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Short note

Molecular characterization of the cyst-forming nematode, *Heterodera sinensis* Chen & Zheng, 1994, from China

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Heterodera sinensis, a cyst-forming nematode collected from Lalong grass, *Imperate cylindrica* (L.) in Luanxian, Hebei, China, was reported by Chen & Zheng (1994). This is the first new cyst-forming nematode species described in China. A detailed description, including morphology, morphometrics, illustrations, and microphotographs of female, male, second-stage juvenile and cyst, were made subsequently by Chen *et al.* (1996). The cyst of this species is characterized by being lemon-shaped, ambifenestrate with bulleae and strong underbridge. The second-stage juvenile has concaved stylet knobs, lateral field consisting of three incisures and the outer margins of lateral pairs of lips are lost, which makes it different from the 6 lip patterns described by Stone (1975). Because the available morphological data did not allow this species to be classified unambiguously to any of the known *Heterodera* morphological groups, it became evident that molecular data might be needed to provide evidence in order to define a position of this species within the genus.

Developed during last decades, DNA-based techniques provide an attractive solution to problems associated with identification and analysis of phylogenetic relationships of organisms. PCR-ITS-rRNA became a reliable tool for a precise and rapid identification of cyst nematodes. Comparisons of RFLP profiles and sequences of the ITS-rRNA of unknown nematodes with those published or deposited in GenBank (Orui, 1997; Subbotin *et al.*, 2000, Tanha Maafi *et al.*, 2003; Mundo-Ocampo *et al.*, 2008) facilitate rapid identification of most species of cyst nematodes. The ITS-rRNA is also considered to be a good marker to study phylogenetic relationships at the species and genus levels. The objectives of this study were: *i*) to molecularly characterize the cyst-forming nematode *Heterodera sinensis* using PCR-ITS-RFLPs and sequence of the ITS region; and *ii*) to study the phylogenetic relationships of this species with other cyst-forming nematodes using Bayesian inference.

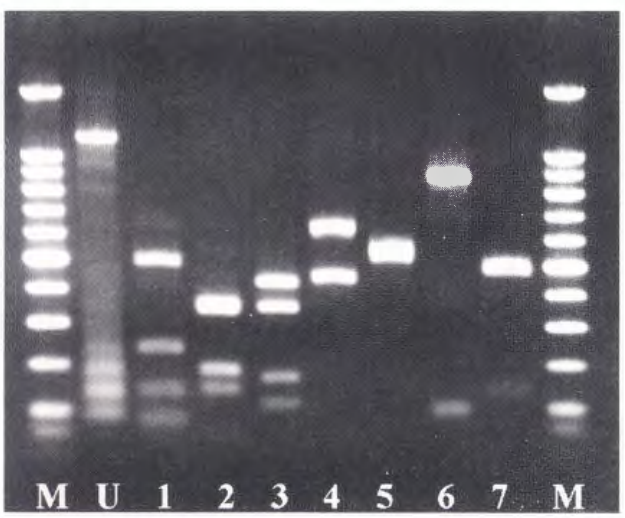

Fig. 1. PCR-ITS-RFLP profiles for *H. sinensis*. M: 100bp DNA ladder (Promega); 1: *Alu*I; 2: *Bsh1236*I; 3:*Cfo*I; 4: *Dde*I; 5: *Hinf*I; 6: *Msp*I; 7: *Rsa*I; U: unrestricted PCR product.

