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Molecular characterisation of Chinese *Heterodera glycines* and *H. avenae* populations based on RFLPs and sequences of rDNA-ITS regions

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Summary. Restriction profiles and sequences of the ITS region of Chinese populations of *Heterodera glycines* and *H. avenae* are given. In *H. glycines*, heterogeneity was detected after restriction of the PCR product with *Ava*I. This is the principal identification enzyme for this species, and usually yields four fragments of 552, 478, 367 and 112 bp. Restriction of the PCR product from the Chinese *H. avenae* population by *Hin*fI and *Tru*9I produced RFLP profiles that differentiated it from other cereal cyst nematode populations.

Key words: *Heterodera glycines*, *H. avenae*, heterogeneity, ITS region, rDNA.

The soybean cyst nematode, *Heterodera glycines* Ichinohe, 1952, was identified occurring in China during the late 19th century. It is distributed in more than ten provinces from North to Southeast China and is the major pathogen of soybean, especially in the northern and north-eastern provinces (Liu *et al.*, 1997). Several biological races that differ in their pathogenicity have been reported from China (Chen *et al.*, 1987). The cereal cyst nematode, *H. avenae* Wollenweber, 1924, is one of the major nematode pathogens affecting wheat in China. It was reported for the first time in China in 1989 and subsequently it has been found in 26 regions, and eight provinces or cities including Hubei, Beijing, Hebei, Henan, Inner Mongolia Autonomous region, Qinghai, Shanxi, and Anhui (Chen *et al.*, 1989; Wang *et al.*, 1991; Zheng *et al.*, 1996; Peng & Moens, 2000). Pathotypes of this nematode occurring in China are different from those reported from Europe and Australia (Peng & Cook, 1996; Zheng *et al.*, 1997).

Accurate and rapid identification of species and pathotypes of cyst nematodes is required for implementing appropriate control methods. Traditional species diagnosis based on the morphology and morphometrics of cysts and juveniles is time consuming and requires taxonomic expertise. rDNA diagnostics provide an alternative solution to overcome problems associated with the traditional identification of cyst forming nematodes (Ferris *et al.*, 1993; Thiéry & Mugniéry, 1996; Bekal *et al.*, 1997; Szalanski *et al.*, 1997; Blok *et al.*, 1998; Subbotin *et al.*, 1997; 1999; 2000). The objective of this study was to characterise Chinese *Heterodera glycines* and *H. avenae* populations based on RFLPs and sequences of their rDNA-ITS regions.

MATERIALS AND METHODS

Nematode populations. The *H. glycines* populations used in this study were from Luobei, Jiamusi, and Haerbin from Heilongjiang Province, Shenyang from Liaoning Province, Taigu, and Yuanqu from Shanxi Province, Xuzhou from Jiangsu Province, Jinan from Shandong Province, Shanghai, and Beijing each from China and a population from the USA. *Heterodera avenae* populations were obtained from Taigu, Shanxi Province, China, Taaken, Germany, and Bet-Dagan, Israel.

