Heterodera sturhani sp. n. from China, a new species of the *Heterodera avenae* species complex (Tylenchida: Heteroderidae)

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Summary. *Heterodera sturhani* sp. n. is described as a new species from the *Heterodera avenae* complex. The new species is morphologically similar to *H. pratensis* and *H. riparia* in many characteristics of cysts and second-stage juveniles. It can be differentiated from *H. pratensis* by smaller cyst sizes and from *H. riparia* by longer body and tail lengths of the second-stage juveniles. The sequence and phylogenetic analysis of the *coxI* mtDNA gene sequences and the PCR-coxI-RFLP separate *H. sturhani* sp. n. from all other species of the *H. avenae* complex. Phylogenetic analysis of the *coxI* gene sequences enables discrimination between A (France) and B (Syria) types of *H. avenae*. The independent species status of *H. australis* is confirmed. The multiple species concept of the cereal cyst nematodes is proposed and discussed.

Key words: *coxI*, *Heterodera australis*, PCR-RFLP, phylogeny.

At present, the *Heterodera avenae* complex includes the following nine species: *H. arenaria* 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 and *H. ustinovi* Kirjanova, 1969. Three species, *H. avenae*, *H. filipjevi* and *H. australis*, from this complex are major nematode pests in cereal growing areas, whereas the other six species only parasitise various grasses. The European cereal cyst nematode, *Heterodera avenae*, and the Filipjev cereal cyst nematode, *H. filipjevi*, were found in many countries in Europe, Asia and North America, where they have often overlapping distributions, whereas the Australian cereal cyst nematode *H. australis* has limited distribution and was reported in Australia and recently in China (Subbotin *et al.*, 2010).

During last few years, special attention was devoted to studies of the cereal cyst nematode in China. In 1989, the cereal cyst nematode was first reported in Hubei Province, China (Chen *et al.*, 1992). Subsequent surveys revealed that this nematode is widely distributed and has now been found in more than 16 provinces (autonomous regions or cities) in China, including Hubei, Shanxi, Henan, Hebei, Beijing, Inner Mongolia, Qinghai, Anhui, Shandong, Shaanxi, Gansu, Jiangsu, Tianjin, Tibet and Xinjiang. More than 4 million ha of the major wheat-producing regions considered be infected by the cereal cyst nematode with annual reduction of wheat yield valued at 1.9 billion Chinese Yuan (Peng *et al.*, 2009; Cui *et al.*, 2015). Molecular and morphological analysis of samples of the cereal cyst nematode revealed the presence of three species: *H. avenae*, *H. filipjevi* (Li *et al.*, 2010; Peng *et al.*, 2010; Fu *et al.*, 2011) and *H. australis* (Fu *et al.*, 2011) in China. However, Subbotin *et al.* (2003, 2010) noticed that that nematode samples identified as *H.* '*avenae*' from China appeared to form a distinct group within the *H. avenae* complex. These populations are close morphologically and genetically to *H. pratensis* and are not similar to *H. avenae* and might represent a separate species. Sturhan & Rumpenhorst (1996) also showed that Chinese cereal cyst nematode populations had a

different IEF protein profile from those of *H. avenae* and other species.

In this paper, the analysis of *coxI* gene sequences provided evidence of the separate status of Chinese *H.* '*avenae*' from other species of the *H. avenae* complex. The samples from China previously identified as *H.* '*avenae*' (type C) are formally described here as a new species under the common name the Sturhan cereal cyst nematode.

