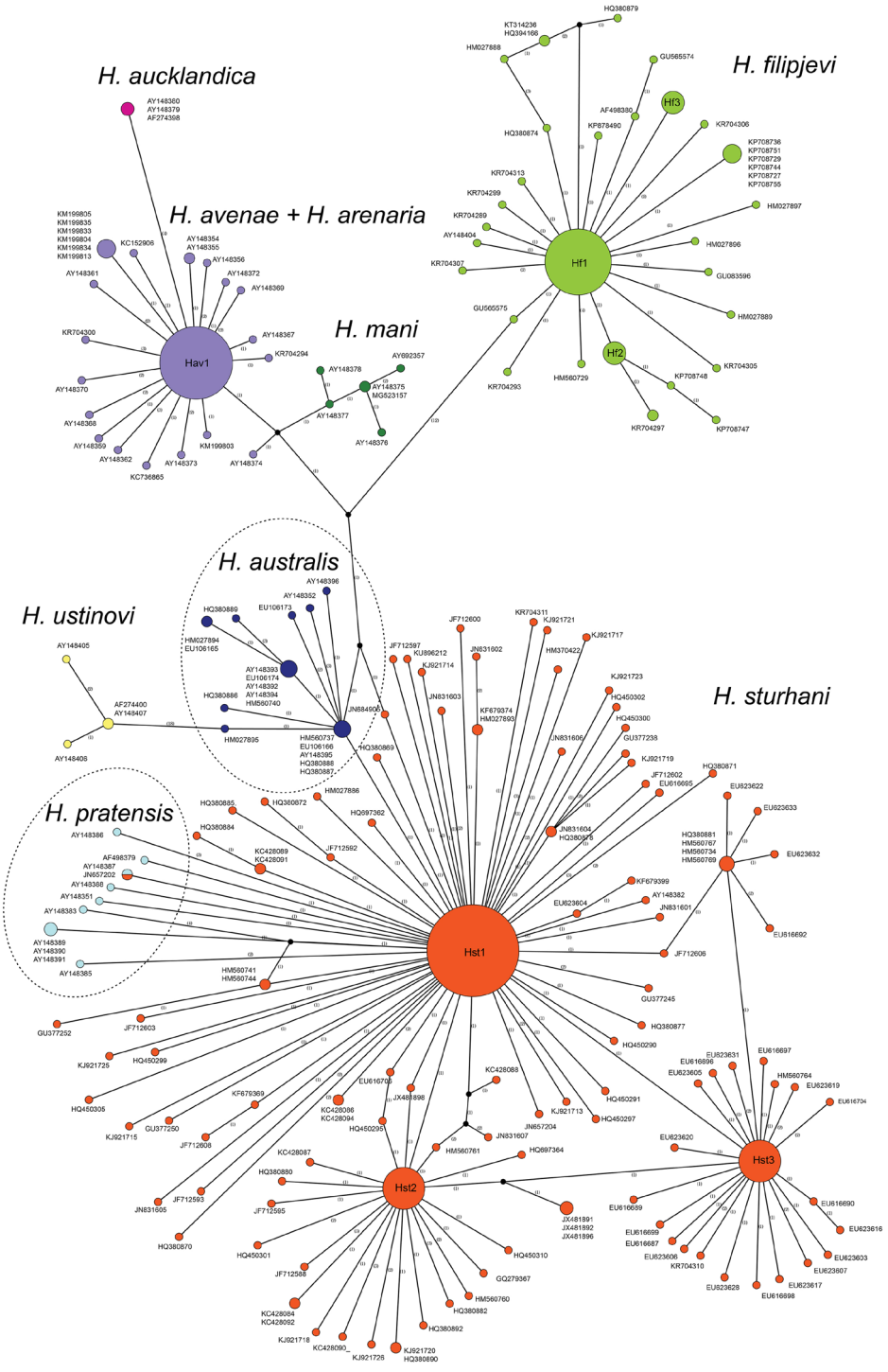


Fig. 2. Statistical parsimony network showing the phylogenetic relationships between ITS rRNA gene haplotypes of species from the *Heterodera avenae* complex. The sequences of each species are marked by different colours. Pies (circles) represent sequences of each species with the same haplotype and 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. Accession numbers for main haplotypes are given in Table 2.

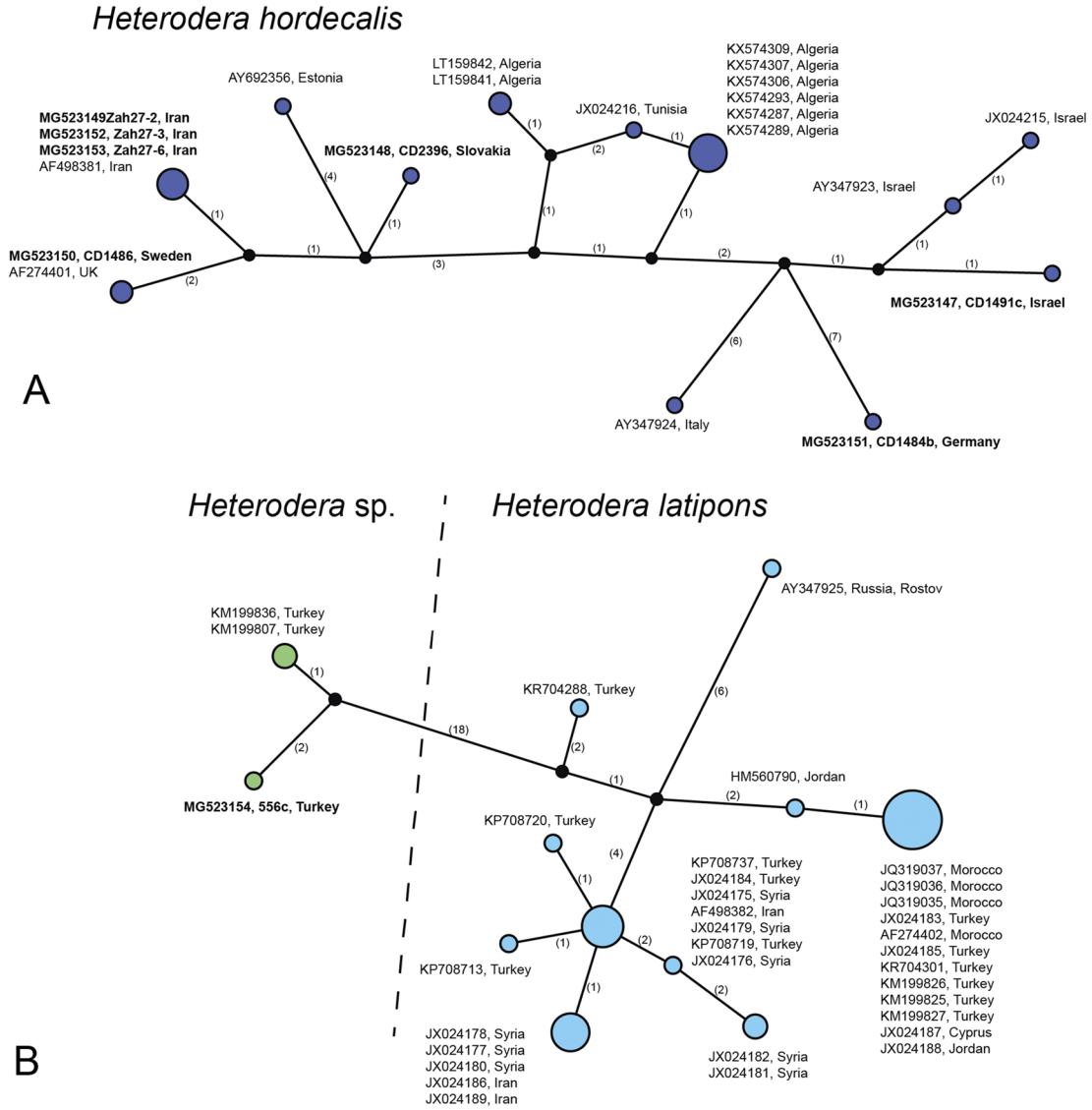


Fig. 3. Statistical parsimony network showing the phylogenetic relationships between ITS rRNA haplotypes. A: *Heterodera hordecalis*; B: *H. latipons* and *Heterodera* sp. The sequences of each species are marked by different colours. Pies (circles) represent the sequences with the same haplotype and 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. Newly obtained sequences are indicated by bold letters.

types. Maximal intraspecific variation for *H. latipons* was 2.8%.