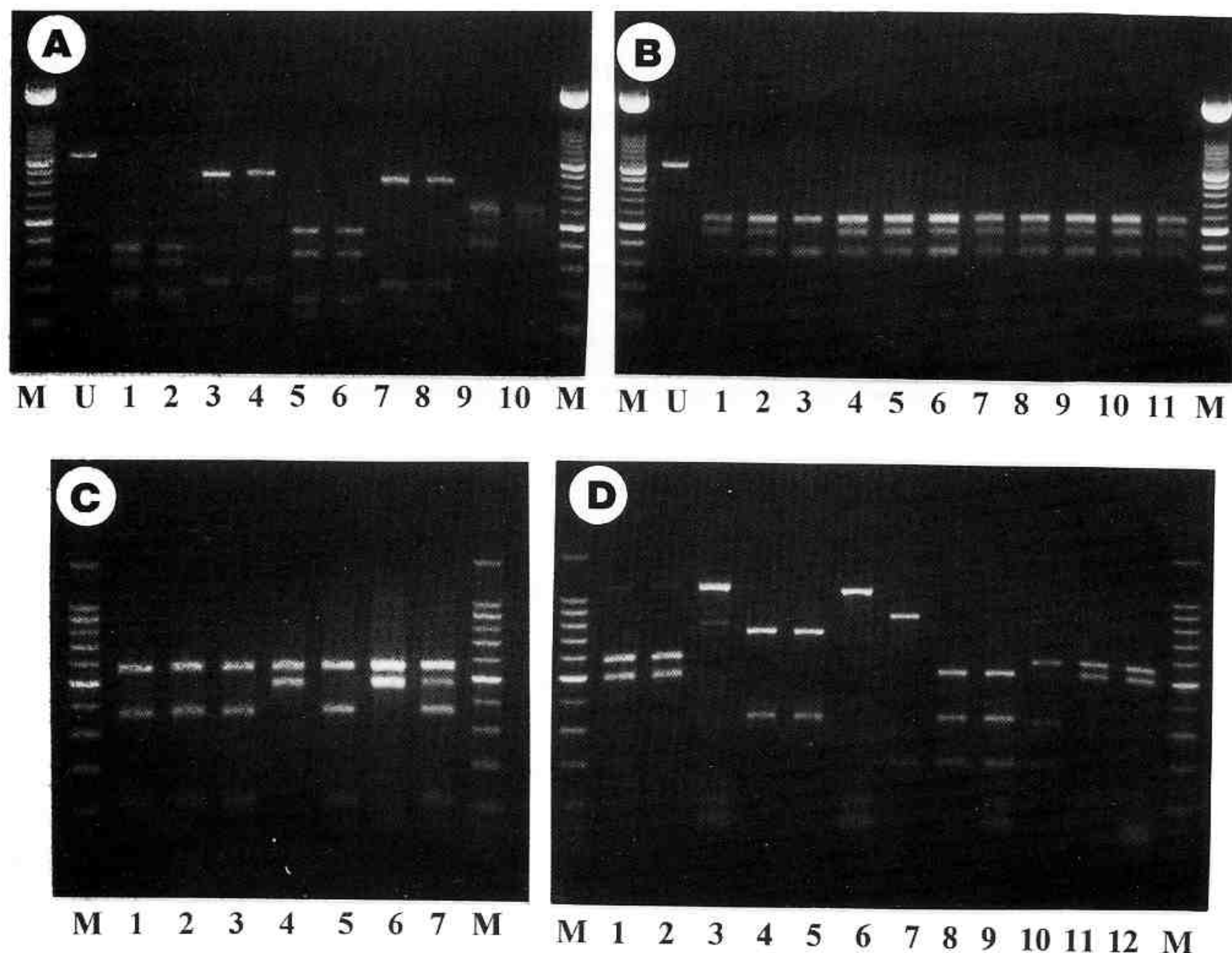

Fig. 1. Molecular variability between and within populations of *Heterodera avenae* and *H. glycines*. Lanes: M: 100 bp DNA ladder, U: unrestricted PCR product. **A.** RFLP profiles produced after digestion (*Alu*I: lanes 1-2; *Rsa*I: lanes 3-4; *Cfo*I: lanes 5-6; *Mva*I: lanes 7-8; *Ava*I: lanes 9-10) of the ITS-PCR products obtained for two Chinese *H. glycines* populations (Jiamusi: lanes 1, 3, 5, 7, 9; Tiagu: lanes 2, 4, 6, 8, 10). **B.** *Ava*I digestion of the ITS region of *H. glycines*. Lanes 1-5: RFLP patterns of individual cysts of the Taigu population; lane 6: RFLP patterns of 25 cysts of the Taigu population; lanes 7-10: RFLP patterns of individual cysts of the Jinan population; lane 11: RFLP patterns of 25 cysts of the Jinan population. **C.** *Ava*I restriction patterns from six (Hgl1-Hgl6) ITS Taigu clones (lanes 1-6) and from the Taigu population of *H. glycines* (7). **D.** Restriction fragments of amplified ITS region of a *H. avenae* population from China (lanes 1, 4, 7, 10), Israel (lanes 2, 5, 8, 11), and Germany (lanes 3, 6, 9, 12); *Alu*I: lanes 1-3: *Rsa*I: lanes 4-6; *Hin*fI: lanes 7-9; *Tru*9I: lanes 10-12.