MATERIALS AND METHODS

DNA extraction, PCR, RFLP and sequencing. DNA was extracted from single cysts. Protocols of DNA extraction with proteinase K, PCR and sequencing were described by Tanha Maafi *et al.* (2003). The primer set with the forward Het-coxiF (5'-TAGTTGATCGTAATTTTAATGG-3') and the reverse Het-coxiR (5'-CCTAAAACATAATGAAA ATGWGC-3') was used for amplification of the partial mitochondrial cytochrome oxidase I (*coxI* mtDNA) gene with the following thermal profile: 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. A few microliters of purified PCR product were digested by restriction enzyme *Bcu*I (Thermo Fisher Scientific, USA) in the buffer stipulated by the manufacturer. The digested DNA was run on a 1.4% TAE buffered agarose gel, stained with ethidium bromide, visualised on UV transilluminator and photographed. Sequencing was made in Quintara Biosciences (San Francisco, USA). New sequences were deposited in the GenBank under accession numbers: KU147188-KU147203.

Phylogenetic analysis. The newly obtained *coxI* sequences were aligned with corresponding published gene sequences of other species from the *Avenae* group (Toumi *et al.*, 2013) using ClustalX 1.83 with default parameters. *Heterodera hordecalis* and *H. latipons* were chosen as outgroups. The sequence dataset was analysed with Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001) under the HKY+G model. BI analysis was initiated with a random starting tree and was run with four chains for 1.0×10^6 generations. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities are given on appropriate clades. The *coxI* sequence alignment was also used to construct phylogenetic network estimation using statistical parsimony with the TCS software (Clement *et al.*, 2000).

RESULTS AND DISCUSSION

Heterodera sturhani sp. n. (Fig. 1, Table 1)

Descriptions and measurements for specimens designated as the paratypes (China, Beijing, Fangshan district, sample 1) are given according to Subbotin *et al.* (2003). Descriptions and measurements for other populations were published by Liu W.Z. *et al.* (2005), Liu J. *et al.* (2009), Liu K. *et al.* (2012) and Wang *et al.* (2013).

Holotype cyst. L (excluding neck) = 595 µm; W (slightly deformed) = 330 µm.

Paratype cysts (n = 9). L = 597 ± 25 (504-696) µm; W = 437 ± 13 (384-504) µm; L/W = 1.4 ± 0.05 (1.2-1.5); fenestral length = 46 ± 0.8 (43-50) µm; mean semifenestral width = 23 ± 1.3 (19-31) µm; vulval bridge width = 10 ± 0.9 (6.6-16) µm; vulval slit length = 8.6 ± 0.4 (6.6-12) µm; vulva-anus distance = 57 ± 1.5 (50-62) µm.

Paratype second-stage juveniles (n = 20). L = 513 ± 3.5 (480-537) µm; a = 25 ± 0.2 (23-27); b = 4.5 ± 0.1 (4.0-4.8); c = 8.1 ± 0.1 (7.5-9.1); stylet length = 24 ± 0.1 (24-25) µm; lip region height = 4.1 ± 0.06 (3.4-4.4) µm; lip region width = 9.3 ± 0.09 (8.8-9.9) µm; DGO = 6.4 ± 0.1 (5.4-6.9) µm; anterior end to valve of median bulb = 72 ± 1.0 (67-87) µm; anterior end to excretory pore = 103 ± 1.0 (98-112) µm; pharynx length = 116 ± 1.2 (107-126) µm; body diam. at mid-body = 21 ± 0.1 (20-22) µm; body diam. at level of anus = 15 ± 0.1 (15-16) µm; tail length = 63 ± 0.9 (55-72) µm; hyaline part of tail length = 40 ± 0.6 (34-45) µm.

Cyst. Lemon-shaped with low vulval cone. Subcrystalline layer distinct; egg sac not observed. Cuticle with irregular zig-zag pattern; colour varying from pale to medium brown. Vulval cone bifenestrate, vulval slit short. Bullae numerous, distinct.

Second-stage juvenile. Body of heat-killed specimens slightly curved ventrally. Labial region slightly offset, flatly rounded, with two indistinct annuli. Labial framework strongly sclerotised. Stylet strong, cone slightly shorter than shaft. Stylet knobs slightly concave, sloping slightly posteriorly. Lateral field with four lines, but outer lines mostly indistinct and outer bands completely areolated. Pharyngeal glands well developed. Tail gradually tapering to a narrow, rounded terminus.