Phylogenetic and sequence analysis with the *COI* gene

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

Avenae *group*

A total of 220 new *COI* gene sequences was obtained in this study. The *COI* gene alignment was 415 bp in a

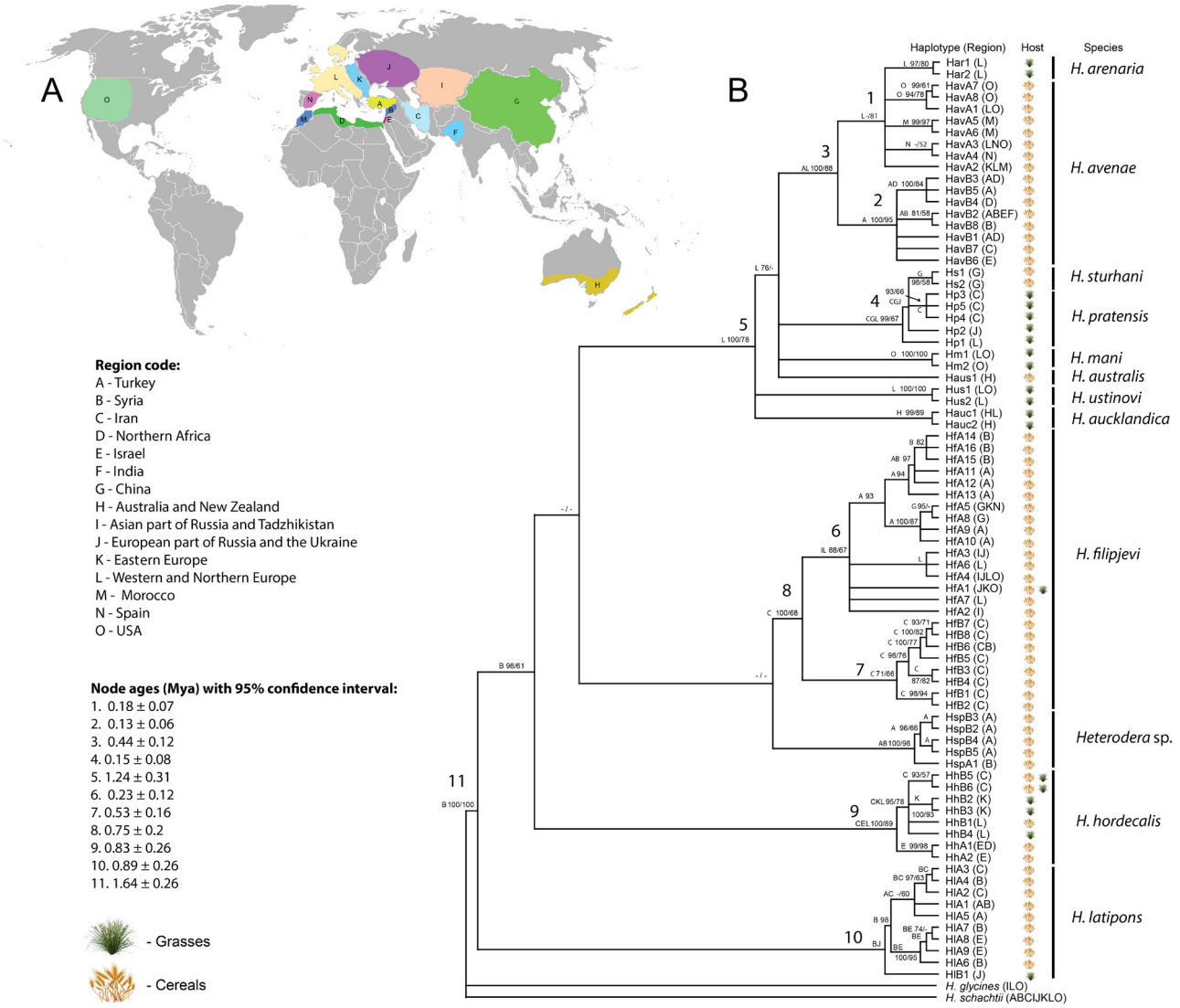


Fig. 4. Phylogenetic relationships between *COI* haplotypes of the *Avenae* group species as inferred from Bayesian analysis with mapping of regions and plant-hosts and indication of node ages. A: World map with region codes; B: Phylogenetic tree. Codes with most probable ancestral regions, posterior probability values for BI analysis and bootstrap values for ML analysis are given to appropriate clades.

length. The majority of nematode populations contained only one *COI* gene haplotype, whereas two haplotypes were found in 21 populations and three haplotypes in two populations. Phylogenetic relationships within the *Avenae* group species, containing 79 reference haplotype sequences and two sequences of outgroup species as inferred from BI and ML analyses, are given in Figure 4. Several distinct clades represented species of the *Avenae* group. *Heterodera latipons* occupied the basal position in the BI tree. Two species-pairs clustered in the same clades: *Heterodera avenae* type A clustered together with *H. arenaria*, and *H. pratensis* clustered with *H. sturhani*.

Heterodera avenae sensu stricto *and* H. arenaria

A total of 82 sequences of *H. avenae* and six sequences of *H. arenaria* were included in the analysis. The haplotype phylogenetic network is given in Figure 5. Haplotypes of *H. avenae* were divided into two