Sample preparation, PCR reaction and RFLP of ITS region. The methods used for DNA extraction and PCR amplification were similar to those described by Subbotin *et al.* (2000). For each population either single or several cysts were used. Primers TW81 (5′-GTTTCCGTAGGTGAACC-TGC-3′) and AB28 (5′-ATATGCTTAAGTT-CAGCGGGT-3′) were used in the PCR reaction (Joyce *et al.*, 1994). After DNA amplification, 5 µl product was run on a 1% agarose gel and the remaining product was stored at − 20°C. Before digestion, PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen). Four to 7 µl of each product was digested with restriction enzymes: *Alu*I, *Ava*I, *Cfo*I, *Hin*fI, *Msp*I, *Mva*I, *Rsa*I and *Tru*9I, each in the buffer stipulated by the manufacturer. The digested DNA was loaded on a 1.5% agarose gel, separated by electrophoresis (100V, 1.5 h), stained with ethidium bromide, visualised on a transilluminator, and photographed. Procedures for obtaining PCR amplified products and endonuclease digestion of these products were

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              *        20         *        40         *        60         *        80         *       100
Hav1 GTTTCCGTAGGTGAACCTGCTGCTGGATCATTACCCAAGTGATTCC-ATTCACCATCTACCTGTGCTGTCCAGTTGAAACGTTGCTTGGCACCACCACAT :   99
Hav3 ..............................................-..................................................... :   99
Hgl5 ..............................................T........C.......--......T....GG-.TAGCG............A.. :   97
Hgl6 ..............................................T........C.......--......T....GG-.TAGCG............A.. :   97

              *       120         *       140         *       160         *       180         *       200
Hav1 GCCCCCGTCTGCTGACGGGCACGGACCGGTCGAGATGGTCTGTGGGCACGGGACAACGCTGAGTGGACGGCTACCCTGCGAGCACTCTGTGCTTTGGGGT :  199
Hav3 .................................................................................................... :  199
Hgl5 .........C.....T......A.GT..T.......ACT......A.G.T.CC....AT.AC.-..G.A...G..TCA.....CA.GC---T........ :  193
Hgl6 .........C.....T......A.GT..T.......ACT......A.G.T.CC....AT.AC.-..G.A...G..TCA.....CA.GC---T........ :  193

              *       220         *       240         *       260         *       280         *       300
Hav1 GTTCTCCGACGATGGTGCT-TGGTATACTGACTCGTTGCTGAGCAAAGTGAAAAGCCTGAGGTTTG--GCTGCGAAGCAATCGAGTTGGTGGCGGACCGC :  296
Hav3 ...................-..............................................--................................ :  296
Hgl5 .C.TC.AT...T...A...G........C.-...AG.....CA..T-......-.....T.TA.G.CT......TG....-..T..C........G.... :  289
Hgl6 .C.TC.AT...T...A...G........C.-...AG.....CA..T-......-.....T.TA.G.CT......TG....-..T..C........G.... :  289

              *       320         *       340         *       360         *       380         *       400
Hav1 CTTGCT-GGTTGGTTTGCTGTACCGTCGTTGGGCGAGCACGCTCGTTGGGTAACCCAACGGCTGTGCTGGTGTCTGCGCGTCGTTGAGCGGTTGTTGTGC :  395
Hav3 ......-.....................................................................T....................... :  395
Hgl5 ..C...T..C.....C....CG..AAT..--...GAT.........G...CG...T..............C.....T......................G :  387
Hgl6 ..C...T..C.....C....CG..AAT..--...GAT.........G...CG...T..............C.....T......................G :  387

              *       420         *       440         *       460         *       480         *       500
Hav1 CTGGCACATGTGACACACTGGCCTGGGAAAGTGGTT-CTTTCCTGGCCTTACGAACCGTAACTAGCGGTGTGTCCGTGCCTGTGTGCTACGTCCGTGGCC :  494
Hav3 ....................................-............................................................... :  494
Hgl5 .A.......--A..........TG....TG......T.G....C..T......TG..................TT....T.C-................. :  484
Hgl6 .A.......--A........A.TG....TG......T.G....C..T......TG..................TT....T.C-................. :  484

              *       520         *       540         *       560         *       580         *       600
Hav1 GTGATGAGACGACGTGGTAGGGCCCGTGCTATGCTTCTGCACGTGGCTTAAGACTTAATGAGTGTCAGCTAGG-CACCGCCAG-----TGTTTTTTTTC :  588
Hav3 .........................................................................-.........-----.........--. :  586
Hgl5 ..............C...............TG..C.A-.................C..........CA...C..G.......CTTTT.C.......... :  583
Hgl6 ..............C...............TG..C.A-.................C..........CA...C..G.......CTTTT.C.......... :  583