Males. Not found.

Etymology. The species was named in honour of Dr Dieter Sturhan for his great contributions in systematics of cyst forming and other nematodes.

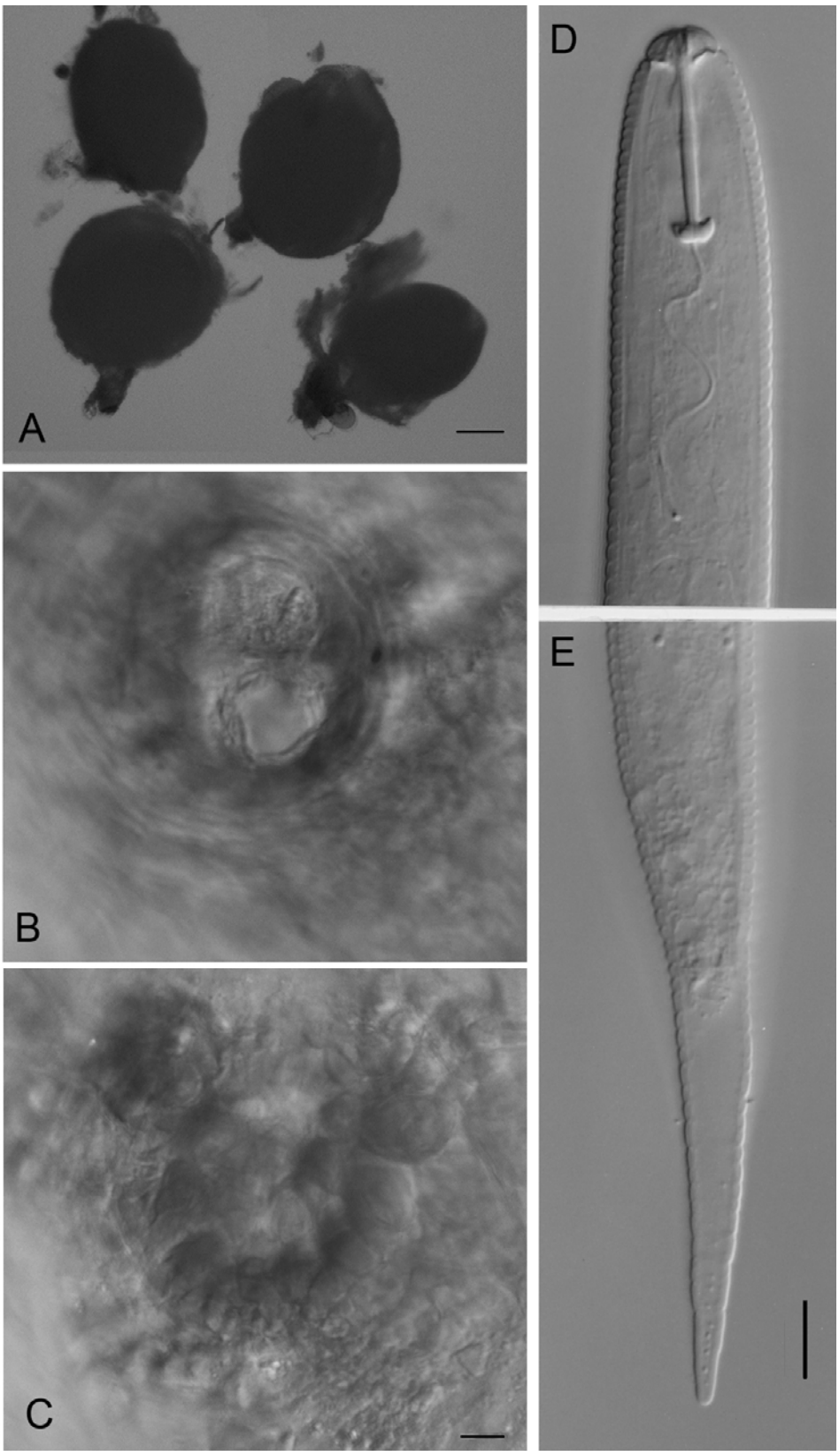

Fig. 1. *Heterodera sturhani* sp. n. A: white females (China, Shanxi province); B, C: vulval plate (Paratype; China, Beijing); D, E: anterior and posterior ends of the second-stage juveniles (Paratype; China, Beijing) (after Subbotin *et al.*, 2003). Scale bars: A = 120 µm, B, C = 10 µm; D, E = 9 µm.