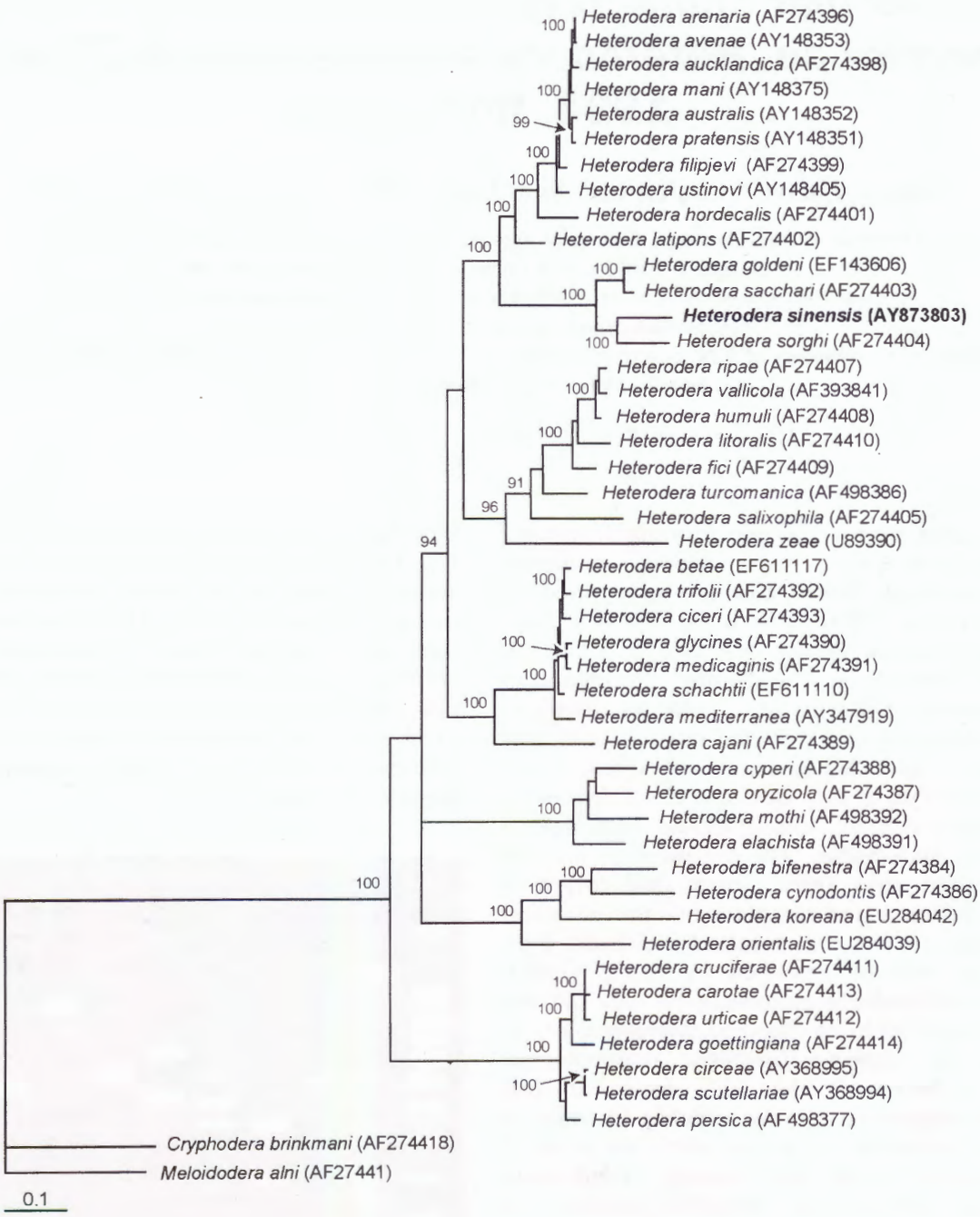

Fig. 2. Phylogenetic relationships among *Heterodera* spp. : Bayesian 50% majority rule consensus tree from two runs as inferred from ITS1-5.8S-ITS2 sequences of rRNA gene alignment under the GTR+I+G model. Newly obtained sequence for *H. sinensis* is indicated in bold.

In the present work, the specimens of *H. sinensis* were re-collected from the type host and locality. Several juveniles from cysts were used for DNA extraction. The methods for DNA extraction, PCR amplification and sequencing have been previously described by Zheng *et al.* (2000). The following primers were used for amplification of the ITS-rRNA: TW81 (5′-GTT TCC GTA GGT GAA CCT GC-3′) and AB28 (5′-ATA TGC TTA AGT TCA GCG GGT-3′). The newly obtained sequence is deposited at the Genbank under the accession number AY873803. PCR product was purified using the QIAquick Gel Extraction Kit (Qiagen). Three µl of purified PCR product was digested by one of the following restriction enzymes: *Alu*I, *Bsh*1236I, *Cfo*I, *Dde*I, *Hin*fI, *Msp*I, and *Rsa*I in the buffer stipulated by the manufacturer. The digested DNA was run on a 1.5% agarose gel, stained with ethidium bromide, visualised on UV transilluminator and photographed. The exact length of each restriction fragment from the PCR products was obtained by a virtual digestion of the sequences using WebCutter 2.0 (www.firstmarket.com/cutter/cut2.html).

The newly obtained sequence was aligned with published gene sequences of cyst-forming nematodes and outgroup taxa (Subbotin *et al.*, 2001; Tanha Maafi *et al.*, 2003; Mundo-Ocampo *et al.*, 2008) using ClustalX 1.83 with default parameters. The sequence dataset was analysed with Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). BI analysis under the GTR+I+G model was initiated with a random starting tree and was run with four chains for 1.0 x 10^6 generations. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. The log-likelihood values of the sample points stabilised after approximately 10^3 generations. 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.