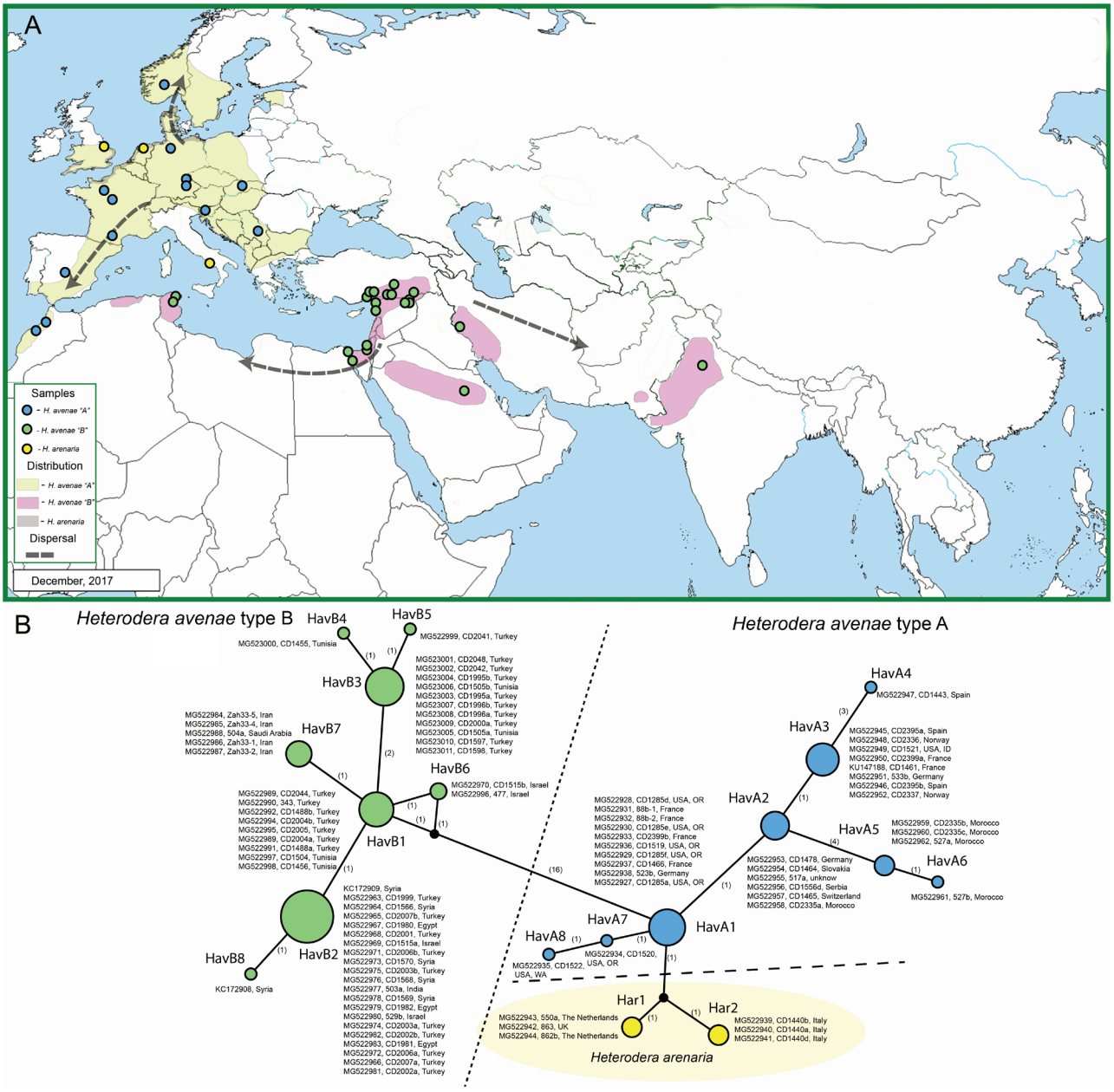


Fig. 5. A: Distribution map of *Heterodera avenae* and *H. arenaria* with indication of the studied samples and putative pathways of dispersal; B: Statistical parsimony network showing the phylogenetic relationships between *COI* haplotypes of *H. avenae* and *H. arenaria*. Small black circles represent missing haplotypes. Pie chart sizes are proportional to the number of samples with a particular haplotype.

groups (Table 3). Type A included eight haplotypes (HavA1-HavA8) from Europe, North America and Northern Africa (Morocco). Type B also included eight haplotypes (HavB1-HavB8) from Asia and Northern Africa (Tunisia, Egypt). HavA1-HavA3 were the most distributed haplotypes in Europe and the USA, whereas HavA5 and HavA6 were found only in Morocco, HavA7 and HavA8 only in the USA, and HavA4 was only reported from Spain. HavB2 was the most distributed haplotype in Asia and North Africa. Four haplotypes: HavB1, HavB2, HavB3 and HavB5, were found in Turkey, three haplotypes: HavB1, HavB3, HavB4, in Tunisia, and HavB2 and HavB8 in Syria. Haplotype HavB7 was reported only from Iran and Saudi Arabia (Fig. 5A). Two haplotypes (Har1 and Har2) of *H. arenaria* differed by two changes from each other and from the related *H. avenae* HavA1 haplotype (Fig. 5B). Maximal intraspecific sequence diversity for *H. avenae* was 8.2% and for *H. arenaria* it was 0.4%. Maximal sequence diversity for *H. avenae* type A was 2.6% and for *H. avenae* type B it was 1.7%.

Heterodera filipjevi

A total of 75 sequences of *H. filipjevi* was analysed. The haplotype network is given in Figure 6. All haplotypes were divided into two groups: type A containing 16 haplotypes (HfA1-HfA16) from Europe, Asia and North America, and type B containing eight haplotypes (HfB1-HfB8) from Iran and Syria (Toumi *et al*., 2013b). Haplotypes of type B were linked with the haplotype HfA2 found in Tadzhikistan and Bashkortostan, Russia. HfA1 and HfA4 were the most distributed haplotypes in Central, Northern and Eastern Europe, the European part of Russia, and North America. HfA3 was found in Tadzhikistan, Ukraine and Bashkortostan. HfA6, and HfA7 were found only from the UK, and HfA8 only in China. HfA9, HfA10, HfA11 HfA12 and HfA13 were reported from Turkey, and HfA14, Hf15, HfA16 were only from Syria. Maximal intraspecific sequence diversity was 10.6%. Maximal sequence diversity for *H. filipjevi* type A was 3.4% and *H. filipjevi* type B was 8.1%.

Heterodera pratensis *and* H. sturhani

A total of 17 sequences of *H. pratensis* and seven of *H. sturhani* were included in this study. The haplotype network is given in Figure 7. Two haplotypes (Hs1, Hs2) for *H. sturhani* and five haplotypes (Hp1-Hp5) for *H. pratensis* were revealed. The haplotypes Hs1 and Hs2 of *H. sturhani* were isolated from China, the only country where this species is known to be present (Fig. 7A). The haplotypes Hp1 and Hp2 came from European samples, whereas Hp3, Hp4 and Hp5 were only found in Iran. The haplotypes of *H. pratensis* differed by two or more changes from those of *H. sturhani* (Fig. 7B). Maximal sequence diversities for *H. pratensis* was 2.1% and for *H. sturhani* was 0.7%.

Heterodera aucklandica

A total of five new sequences of *H. aucklandica* was analysed. Two haplotypes were revealed: Hauc1 from New Zealand and UK, and Hauc2 from New Zealand, only differing by one change from other with sequence diversity equal to 0.2%.

Heterodera australis

A total of five sequences of *H. australis* showing a single haplotype (Haus1) was analysed.

Heterodera mani

A total of six sequences of *H. mani* was analysed. Two haplotypes (Hm1 and Hm2) were revealed: Hm1 from Germany and the USA, and Hm2 from the USA differing by one change. Sequence diversity was 0.2%.

Heterodera ustinovi

A total of five new sequences of *H. ustinovi* was analysed. Two haplotypes were revealed: Hus1 from Belgium and the USA, and Hus2 from Belgium, being distinguished by one change with sequence diversity equal to 0.2%.

Heterodera hordecalis

A total of 11 sequences of *H. hordecalis* was analysed. The haplotype network is given in Figure 8. All haplotypes were divided into two groups: type A containing two haplotypes (HhA1, HhA2) from Israel and Tunisia, and type B containing six haplotypes (HhB1-HfB6) from Europe and Iran (Fig. 8A). These two groups differed by 32 changes. Haplotypes from type B were reported for certain regions: HhB1 from Sweden, HhB2, HhB3 from Slovakia, HhB4 from Germany, and HhB5, HhB6 from Iran (Fig. 8B). In two samples from Iran, *H. goldeni* was found in a mixture with *H. hordecalis*. Maximal intraspecific sequence diversity was 11.4%. Maximal sequence diversity for *H. hordecalis* type A was 1.2% and *H. hordecalis* type B was 4.1%.

Heterodera latipons *and an unknown* Heterodera *sp.*

A total of 16 sequences of *H. latipons* and five sequences of an unknown *Heterodera* sp. were analysed.

Table 3. Variable positions of nucleotides in the partial *COI* gene defining *Heterodera avenae* (Hav) and *H. arenaria* (Har) haplotypes.

Haplotype	Positions in alignment																								
	2	7	11	41	47	53	56	66	69	92	95	107	131	140	143	146	147	157	164	173	179	197	200	209	215
HavA1	C	A	C	C	A	T	C	G	G	A	T	G	T	T	T	C	C	C	T	A	T	A	T	A	T
HavA2	T	A	C	C	G	T	C	G	G	A	T	G	T	T	T	C	C	C	T	A	T	A	T	A	T
HavA3	C	A	C	C	G	T	C	G	G	A	T	G	T	T	T	C	T	C	T	A	T	A	T	A	T
HavA4	C	A	C	C	G	T	C	G	G	A	T	T	T	T	T	T	T	C	T	A	T	A	T	A	T
HavA5	C	G	C	C	G	T	C	G	G	A	T	G	T	T	T	C	C	C	C	A	T	A	T	A	T
HavA6	C	G	C	C	G	T	C	G	G	A	T	G	T	C	T	C	C	C	C	A	T	A	T	A	T
HavA7	C	A	C	C	A	T	C	G	G	A	T	G	T	T	T	C	C	C	T	A	A	A	T	A	T
HavA8	C	A	C	C	A	T	C	G	G	A	T	G	T	T	A	C	C	C	T	A	A	A	T	A	T
Har1	C	A	C	C	G	T	C	G	G	A	T	G	T	T	T	C	C	C	T	A	T	A	T	A	T
Har2	C	A	C	C	G	T	C	G	G	A	T	G	T	T	T	C	C	C	T	A	G	A	T	A	T
HavB1	C	G	T	T	G	C	A	A	G	T	C	G	C	T	T	C	C	A	T	G	T	A	C	G	C
HavB2	C	G	T	T	G	C	A	A	G	T	C	G	C	T	T	C	C	A	T	G	T	G	C	G	C
HavB3	C	A	T	T	A	C	A	A	A	T	C	T	C	T	T	C	C	A	T	G	T	A	C	G	C
HavB4	C	A	T	T	A	C	A	A	A	T	C	T	C	T	T	C	C	A	T	G	T	A	C	G	C
HavB5	C	A	T	T	A	C	A	A	A	T	C	T	C	T	T	C	C	A	T	G	T	A	C	G	C
HavB6	C	G	T	T	G	C	A	A	G	T	C	G	C	T	T	C	C	A	T	G	T	A	C	G	C
HavB7	C	G	T	T	G	C	A	A	G	T	C	G	C	T	T	C	C	A	T	G	T	A	C	G	C
HavB8	–	–	–	–	–	–	–	–	–	T	C	G	C	T	T	C	C	A	T	G	T	A	C	G	C

Table 3. (Continued.)