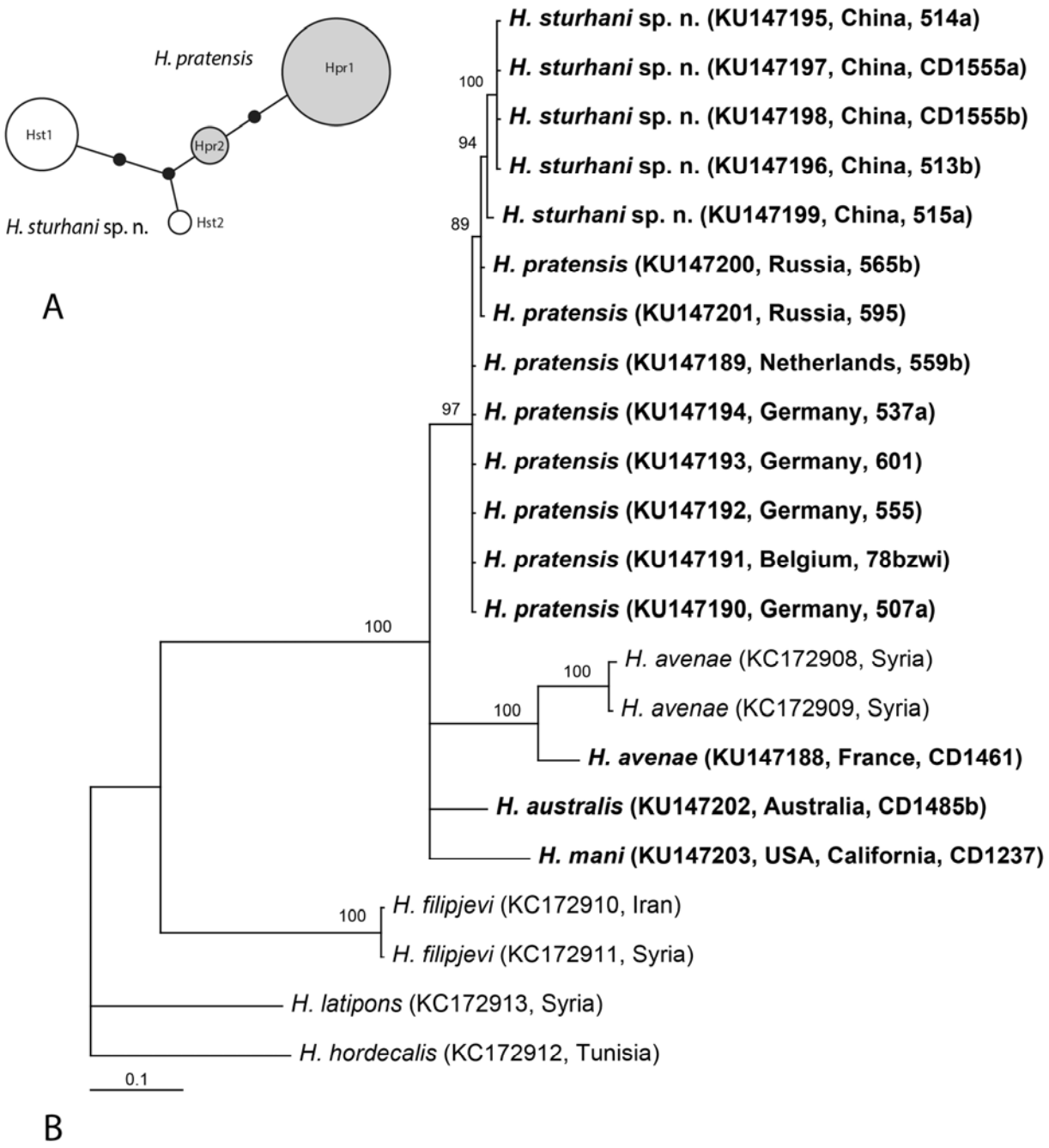

Fig. 2. Phylogenetic relationships between cyst nematode species of the *Avenae* group as inferred from the analysis of the *coxI* mtDNA gene sequences. A. Statistical parsimony network showing the phylogenetic relationships between haplotypes of *H. sturhani* sp. n. and *H. pratensis.* Small black cycles represent missing haplotypes. Pie chart sizes are proportional to the number of samples with a particular haplotype. B: Bayesian majority rule consensus tree inferred from the analysis of the *coxI* gene sequence alignment under the HKY + G model. Newly obtained sequences are indicated by bold letters.

Table 1. Species and populations of the *Heterodera avenae* complex used in the present study

Species	Location	Host	Sample code	Accession number for *coxI* sequence	Source or reference
H. australis	Australia, Yorke Peninsula	Cereals	CD1485	KU147202	Subbotin *et al.* (2003)
H. avenae	Syria, Deir Al-Zor	Wheat	–	KC172908	Toumi *et al.* (2013)
H. avenae	Syria, Al-Hasakah	Wheat	–	KC172909	Toumi *et al*. (2013)
H. avenae	France, St Georges du Bois	Cereals	CD1461	KU147188	R. Rivoal; Subbotin *et al.* (2003)
H. filipjevi	Iran, Aligoudarz	Wheat	–	KC172910	Toumi *et al*. (2013)