              *       620         *       640         *       660         *       680         *       700
Hav1 ATTTACTTTTTGACCACTTCTTTGTTGAAGAAAGAAATTCTAGTCTTATCGGTGGATCACTCGGCTCGTGGATCGATGAAGAACGCAGCCAACTGCGAT :  688
Hav3 .....T.............................................................................................. :  686
Hgl5 ...ATT......TTA......--..........T...-.............................................................. :  680
Hgl6 ...ATT......T-A......--..........T...-.............................................................. :  679

              *       720         *       740         *       760         *       780         *       800
Hav1 AATTAGTGCGAACTGCAGAAACCTTGAACACAAAACTTTCGAATGCACATTGCGCCATTGGAGTTACATCCATTGGCACGCCTGGTTCAGGGTCGTTATC :  788
Hav3 .................................................................................................... :  786
Hgl5 ....................................A.............................................................C. :  780
Hgl6 ....................................A.............................................................C. :  779

              *       820         *       840         *       860         *       880         *       900
Hav1 ATAAAAGGCACTGCTGTGCCTGTTATGTTGGTGAGATCATGTCGGCTTG-ACGTGTTCTTGCGCTATTCTTAAAAATGCTCGGCCGTGGAGTGTGGTTGT :  887
Hav3 .................................................-.................................................. :  885
Hgl5 ......T.......-..TGC...GC-..C...G........--A.....T.........A..T..--...GCTC.-.......T.....G..T.T.G... :  873
Hgl6 ......T.......-..TGC...GC-..C...G........--A.....T.........A..T..--...GCTC.-.......T.....G..T.T.G... :  872

              *       920         *       940         *       960         *       980         *      1000
Hav1 GTTGGCGCGAAACTGTCAGGTTCT--TTCGCGTTTTACGGTCCGTAACTAGGACGCAACTGCTCGCCATGTGTGCTG-GGTGGAATGCTTCGCTGGTAGG :  984
Hav3 ........................--.....................................................-...................... :  982
Hgl5 .C........CT...--......AA..............A........C..G....C.AAT--..TT..CA.....T..C...G...C.G.A.TACT... :  969
Hgl6 .C........CT...--......AA..............A........CAAG....C.AAT--..TT..CA.....T..C...G...C.G.A.TACT... :  968

              *      1020         *      1040         *      1060
Hav1 CATTCCGTGTTTGAATTTTCGACCTGAACTCAGACGTGAGTACCCGCTGAACTTAAGCATAT : 1046
Hav3 .............................................................. : 1044
Hgl5 ......TGC.....T........................AC..................... : 1031
Hgl6 ......TGC.....T.....................A..AC..................... : 1030
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Fig. 2. Alignment of rDNA sequences of two clones (Hav1 and Hav3) of *Heterodera avenae* and two clones (Hgl5 and Hgl6) of *H. glycines* from China. Positions of primers are indicated in italics and 18S, 5.8S and 28S genes in bold fonts. Restriction sites in sequences recognised by *Alu*I (AG/CT) are underlined; those recognised by *Ava*I (C/YCGRG) are double underlined.

repeated several times to verify the results.

Cloning and sequencing. Prior to sequencing, PCR products from *H. glycines* and *H. avenae* were excised from 1% TBE buffered agarose gels using the QIAquick Gel Extraction Kit (Qiagen), cloned into the pGEM-T vector and transformed into JM109 High Efficiency Competent Cells (Promega Corporation, USA). Two clones of each species were isolated using blue/white selection. DNA fragments were sequenced in both directions using two vector primers, one internal forward

primer 5.8SM2 (5′-CTTATCGGTGGATCACT-CGG-3′) and one internal reverse primer 5.8SM5 (5′-GGCGCAATGTGCATTCGA-3′) with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, UK) according to the manufacturer's instructions using a 377 DNA sequencer (PE Applied Biosystems). The DNA sequences were edited with the Chromas program (version 1.3) and aligned using the ClustalX program and GeneDOC (version 2.5.0; Nicholas and Nicholas, 1997).