Haplotype	Positions in alignment																						
	218	230	239	242	245	247	257	261	287	299	329	341	344	347	356	357	359	362	374	377	378	395	401
HavA1	T	A	G	T	T	G	A	T	G	T	T	T	A	T	G	T	A	T	T	A	T	T	T
HavA2	T	A	G	T	T	G	A	T	G	T	T	T	A	T	G	T	A	C	T	A	T	T	C
HavA3	T	A	G	T	T	G	A	T	G	T	T	T	A	T	G	T	A	C	T	A	T	T	C
HavA4	T	A	G	T	T	G	A	T	G	T	T	T	A	T	G	T	A	C	C	A	T	T	C
HavA5	T	G	A	T	T	G	A	T	G	T	T	T	A	T	G	C	A	C	T	A	T	T	C
HavA6	T	G	A	T	T	G	A	T	G	T	T	T	A	T	G	C	A	C	T	A	T	T	C
HavA7	T	A	G	T	T	G	A	T	G	T	T	T	A	T	G	T	A	T	T	A	T	T	T
HavA8	T	A	G	T	T	G	A	T	G	T	T	T	A	T	G	T	A	T	T	A	T	T	T
Har1	T	A	G	T	T	G	A	T	G	T	C	T	A	T	G	T	A	T	T	G	C	T	C
Har2	T	A	G	T	T	G	A	T	G	T	C	T	A	T	G	T	A	C	T	G	C	T	C
HavB1	A	A	C	G	A	A	A	C	G	T	T	A	G	T	T	T	A	T	T	A	T	C	C
HavB2	A	A	C	G	A	A	A	C	G	T	T	A	G	T	T	T	A	T	T	A	T	C	C
HavB3	A	A	C	G	A	A	C	C	G	T	T	A	G	T	T	T	A	T	T	A	T	C	C
HavB4	A	A	C	G	A	A	C	C	G	C	T	A	G	T	T	T	A	T	T	A	T	C	C
HavB5	A	A	C	G	A	A	C	C	G	T	T	A	G	T	T	T	G	T	T	A	T	C	C
HavB6	A	A	C	G	A	A	A	C	G	T	T	A	G	T	T	T	A	T	T	A	T	C	C
HavB7	A	A	C	G	A	A	A	C	G	T	T	A	G	G	T	T	A	T	T	A	T	C	C
HavB8	A	A	C	G	A	A	A	C	T	T	T	A	G	T	T	T	A	T	T	A	–	–	–

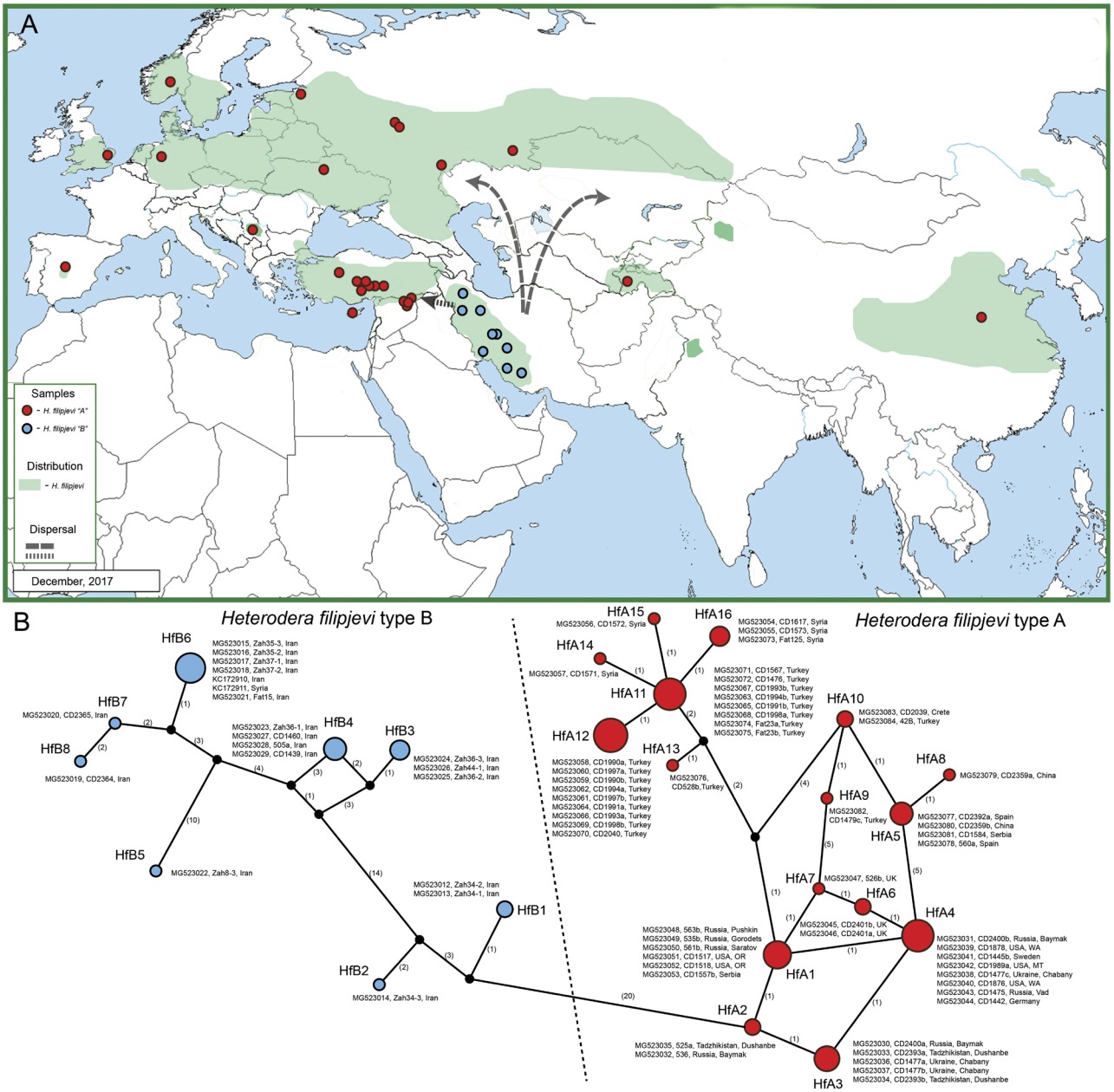


Fig. 6. A: Distribution map of *Heterodera filipjevi* with indication of the studied samples and putative pathways of dispersal; B: Statistical parsimony network showing the phylogenetic relationships between *COI* haplotypes of *H. filipjevi*. Small black circles represent missing haplotypes. Pie chart sizes are proportional to the number of samples with a particular haplotype.