H. filipjevi	Syria, Al-Hasakah	Wheat	–	KC172911	Toumi *et al*. (2013)
H. hordecalis	Tunisia	Wheat	–	KC172912	Toumi *et al*. (2013)
H. sturhani sp. n.	China, Beijing, Tongzhou district	Cereals	514a	KU147195	D. Peng; Subbotin *et al.* (2003)
H. sturhani sp. n.	China, Beijing, Pinggu district	Cereals	515a	KU147199	D. Peng; Subbotin *et al.* (2003)
H. sturhani sp. n.	China, Beijing, Fangshan district	Cereals	513b	KU147196	D. Peng; Subbotin *et al.* (2003)
H. sturhani sp. n.	China, Shanxi province, Wenxi	Cereals	CD1555	KU147197, KU147198	J. Zheng
H. latipons	Syria, Deir Al-Zor	Wheat	–	KC172913	Toumi *et al*. (2013)
H. mani	USA, California	Grasses	CD1252	KU147203	S.A. Subbotin
H. pratensis	Russia, Leningrad region, Putilovo	Grasses	565, 595	KU147200, KU147201	Subbotin *et al.* (2003)
H. pratensis	The Netherlands, near Rotterdam	Grasses	559	KU147189	Subbotin *et al.* (2003)
H. pratensis	Germany, Missunde, near Schleswig	Grasses	537a	KU147194	D. Sturhan; Subbotin *et al.* (2003)
H. pratensis	Germany	Grasses	555	KU147192	D. Sturhan; Subbotin *et al.* (2003)
H. pratensis	Belgium, Zwin	Grasses	Zwi78	KU147191	S.A. Subbotin
H. pratensis	Germany	Grasses	601	KU147193	D. Sturhan
H. pratensis	Germany	Grasses	507a	KU147190	D. Sturhan