RESULTS

Soybean cyst nematodes. Amplification of the rDNA-ITS region yielded a single PCR fragment of approximately 1030 bp (including primers) for each of the 11 populations and isolates of *H. glycines*. The *Alu*I, *Ava*I, *Cfo*I, *Mva*I, and *Rsa*I digestions each showed identical restriction profiles for all populations. Restriction profiles for two of the populations are shown in Fig. 1A. The *Ava*I digestion yielded four fragments that were revealed on a 1.5% agarose gel (Fig. 1A, B & C). The sum of the restriction fragments obtained by this enzyme was higher than the length of the unrestricted PCR product, which indicates the occurrence of heterogeneity in the ITS regions of each of these populations. An identical restriction pattern was obtained for four out of five cysts of the Taigu population, and for four cysts of the Jinan population. However, *Ava*I digestion of the PCR product from one cyst of the Taigu population yielded only three fragments (Fig. 1B, lane 3).

After cloning of the Taigu PCR product, two ITS haplotypes were selected using the *Ava*I digestion. Restriction profiles produced by this enzyme with the first haplotype contained three fragments of 552, 367 and 112 bp, whereas those produced from a second haplotype contained only two fragments of 552 and 478 bp (Fig. 1C). Aligned sequences of these two haplotypes are shown in Fig. 2.

Cereal cyst nematodes. The PCR amplification product (including primers) obtained from the Chinese population was approximately 1045 bp (Fig. 2). The two enzymes *Alu*I and *Rsa*I produced restriction profiles that were identical for the Chinese and the Israel population, whereas *Hin*fI and *Tru*9I produced RFLPs unique for the Chinese population (Fig. 1D). Aligned sequences of two clones obtained from the Chinese population are shown in Fig. 2.

DISCUSSION

In a previous study it was shown that a combination of seven restriction enzymes clearly differentiated different cyst forming nematode species (Subbotin *et al.*, 2000). The present study confirms that ITS-RFLPs are useful for identifying *H. glycines* and *H. avenae*.

RFLPs obtained by *Ava*I digestion distinguish *H. glycines* (Arkansas, USA) from other species of the *H. schachtii sensu stricto* group by the presence of two fragments of *ca.* 560 and 510 bp (Subbotin *et al.*, 2000). The results of the present sequencing study, and of the RFLP analysis, reveal the exact length of these restriction fragments. They also explain that the heterogeneity detected in the ITS region of the Chinese populations of this species is caused by the existence of two ITS haplotypes. The presence of the two longest fragments may be typical for some individuals or populations of *H. glycines* containing only one ITS haplotype, as is the case for the population from Arkansas. The occurrence of a mixture of the two haplotypes appears typical for genomes of the Chinese populations examined in this study.

ITS-RFLP also enabled observation to be made of molecular intraspecific polymorphism in *H. avenae,* in which two ITS types were detected: i) type A, which remained unrestricted by *Alu*I and *Rsa*I and was typical for most of the European populations and ii) type B, which was restricted by both of these enzymes and was typical for a population from India (Subbotin *et al.,* 1999). The present study revealed that the Taaken and the Bet-Dagan populations belong to ITS type A and B, respectively. The *Hin*fI and *Tru*9I RFLP patterns of the Taigu population were clearly different from those of the two other populations, therefore this population can be considered to belong to a new ITS type C.

This is the first report of molecular studies with Chinese cyst forming nematode populations. It provides important information from which, it can be concluded that a robust molecular diagnostic protocol can only be developed if based on examination of many populations obtained from widely disparate geographic origins.

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REFERENCES

Bekal, S., Gauthier, J.P. & Rivoal, R. 1997. Genetic diversity among a complex of cereal cyst nematodes inferred from RFLP analysis of the ribosomal internal transcribed spacer region. *Genome* 40: 479-486.

Blok, V.C., Malloch, G., Harrower, B., Phillips, M.S. & Vrain, T.C. 1998. Intraspecific variation in ribosomal DNA in populations of the populations of the potato cyst nematode *Globodera pallida*. *Journal of Nematology* 30: 262-274.

Chen, P., Zhang, D. & Chen S. 1987. [First report on a new physiological race (race 7) of soybean cyst nematodes (*Heterodera glycines*)]. *Scientia Agricultura Sinica* 20: 94.