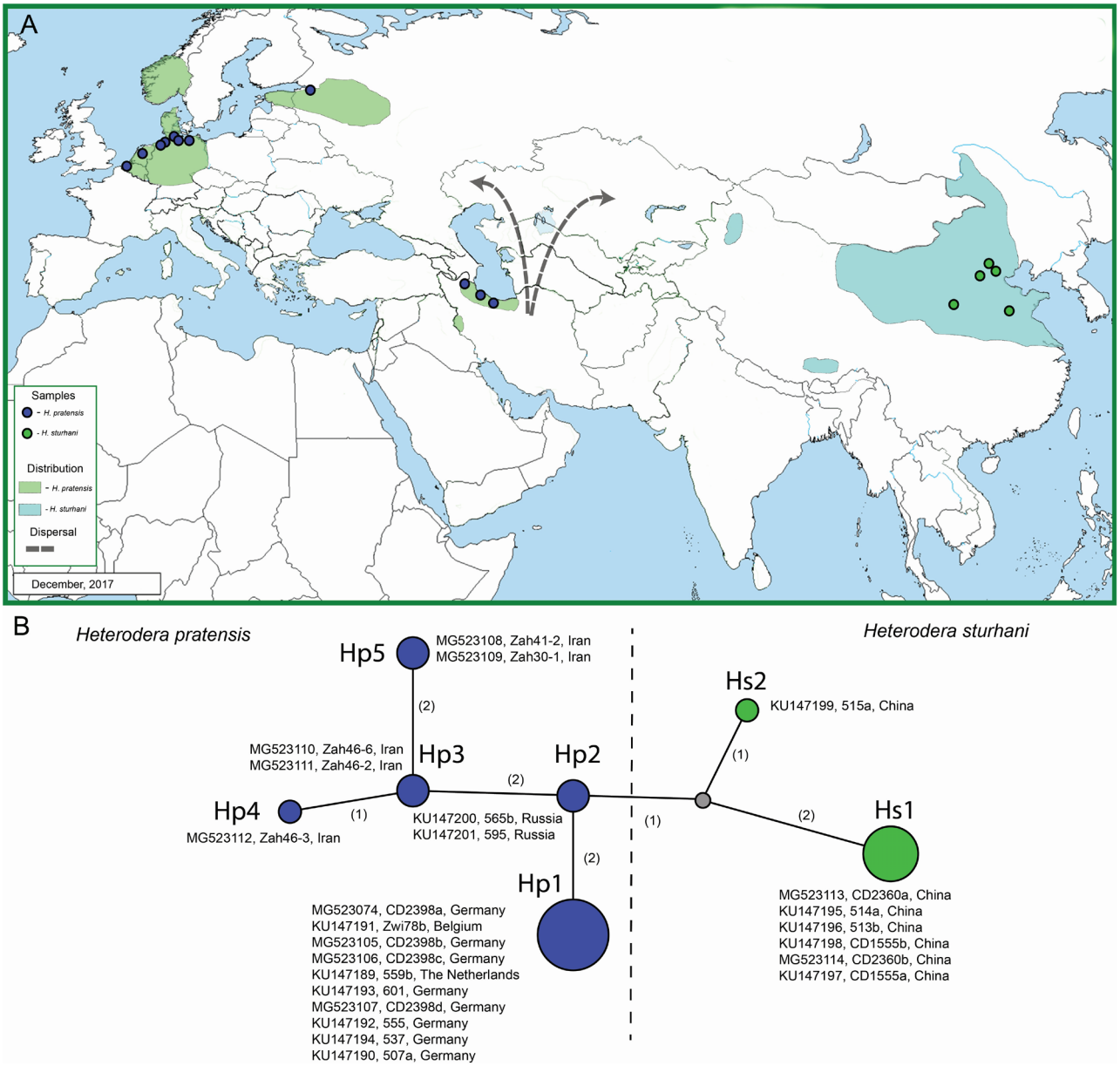


Fig. 7. A: Distribution map of *Heterodera pratensis* and *H. sturhani* with indication of the studied samples and putative pathways of dispersal; B: Statistical parsimony network showing the phylogenetic relationships between *COI* haplotypes of *H. pratensis* and *H. sturhani*. Small black circles represent missing haplotypes. Pie chart sizes are proportional to the number of samples with a particular haplotype.

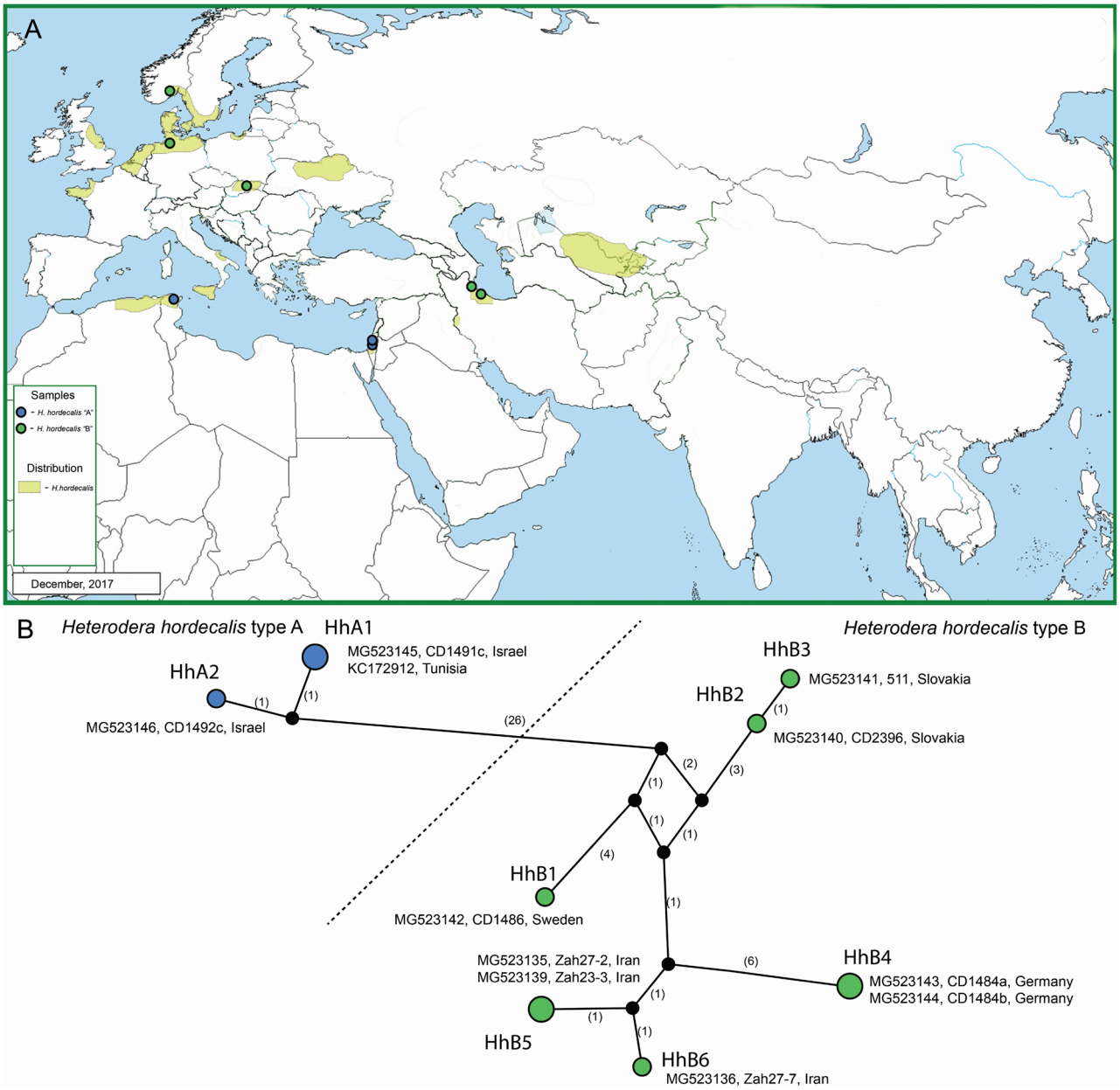


Fig. 8. A: Distribution map of *Heterodera hordecalis* with indication of the studied samples; B: Statistical parsimony network showing the phylogenetic relationships between *COI* haplotypes of *H. hordecalis*. Small black circles represent missing haplotypes. Pie chart sizes are proportional to the number of samples with a particular haplotype.

The haplotype network is given in Figure 9. The haplotypes were divided into two groups: A and B (Fig. 9A). Type A contained nine haplotypes from certain regions: HlA1 in Syria and Turkey, HlA2, HlA3 in Iran, HlA4, HlA6, HlA7 in Syria, HlA5 in Turkey, and HlA8, HlA9 in Israel. The type B haplotype group was only found in Russia and significantly differed from all the others. *Heterodera* sp. was represented by two distinct haplotype groups (A and B) – HspA1 from Syria, and HspB2-HspB5 from Turkey (Fig. 9B). Maximal intraspecific sequence diversity for *H. latipons* was 12.0% and for *Heterodera* sp. was 9.4%. Maximal sequence diversity for *H. latipons* type A was 7.7%.