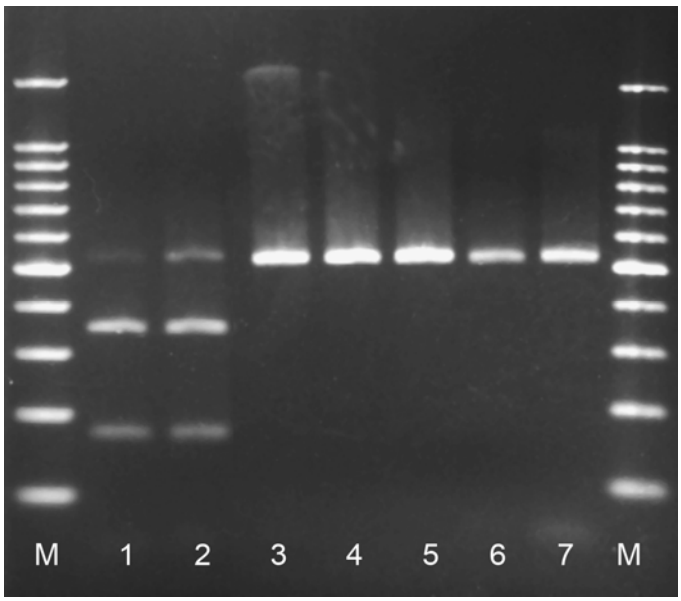

Fig. 3. PCR-coxI-RFLP profiles generated by *Bcu*I for several *Heterodera* species. Lanes – M: 100 bp DNA marker (Promega, USA); 1: *H. sturhani* sp. n. (China, Shanxi province, CD1555a); 2: *H. sturhani* sp. n. (China, Beijing, CD515a); 3: *H. pratensis* (Germany, 537); 4: *H. pratensis* (Germany, 555); 5: *H. mani* (USA, California, CD1252); 6: *H. australis* (Australia, CD1485); 7: *H. avenae* (France, CD1461).

Type locality and host. Fangshan district, Beijing, China; cereals.

Type materials. Holotype and paratypes are deposited at the Nematode collection of Julius Kühn-Institut, Bundesforschungsinstitut für Kulturpflanzen Institut für Epidemiologie und Pathogendiagnostik, Münster, Germany.

Differential diagnosis. *Heterodera sturhani* sp. n. is morphologically similar with *H. pratensis* and *H. riparia* and overlaps with these species in many characteristics of cysts and second-stage juveniles. *Heterodera sturhani* sp. n. can be differentiated from *H. pratensis* by smaller average cyst size (L = 540-641 µm; W = 324-480 µm *vs* L = 650-760 µm; W = 490-570 µm) and from *H. riparia* by longer average body length (488-570 *vs* 446-486 µm) and longer average tail length (62-67 *vs* 57-61 µm) of the second-stage juveniles. The new species differs from *H. avenae* by smaller average cyst size (L = 540-641 µm; W = 324-480 µm *vs* L = 600-808 µm; W = 465-507 µm) and smaller average fenestral length (40-46 *vs* 43-55 µm). *Heterodera sturhani* sp. n. can be distinguished from *H. mani* by the structure of stylet knobs, which are flat or slightly concave *vs* strongly developed and deeply concave in second stage juveniles.

Molecular characterisation of *Heterodera sturhani* sp. n. and its relationships with other species. The *coxI* mtDNA gene alignment included 22 sequences and was 425 bp in length. Intraspecific variation for *H. sturhani* sp. n. was 0-3 bp (0.7%) and for *H. pratensis* was 0-3 bp (0.7%). Two *coxI* haplotypes (Hst1 and Hst2) were found for *H. sturhani* sp. n. and two haplotypes (Hpr1 and Hpr2) were revealed for *H. pratensis.* The *coxI* gene sequences of *H. sturhani* sp. n. differ from those of *H. pratensis* by up to 5 bp (1.2%). Differences between *H. avenae* type A (France) and type B (Syria) were 21-26 bp (6.4-7.0%) and between *H. australis* and *H. avenae* types were 36-46 bp (9.1-12.0%). Phylogenetic relationships of *H. sturhani* sp. n. with other species are given in Figure 2. *Heterodera sturhani* sp. n. showed close relationships with *H. pratensis* (Fig. 2A & B) and, perhaps, originated from the later species. *Heterodera australis* was presented as a separate lineage from *H. avenae* (types A and B) and other species in the phylogenetic tree. Thus, the *coxI* mtDNA sequence analysis confirms the validity of *H. australis* as was previously proposed based on other biochemical and molecular data (Subbotin *et al.*, 2002).