The amplification of the ITS1-5.8s-ITS2 using TW81 and AB28 primers yielded a single product of approximately 1,092 bp in length for *H. sinensis*. RFLP of the ITS region revealed that the digestion patterns generated by seven restricted enzymes, *Alu*I, *Bsh*1236I, *Cfo*I, *Dde*I, *Hin*fI, *Msp*I, and *Rsa*I differed from those profiles previously reported for cyst-forming nematodes (Subbotin *et al.*, 2000; Tanha Maafi *et al.*, 2003; Mundo-Ocampo *et al.*, 2008). RFLP profile and the lengths for restriction fragments generated by the seven enzymes are given in Fig. 1 and Table 1, respectively.

Table 1. Approximate sizes (in bp) of restriction fragments generated by seven diagnostic enzymes after digestion of PCR product of the ITS-rRNA regions amplified with TW81 and AB28 primers for *Heterodera sinensis*.

Enzyme	Fragment length
*Alu*I	488, 233, 141, 86, 72, 47, 25
*Bsh1236*I	347, 338, 185, 148, 52, 22
*Cfo*I	425, 346, 170, 109, 42
*Dde*I	658, 434
*Hin*fI	533, 518, 41
*Msp*I	857, 104, 93, 38
*Rsa*I	482, 472, 138

The length of the sequence alignment for ITS1-5.8S-ITS2 was 1258 nucleotides and included 47 taxa. Phylogenetic BI tree (Fig. 2) was congruent to those published by Subbotin *et al.* (2001) and Tanha Maafi *et al.* (2003). In the BI tree *H. sinensis* was a sister species of *H. sorghi*. This two species together formed a highly supported clade and larger clade with *H. sacchari* and *H. goldeni* with posterior probabilities equal to 100%. Thus, based on the ITS-rRNA dataset, *H. sinensis* could be considered as a representative of *H. sacchari* group together with *H. sacchari, H. goldeni* and *H. sorghi*.

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REFERENCES

CHEN, P. & ZHENG, J. 1994. [Primary report on a new species *Heterodera sinensis* sp. nov. from China]. *Scientia Agricultura Sinica* 27: 88-89, (In Chinese).

CHEN, P., ZHENG, J. & PENG D. 1996. [A new species of the genus *Heterodera* from China (Nematoda: Tylenchoida: Heteroderidae)]. *Acta Zootaxonomica Sinica* 21: 18-24, (In Chinese).

HUELSENBECK, J.P. & RONQUIST, F. 2001. MrBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754-755.

MULVEY, R.H. 1972. Identification of *Heterodera* cysts by terminal and cone top structure. *Canadian Journal of Zoology* 50: 1277-1292.

MUNDO-OCAMPO, M., TROCCOLI, A., SUBBOTIN, S.A., DEL CID, J., BALDWIN, J.G. & INSERRA, R.N. 2008. Synonymy of *Afenestrata* with *Heterodera* supported by phylogenetics with molecular and morphological characterisation of *H. koreana* comb. n. and *H. orientalis* comb. n. (Tylenchida: Heteroderidae). *Nematology* 10: 611-63.

ORUI, Y. 1997. [Discrimination of *Globodera rostochiensis* and four *Heterodera* species (Nematoda: Heteroderidae) by PCR-RFLP analysis]. *Japanese Journal of Nematology* 27: 67-75.

STONE, A.R. 1975. Head morphology of second-stage juveniles of some Heteroderidae (Nematoda: Tylenchida). *Nematologica* 21: 81-88.

SUBBOTIN, S.A. WAEYENBERGE, L. & MOENS, M. 2000. Identification of cyst-forming nematodes of the genus *Heterodera* (Nematoda:Heteroderidae) based on the ribosomal DNA-RFLPs. Nematology 2: 153-164.

SUBBOTIN, S. A., VIERSTRAETE, A., DE LEY, P., ROWE, J., WAEYENBERGE, L., MOENS, M. & VANFLETEREN, J.R. (2001). Phylogenetic relationships within the cyst-forming nematodes (Nematoda, Heteroderidae) based on analysis of sequences from the ITS regions of ribosomal DNA *Molecular Phylogenetics and Evolution* 21: 1-16.

TANHA MAAFI Z., STURHAN, D., HANDOO, Z., MORDEHAI M., MOENS, M. & SUBBOTIN, S.A. 2007. Morphological and molecular studies of *Heterodera sacchari*, *H. goldeni* and *H. leuceilyma* (Nematoda: Heteroderidae). *Nematology* 9: 483-497.

ZHENG J., SUBBOTIN, S.A. WAEYENBERGE, L. & MOENS, M. 2000. Molecular characterisation of Chinese *Heterodera glycines* and *H. avenae* populations based on RFLPs and sequences of rDNA-ITS regions. *Russian Journal of Nematology* 8: 109-113.