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 and are given in Figure 4. Ancestral areas for clades with *H. latipons*, *H. hordecalis*, *Heterodera* sp. and *H. filipjevi* were Turkey, Syria and Iran. This world region could be also considered likely as ancestral for *H. filipjevi*, *H. avenae* type B and *H. pratensis*, *H. latipons* and *Heterodera* sp.

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 *H. hordecalis* and *H. latipons*. Estimated node ages for some main clades are given in Figure 4. The earliest divergence within the *Avenae* group was estimated at 1.64 ± 0.26 Mya (million years ago). It further split into clades with *H. avenae* A and B at 0.44 ± 0.12 Mya; *H. filipjevi* – 0.75 ± 0.2 Mya; *H. hordecalis* – 0.83 ± 0.26 Mya, and *H. latipons* – 0.89 ± 0.26 Mya (Fig. 4).

Discussion

Species delimiting and DNA barcoding

The results of the present study suggest that the main sequence groups are generally congruent with the currently recognised species. The ITS rRNA gene marker was able to differentiate the majority of species in the *Avenae* group, but failed to separate two species-pairs: *i*) *H. avenae* and *H. arenaria*; and *ii*) *H. pratensis* and *H. sturhani*. Analysis of the *COI* gene sequences, however, was able to differentiate all species of the group.

Heterodera avenae and *H. arenaria*, *H. pratensis* and *H. sturhani*

Our study showed that *H. arenaria* colonising grasses growing exclusively in coastal areas is closely related to *H. avenae*, and represents a species recently diverged from *H. avenae* type A somewhere in Europe. Molecular analysis also showed that *H. pratensis* and *H. sturhani* are closely related. We hypothesised that *H. sturhani* represents a species recently derived from *H. pratensis* in the East Asia region with an obtained ability to parasitise cereals. The *COI* gene phylogenetic and sequence analyses revealed that *H. arenaria* nested within the group of sequences of the *H. avenae* type A and that *H. sturhani* nested within the *H. pratensis* group of sequences, the differences in *COI* gene sequences within each pair being within the range of intraspecific variations for most of the other *Avenae* group species. However, both *H. arenaria* and *H. sturhani* represent separate evolutionary lineages and we believe that these species should be considered as valid species. Morphological, biochemical and molecular differentiations of *H. arenaria* from *H. avenae* and *H. sturhani* from *H. pratensis* and other species are discussed in detail in several publications (Robinson *et al*., 1996; Subbotin *et al*., 2001, 2003, 2010; Subbotin, 2015) and here we can only underline that their taxonomic differentiation is necessary for phytosanitary regulatory reasons. In fact, *H. sturhani* and *H. avenae* are economically important parasites of cereals and should be subjected to phytosanitary actions, whereas *H. pratensis* and *H. arenaria* have less economic relevance since they only infest grasses. Furthermore, a proper management of *H. sturhani* and *H. avenae* using resistant cereal varieties in nematode-infested regions should also include the identification of their haplotypes.

Heterodera australis in China

Australian CCN was considered an endemic in Australia (Subbotin *et al*., 2010). It has been shown by Subbotin *et al*. (2003) and Subbotin (2015), and confirmed by the present study, that *H. australis* can be differentiated from other species by ITS rRNA and *COI* gene sequences, the latter gene marker giving the best discrimination. Several ITS rRNA sequences submitted from samples collected in the Henan and Hebei Provinces of China, and described as *H. avenae* by Ou *et al*. (2008) and Fu

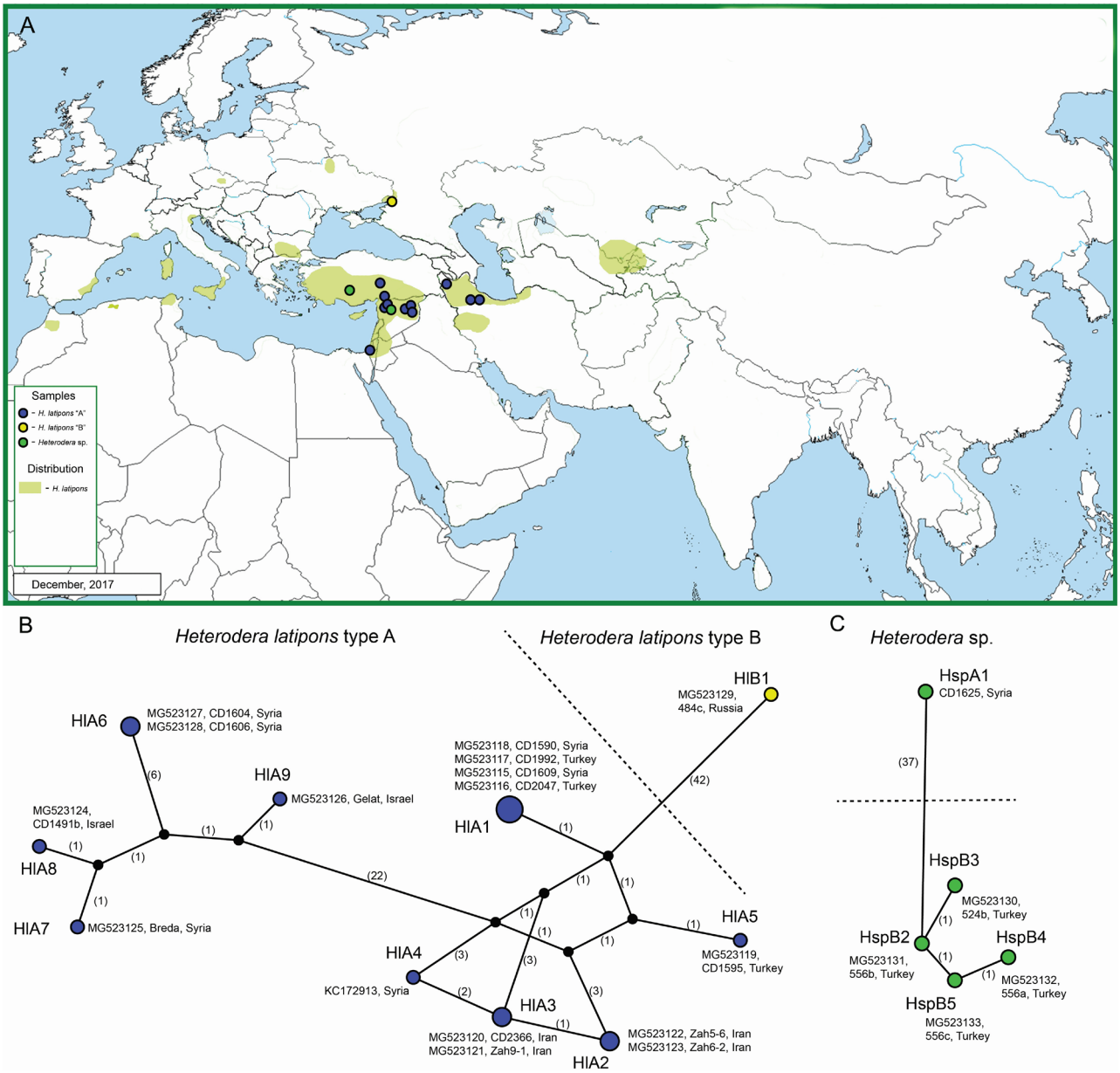


Fig. 9. A: Distribution map of *Heterodera latipons* and *Heterodera* sp. with indication of the studied samples; B: Statistical parsimony network showing the phylogenetic relationships between *COI* haplotypes of *H. latipons*; C: Statistical parsimony network showing the phylogenetic relationships between *COI* haplotypes of *Heterodera* sp. Small black circles represent missing haplotypes. Pie chart sizes are proportional to the number of samples with a particular haplotype.

et al. (2011), were identified in our analysis as representatives of *H. australis*. Fu *et al*. (2011) have already reported on the detection of the 'Australis type' in the datasets and noticed that the CCN in China was more genetically diverse than previously known and that this diversity can occur within a relatively restricted geographic range. In another study, Yuan *et al*. (2010) proposed the new pathotype Ha43 for the CCN population from Henan, near Zhengzhou, which made it different from all other *H. avenae* pathotypes and similar to the *H. australis* pathotype. Although, the phylogenetic and sequence analysis of ITS rRNA gene sequences clearly showed the presence of *H. australis* in China, these findings still need to be confirmed by the *COI* gene marker and morphometric analysis of nematodes. If this identification is supported, the hypothesis of the introduction of this species from Asia to Australia would appear to be more plausible than the alternative, as suggested by Meagher (1977), of a European origin of the CCN occurring in Australia.