Chen, P.S., Whang, M.Z. & Peng, D.L. 1989. [Preliminary report of identification of cereal cyst nematode of wheat in China]. *Scientia Agricultura Sinica* 24: 89.

Ferris, V.R., Ferris, J.M. & Faghihi, J. 1993. Variation in spacer ribosomal DNA in some cyst-forming species of plant parasitic nematodes. *Fundamental and Applied Nematology* 16: 177-184.

Joyce, S.A., Reid, A., Driver, F. & Curran, J. 1994. Application of polymerase chain reaction (PCR) methods to identification of entomopathogenic nematodes. In: *COST 812 Biotechnology: Genetics of entomopathogenic nematode-bacterium complexes.* (A.M. Burnell, R.-U. Ehlers & J.P. Masson. Eds.). pp. 178-187. European Commission, DG XII.

Liu, X.Z., Li, J.Q. & Zhang, D.S. 1997. History and status of soybean cyst nematode in China. *International Journal of Nematology* 7: 18-25.

Nicholas, K.B. & Nicholas, H.B.Jr. 1997. *GeneDoc: Analysis and Visualization of Genetic Variation.* http://www.cris.com/~Ketchup/genedoc.shtml

Peng, D.L. & Cook, R. 1996. Observations on the pathotypes of cereal cyst nematodes (*Heterodera avenae*) in China. *Afro-Asian Journal of Nematology* 6: 176-178.

Peng, D. & Moens, M. 2000. Cereal cyst nematode *Heterodera avenae*, a potential threat to wheat production in China. *Abstracts of the 52nd International Symposium on Crop Protection*, Gent, Belgium, 156.

Subbotin, S.A., Sturhan, D., Waeyenberge, L. & Moens, M. 1997. *Heterodera riparia* sp. n. (Tylenchida: Heteroderidae) from common nettle, *Urtica dioica* L., and rDNA-RFLP separation of species from the *H. humuli* group. *Russian Journal of Nematology* 5: 143-157.

Subbotin, S.A., Waeyenberge, L., Molokanova, I.A. & Moens, M. 1999. Identification of species from the *Heterodera avenae* group by morphometrics and ribosomal DNA-RFLPs. *Nematology* 1: 195-207.

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.

Szalanski, A., Sui, D.D., Harris, T.S. & Powers, T.O. 1997. Identification of cyst nematodes of agronomic and regulatory concern with PCR-RFLP of ITS1. *Journal of Nematology* 29: 255-267.

Thiéry, M. & Mugniéry, D. 1996. Interspecific rDNA restriction fragment length polymorphism in *Globodera* species, parasites of solanaceous plants. *Fundamental and Applied Nematology* 19: 471-479.

Wang, M., Peng, D. & Wu, X. 1991. [Studies on a wheat cyst nematode disease. I. Identification of pathogen]. *Journal of Huazhong Agricultural University* 10: 352-356.

Zheng, J., Cheng, H. & Fang, Z. 1997. [Pathotype of cereal cyst nematodes (*Heterodera avenae*) on wheat in Shanxi and Anhui, China]. *Acta Phytopathologica Sinica* 27: 309-314.

Zheng, J., Lin, M., Cheng, H. & Fang, Z. 1996. [Identification of two cyst nematode populations parasitizing wheat in Anhui, China]. *Jiangsu Journal of Agricultural Sciences* 12: 31-35.

Zheng J., Subbotin S.A., Waeyenberge L., Moens M. Молекулярно-биологическая характеристика популяций *Heterodera glycines* и *H. avenae* из Китая методами RFLP и секвенирования ITS участка рибосомальной ДНК.
Резюме. Представлены рестрикционные спектры и нуклеотидные последовательности ITS участка рибосомального гена популяций *Heterodera glycines* и *H. avenae* из Китая. У *H. glycines* была выявлена гетерогенность по результатам рестрикции амплифицированного продукта рестриктазой *Ava*I. Это основная рестриктаза для идентификации этого вида продуцирует обычно 4 фрагмента в 552, 478, 367 и 112 bp. Рестрикция амплифицированного продукта, полученного от популяций *H. avenae* из Китая, рестриктазами *Hin*fI и *Tru*9I выявила RFLP спектры, отличающие их от остальных популяций овсяных цистообразующих нематод.