The PCR-coxI-RFLP profiles generated by *Bcu*I for *H. sturhani* sp. n. and several other species from the *H. avenae* complex are given in Figure 3. This restriction enzyme cut only PCR products obtained from *H. sturhani* sp. n. samples.

The results of previous published analyses of the ITS rRNA gene sequences (Subbotin *et al.*, 2003; Fu *et al.*, 2011) and the present analysis of the *coxI* mtDNA gene sequences showed that the cereal cyst nematode from China is different from *H. avenae* and represents a separate evolutionary lineage. Moreover, several studies (Peng & Cook, 1996; Zheng *et al.*, 1997; Yuan *et al.*, 2010; Cui *et al.*, 2015) demonstrated that these populations belong to new pathotypes, which are distinct from European ones according to the pathotype scheme proposed by Andersen & Andersen (1982). Thus, molecular, biological and morphological results justify the erection of a new species *H. sturhani* sp. n. and its separation from *H. avenae*. Although molecular results show close relationships of *H. sturhani* sp. n. and *H. pratensis*, they are different in plant-host range. *Heterodera sturhani* sp. n. parasitises cereals, whereas *H. pratensis* is presently known only as a parasite of grasses (Subbotin *et al.*, 2010).

The ability of nematodes to parasitise cereals might have appeared independently in several evolutionary lineages of the *H. avenae* species complex and different geographical regions. Nematodes belonging to these lineages also showed differences in their virulence and pathogenicity for cereal crops. Thus, under the common name of "the cereal cyst nematode" there presently exists several species, which could be named as the European cereal cyst nematode, *H. avenae*, the Filipjev cereal cyst nematode, *H. filipjevi*, the Australian cereal cyst nematode, *H. australis* and now the Sturhan cereal cyst nematode, *H. sturhani* sp. n. Although, morphological characters can fail to differentiate species of the cereal cyst nematodes from each other or from sibling species parasitising grasses, the molecular markers (ITS rRNA and *coxI*) presently provide reliable differentiation of this complex at a species level. Acceptance of the multiple species concept of the cereal cyst nematodes raises an important question about reconsidering the present quarantine regulations and other measures, which prevent dispersal of some nematode species that are present only in geographically isolated regions. Special concern should be directed to *H. sturhani* sp. n., which could be considered as a potential invasive species for Europe, America and Australia.

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S.A. Subbotin. *Heterodera sturhani* sp. n. из Китая, новый вид из группы видов '*Heterodera avenae*' (Tylenchida: Heteroderidae).

Резюме. *Heterodera sturhani* sp. n. описан как новый вид из группы видов '*Heterodera avenae*'. Новый вид по многим морфологическим признакам цисты и личинки второго возраста схож с *H. pratensis* и *H. riparia*. Он может быть дифференцирован от *H. pratensis* меньшими размерами цист и от *H. riparia* большей длиной тела и длиной хвоста личинки. Филогенетический анализ нуклеотидных последовательностей гена цитохром с-оксидазы I и рестрикционный анализ ПЦР продуктов этого гена позволяют четко отличать *H. sturhani* sp. n. от всех близких видов. Филогенетический анализ нуклеотидных последовательностей гена цитохром с-оксидазы I также позволил разделить два типа (A и B) *Heterodera avenae* друг от друга и подтвердить видовой статус *H. australis*. Таким образом, под названием "овсяная цистообразующая нематода" следует рассматривать не один, а несколько видов.