Heterodera sp. from Turkey and Syria

The analysis of two ITS rRNA gene sequences published by İmren *et al*. (2015) from Turkey and originally obtained ITS rRNA and *COI* gene sequences from samples collected in Turkey and Syria facilitated the molecular delimitation of an unknown species within the *Avenae* group. This species, being genetically similar to *H. latipons*, can be considered as putatively new to science. Mor & Sturhan (2000) reported that re-examination of *H. latipons* type specimens revealed that it consisted of two morphological types that were distinguishable on cyst characteristics. One type agreed with that figured in the original description of *H. latipons* and the other resembled the related species, *H. hordecalis*. These authors considered these differences as evidence of an additional and undescribed species within the *H. latipons* species complex (Subbotin *et al*., 2010). Rivoal *et al*. (2003) also mentioned morphological divergences in Syrian populations of *H. latipons* from other populations of this species. Additional morphological variations, including a longer cyst vulval slit and J2 stylet, were reported by Abidou *et al*. (2005a) for Turkish populations of putative *H. latipons*. The morphological variability of the Turkish populations corresponds to their genetic variability in this study. These findings suggest that these Turkish populations belong to an undescribed species. Evidently, the samples with aberrant putative *H. latipons* populations should be further carefully studied and used for the formal description of a new *Heterodera* species.

ITS rRNA sequence variation

Our analysis revealed a relatively high intraspecific ITS sequence diversity for *H. latipons* and *H. hordecalis*, which are the two oldest species in the *Avenae* group, whereas for the *H. avenae* species complex the intraspecific variation was relatively low. Sequence variation was observed within *H. sturhani* samples. The relatively high number of haplotypes with single point mutations in *H. sturhani* compared with those of *H. avenae* and *H. filipjevi* may be not reflect a higher mutation rate for the ITS rRNA gene for this species, but may be explained by a higher number of mistakes during sequence reading of some of the Chinese samples.

DNA barcoding

DNA barcoding is a powerful tool for identification of many nematode groups. In this study we generated a large number of *COI* sequences of the *Avenae* group species from different world regions. The study showed that each species has a unique *COI* sequence set or DNA barcode that enables its identification and separation from all other species. The DNA sequences of this gene might serve as an important information basis for developing specific diagnostics tools, including PCR-RFLP, conventional PCR and Real Time PCR with specific primers and other techniques.

Phylogenetic relationships

Phylogenetic relationships reconstructed using ITS rRNA and *COI* gene sequences for species of the *Avenae* group in this study are generally congruent to each other and similar to those published in previous works (Subbotin *et al*., 2001, 2003; Tanha Maafi *et al*., 2003; Subbotin, 2015). Differences in the position of some species between phylogenetic trees were mainly in weakly supported clades. According to our phylogenetic analyses, the *Avenae* group constituted a well-supported monophyletic assemblage and contained several distinct evolutionary lineages.

For the first time in this study we have applied a phylogenetic network approach to analyse massive sequence datasets of the *Avenae* group. This approach gives a clearer picture of discrimination between sequences than a traditional reconstruction of evolutionary relationships using a phylogenetic tree, which is employed by many researchers. Although in this study we described the results of analysis obtained with a statistical parsimony, the min-

imum spanning and median joining algorithms also generated a network with a similar structure from the same sequence dataset.

Biogeography

An important step in the analysis of the *Avenae* group in our study was to describe distribution patterns and understand how these distributions were achieved. Two competing hypotheses, namely, repeated intercontinental dispersal and continental drift, have traditionally been proposed to explain the broad distributions of many organisms, including nematode species. Statistical techniques combining phylogenetic information and distributions of extant taxa and molecular dating have therefore been developed to resolve controversy between these approaches.

In this paper we present the results of two genetic markers, ribosomal and mitochondrial genes. Although ITS-rRNA gene can be used for species delimiting it provides very limited information for phylogeographic studies. Although, we consider our results with mitochondrial marker indicative, it should be taken into account that a single marker may not accurately reflect species history. In fact, we cannot completely exclude the possibility of asymmetrical introgression of mitochondrial DNA between some populations, a phenomenon that may be observed in some nematode species (Subbotin *et al*., 2015).

In our study we applied the molecular clock hypothesis to estimate divergence time between species and populations in the *Avenae* group. Although in the analysis a global molecular clock assumption is rejected, a relaxed molecular clock could be applied. Because of the lack of fossils for this nematode group we used a mitochondrial substitution genome rate for molecular calibration. In our study we used a mitochondrial substitution genome rate equal to 7.2×10^{-8} per site per generation, as calculated by Howe e*t al*. (2010) for *Caenorhabditis briggsae*. This rate was considered as an average for several nematode species: *Pristionchus pacificus* – 4.5×10^{-8} ($\pm$ 1.7×10^{-8}) site/generation (Molnar *et al*., 2011), *C. elegans* – 9.7×10^{-8} site/generation (Denver *et al*., 2000), and close to the mutation rate reported for an insect, the vinegar fly, *Drosophila melanogaster* – 6.2×10^{-8} site/generation (Haag-Liautard *et al*., 2008). Because many controversies have been focused on the most common use of the clock assumptions, such as the dating of divergence times, we wish to emphasise that our results using estimation divergence dates should be viewed with caution.

Origin and dispersal of the *Avenae* group species

Several authors have proposed possible centres of origin for the *Avenae* group species. Krall & Krall (1978) suggested a rather wide area of origin for representatives of *Heterodera*, including the *Avenae* group, namely Mediterranean, Caucasus and some Middle Asian regions. Bekal *et al*. (1997) hypothesised that the most likely site of origin of *H. avenae* was within the cereal production areas in the Middle East and that this nematode could have been introduced to Australia from Western Asia. The result of the S-DIVA analysis, species and haplotype distribution and haplotype diversity analysis showed that the Irano-Anatolian hotspot (Fig. 10) is likely the primary centre of origin and diversification of the *Avenae* group. The hotspot covers almost 900 000 km^2, including major parts of central and eastern Turkey, a small part of southern Georgia, the Nakhchivan Autonomous Republic of Azerbaijan, Armenia, north-eastern Iraq, northern and western Iran, and the Northern Kopet Dag Range in Turkmenistan. It has been shown that the Irano-Anatolian hotspot contains many centres of local endemism (Myers *et al*., 2000). Our estimation of haplotype diversities may indicate that species of the *Avenae* group originated from different regions of this hotspot during the Pleistocene (2.58-0.117 Mya).

Subbotin *et al*. (2016) noticed that mountain areas are possible regions for species diversification of cyst nematodes of the genus *Globodera* Skarbilovich, 1959. This hypothesis can also be applicable to cyst nematodes of the genus *Heterodera*. The Irano-Anatolian hotspot consists of mountainous and inter-mountain regions that could have been crucial for the long-term survival and ability



Fig. 10. Irano-Anatolian hotspot region (after Kiani *et al*., 2017, with modification).

to accumulate genetic diversity for species of the *Avenae* group. The fluctuating climate during the Quaternary period has enhanced patterns of genetic diversity of these species. Populations occurring in this area are characterised by high levels of genetic diversity, whereas populations established in other region show relatively low genetic variation.

Two main regions in the Irano-Anatolian hotspot are proposed herein as subcentres of origin and diversification, and correspond to two hypotheses of dispersals for this group, namely, 'Out of Anatolia' and 'Out of Persia (Iran)'. The Anatolian part of the Irano-Anatolian hotspot is likely to be the centre of origin of *H. latipons* as the highest number of ITS and *COI* haplotypes are presently recorded from Turkey and Syria. This species occupies a basal position in the *Avenae* group phylogeny and can be considered as the oldest taxon in this group. From Anatolia, *H. latipons* likely dispersed in different directions to Europe, Syria, Jordan, Israel and North Africa (Morocco), and Iran.

Anatolia is also a possible subcentre of origin of *H. avenae* from which this species (type B) dispersed across Western Asia and North Africa and South Asia (Pakistan, India). The global haplotype arrangement was achieved by expansions from already internally structured Anatolian diversity. Another evolutionary lineage, *H. avenae* type A, was diversified and distributed across Europe, reaching Morocco *via* the Iberian Peninsula and colonising North Africa during the Holocene. It is interesting to note that there is no overlap in distribution of *H. avenae* groups (type A and B). A plausible explanation for the separation of these two groups is that two mountain systems, one in Asia (Anatolian Mountains) and another in Africa (Atlas Mountains), might have served as environmental barriers to prevent gene flow between these groups.

The Iranian mountains (Alborz Mountains and Zagros Mountains) are possibly another subcentre of origin of *Avenae* group species, namely, *H. filipjevi*. Indeed, the highest genetic diversity of *H. filipjevi* was observed in Iran. The presence of spatially circumscribed haplotypes of *H. filipjevi* in Iran separated by large mutation distances can be explained as a consequence of a long-term extrinsic barrier to genetic exchange in this region. The Iranian *COI* haplotypes, which are linked with Eastern and Central European and Middle Asia ones and not with the Turkish haplotypes, may indicate the presence of significant environmental barriers for distribution. It has been suggested that the so-called Anatolian Diagonal (Ekim & Güner, 1989) may serve as a significant environmental or geophysical barrier for distribution of species and acts as an effective barrier to gene flow between the western and eastern regions of Anatolia. It is interesting to note that the Anatolian Diagonal might also serve as a barrier for *H. avenae* type B by preventing its distribution to the western regions of Anatolia.

The meadow cyst nematode *H. pratensis* also most likely originated in Iran and became relatively recently distributed along the Holarctic region. *Heterodera sturhani* originated from one of the Eastern Asian *H. pratensis* population groups and, due to its ability to parasitise cereals, became the most widely distributed species of the *Avenae* group in agricultural fields in China. Further study of species parasitising grasses in Eastern Asia, such as cysts recently found in alpine meadow steppe from *Kobresia* spp. in Gansu, China (Li *et al*., 2015), could help to identify a secondary centre of speciation for this nematode group.

Iran is also a possible place for origin for the barley CCN, *H. hordecalis*. This species likely colonised Europe *via* different routes, one from Asia and another one from North Africa. Unfortunately, the limited numbers of sources for *H. australis*, *H. aucklandica*, *H. ustinovi* and *H. mani* did not allow us to propose the centres of origin for these species.

The patterns of haplotype distributions for *H. filipjevi* and *H. avenae* type A parasitising cereals and *H. mani* and *H. ustinovi* parasitising grasses are very similar in Europe and North America. This might suggest a scenario involving long-distance and trans-Atlantic dispersals of these species from Western and Northern Europe to North America after the last glaciation across an ocean barrier. We also believe that distribution of these species to North America was not due to the human colonisation of this continent and recent agricultural activity, but was due to earlier natural dispersal events that likely occurred at different time lines. The absence of any unique *COI* haplotypes of *H. filipjevi* for North America and the presence of such haplotypes for *H. avenae* (Hav7 and Hav8) with a wider distribution for the latter species may suggest that *H. avenae* occurred in this continent at an earlier time than *H. filipjevi*.

Although it has been shown that cysts can be dispersed over short distances by wind (Petherbridge & Jones, 1944; Chitwood, 1951; White, 1953), no studies have been done to verify a long distance movement of these nematodes. A possible scenario of a global transport of cysts with dust storms, which may disperse cysts over northern Europe

and across the Atlantic to Canada and the USA, should be considered and tested. However, the conditions for long-distance cyst dispersal may have been more favourable in the late glacial and early postglacial period than the present time.

Although agricultural practice and human activity may facilitate nematode dispersal at a local level, it seems that they might not have played a significant role in the global distribution of species of the *Avenae* group. Different environmental barriers, centres and time of origin as well as different adaptivity of species to climate conditions might explain the current known distribution patterns for *H. filipjevi* and *H. avenae* and other species of the *Avenae* group.

COI HAPLOTYPES AND CCN PATHOTYPES

Presently, there are no resistant cereal cultivars available that can be applied to control all CCN species and which are effective against all pathotypes. That is why the use of resistance cultivars as a control measure against CCN requires precise knowledge of the distribution of species and pathotypes in each region. The pathotype scheme for the cereal cyst nematode *H. avenae* and related species is based on their capacity to reproduce on cereal cultivars having different resistance genes. The pathotypes are characterised based on the International Cereal Cyst Nematode Test Assortments consisting of several barley, oat and wheat lines – the so-called differentials. The Test Assortments was initially proposed by Andersen & Andersen (1982) and later improved by several authors (Rivoal & Cook, 1993; Cook & Rivoal, 1998; Cook & Noel, 2002; Smiley *et al*., 2011; Cui *et al*., 2015). Presently, three pathotypes groups have been identified: Group 1 contains nine pathotypes (Ha11-Ha91); Group 2 – two pathotypes (Ha12, Ha22); and Group 3 – four pathotypes (Ha13-Ha43). Identification of pathotypes is time-consuming and requires numerous repetitive tests. After analysis of our datasets, we suggest that the CCN pathotypes might correspond to certain species having distinct *COI* haplotypes. Taking into consideration the species concept for the *Avenae* group applied here, many pathotypes seem to belong to several valid species.

Pathotypes of Groups 1 and 2 are the most numerous and widely distributed in Europe, North Africa, America and Asia (Andersen & Andersen, 1982; Mor *et al*., 1992; Valocká *et al*., 1994; Al-Hazmi *et al*., 2001; Mokabli *et al*., 2002; Smiley *et al*., 2011; Haddadi *et al*., 2013; İmren *et al*., 2013; Singh & Kaur, 2015) and mainly belong to both haplotype groups of *H. avenae* or *H. sturhani*. Pathotype identification of some populations from these geographical regions remains uncertain. For example, five Pacific Northwest CCN populations exhibited affinities with Group 2, but were not defined as Ha12 and Ha22 pathotypes (Smiley *et al*., 2011). According to our results, several Pacific Northwest CCN populations contain unique *COI* haplotypes (haplotypes HavA7 and HavA8) on peripheral positions in the *COI* network. A unique Spanish haplotype occupying a peripheral position in the *COI* network might also correspond to pathotypes that are not found in other regions. Sanchez & Zancada (1987) designated Ha22, Ha71 and Ha81 to *H. avenae* isolates from different regions of Spain, and recently Cui *et al*. (2015) designated a new Ha91 pathotype to CCN from Daxing and Huangyuan, China, which likely belongs to *H. sturhani*.

Pathotype Group 3 included representatives of two species: *H. australis* and *H. filipjevi*. Pathotype Ha13 from South Australia and Victoria (Subbotin *et al*., 2010) and a recently designated pathotype Ha43 from Xushui and Xingyang, villages near Zhengzhou City (Yuan *et al*., 2010), likely belong to *H. australis*, whereas pathotypes Ha23 and Ha33 belong to *H. filipjevi* (Subbotin *et al*., 2010; Toktay *et al*., 2013). Several pathotypes, likely belonging to *H. sturhani*, and differing from those already known, were found in China (Yuan *et al*., 2010). We suggest that the correspondence between *COI* haplotypes and the CCN pathotypes should be widely tested and analysed. We also propose that all genetic researches and trials with the CCN should include information on *COI* haplotypes of studied populations.

In the present study, we have proposed several hypotheses based on some assumptions. The main one is that the biogeographic datasets used in our study for the *Avenae* group species reflect actual distribution rather than differences in worldwide sampling. Unfortunately, most published and original results came from surveys conducted in agricultural but not in natural areas. Evolution and distribution of the *Avenae* group species are associated with wild grasses and only relatively recently, after the agricultural revolution, did some species of this group became parasites of cereals. It is especially important to obtain results from nematological surveys of grasses in natural areas where we can expect to find higher diversity of *COI* haplotypes, including the discovery of ancestral haplotypes. New datasets could give more light on the time of origin and locations of centres and subcentres of diversification of species. The conclusions made in this study are based on analysis of two genes and the assumption that

the evolution of these genes reflects species evolution. Alternative datasets and other methods of analysis should be used to increase the robustness of biogeographic hypotheses and further test the findings made in the present study.

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