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The identity of a Swedish *Globodera* (Nematoda: Heteroderidae) population, following comparisons with known populations of *G. artemisiae* (Eroshenko and Kazachenko, 1972) Behrens, 1975

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Summary. Morphological/morphometric identification and sequencing of ribosomal DNA internal transcribed spacers (ITS) were used to provide a proper determination of a *Globodera* population from Sösdala, southernmost Sweden. Data of cysts and juveniles corresponded well morphologically with those of the original description of *G. artemisiae* (Eroshenko & Kasachenko, 1972) Behrens, 1975, except for total juvenile length. In relation to a German *G. artemisiae* population and a Chinese one, the Swedish population displayed a genetic divergence of 0.24 and 0.36 %, respectively, along 842 nucleotides of ITS sequences. The combination of similarities in host choice, morphology and DNA sequences justifies the classification of the Sösdala population as belonging to *G. artemisiae*, the first record in Sweden. However, it is felt that international cooperative studies are urgently required, as data from reports on *G. artemisae* from different countries diverge to some extent and there are insufficient comparisons with the other known *Globodera* species, hosted by Asteraceae.

Key words: DNA, identification, morphology, morphometry, sequencing.

On examining soil samples in autumn 1980 from a field in the neighbourhood of Sösdala, in the province of Skåne, southernmost Sweden, where the farmer intended to grow seed potatoes in the next year, a few rounded cysts were found, similar to those of the potato cyst nematodes (PCN), *Globodera rostochiensis* and *G. pallida*. However, on average they seemed to be smaller than PCN cysts, and it also appeared that the number of ridges between anus and vulva in most of the cysts was less than that common to cysts of PCN. It was therefore thought that the cysts found might not belong to either of these species.

At a visit to the field in spring 1981, it was noted that specimens of *Artemisia vulgaris* L. grew abundantly in the borders of the field, and there were also several rhizomes of this plant present in the field. For this reason it was suspected that *A. vulgaris* might be a host of the unknown nematode. Soil and rhizomes were collected from all over the field, and cultures were established in a glasshouse. In autumn 1981 it was found that there was a high rate of multiplication on *A. vulgaris*. Because of this, it was thought that the cysts might belong to *G. artemisiae* (Eroshenko and Kazachenko, 1972) Behrens, 1975, but no definite identification was made. The aim of this work was to characterize our population morphologically, based on the most commonly used cyst, egg and second-stage juvenile differentials, and also genetically, and to compare it with known *G. artemisiae* populations.

MATERIALS AND METHODS

The nematode population, obtained as described above, was kept during the years in a cool store, with multiplications on *A. vulgaris* in a

glasshouse at a temperature of about 20°C every second - third year. Cysts from a cultivation in 1984 were extracted in 1985 and cysts, eggs and second-stage juveniles were investigated morphologically and morphometrically. Cysts from a multiplication in 2001 were extracted in spring of 2003 for molecular analysis. Such an analysis was also performed on cysts from a German population named Lühe, that was collected, identified as *G. artemisae*, and provided to us by Dr. D. Sturhan, Münster, Germany. This population was found in the 1980s (Sturhan, 1988).

Morphological and morphometric analysis. Altogether 100 eggs, 50 second-stage juveniles and 20 (60) cysts were examined for a number of characters of specific taxonomic value for rapid identification. Eggs were examined in water on temporary microscope glass slide mounts. Juveniles were killed and fixed in 4% formaldehyde and mounted permanently in glycerine on aluminium double-coverglass slides according to the method of Seinhorst (1962). Vulval cones were prepared as described by Hooper (1970). Observations and measurements were made using a Leitz Dialux light microscope with interference contrast and camera lucida measurement equipment. All microscope slides have been deposited in the Department of Crop Science, SLU, Alnarp, Sweden. The following statistical analyses were performed in parsing of morphometric data: mean, standard deviation and range. The analyses were done with the Minitab version 13.31 statistical software (Minitab Inc., State College, PA).

DNA extraction and sequencing. DNA was isolated from 40 dry cysts from each of the Swedish and the German populations, as described by Phillips et al. (1992). RNA was removed by incubation with Rnase (1 mg/ml) at 65°C for 30 min, followed by a sodium acetate, phenol/chloroform/isoamyl alcohol (PCI; 25:24:1 v/v/v) and ethanol precipitation. The concentration of extracted DNA was spectrophotometrically determined. DNA was stored at -20°C until required. The 5.8S rDNA and flanking ITS1 and ITS2 regions were amplified from total DNA by the use of primers described by Subbotin *et al.* (2001). The amplified fragments were then sequenced directly in both directions using the fluorescent labeled primers TF1, 28R1, MITSF and MITSR (Subbotin *et al.*, 2001). Finally, the sequence reaction was performed using the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Sequence analysis. Raw sequence data were first imported into the Contig Express component of the Vector NTI 7.1 suite of sequence analysis programs (InforMax, Inc. Frederick, MD) and consensus sequences were then generated. In addition, NCBI Blast Search was used to find a complimentary sequence found in the general databank of known nucleotide sequences (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence analysis was performed using Clustal W software (Thompson *et al.*, 1994). The sequences of the Sösdala and Lühe populations were then aligned with sequences of a Chinese *G. artemisiae* (AF274415) population (Subbotin *et al.*, 2001), *G. rostochiensis* (Bulman and Marshall, 1997) and *G. pallida* (Bulman and Marshall, 1997). Regions that could not be aligned were excluded from the phylogenetic analysis. Genetic distance calculations with uncorrected "p" and HKY85 distance measures (Hasegawa *et al.*, 1985) were performed using PAUP* 4.0b10 (Swofford, 2002).

RESULTS

Morphological and morphometric analysis. Except for a few instances, the cysts were not quite spherical but tended to be oval or otherwise rounded. Cysts of this shape also may be found in PCN but in PCN the proportion of round cysts is much larger. The neck was not found to be as offset as in PCN cysts. The second-stage juvenile stylet basal knobs were mostly slightly forward pointing but we found some specimens with rounded knobs and some with an intermediary shape as well. The lips had four annules.

Morphometric data on eggs, juveniles and cysts are shown in Table 1 together with those of three foreign populations, including data from the original description. In most respects, the data from our population are similar to those of the original description. An obvious exception is total juvenile length with means of 462 and 413 µm for the Swedish and Russian populations, respectively. The juvenile body length in our population is about the same as that of the German and slightly less than in the Chinese population. There seem to be clear differences between the Swedish and the Russian populations on one hand and the German and the Chinese on the other with regard to juvenile stylet length and hyaline tail length. The stylet is, on average, about 1.5 µm and hyaline tail about 2.5 µm longer in the Chinese and German populations than in the Swedish and Russian populations.

Table 1. Morphometric characteristics of the Swedish population supposed to be *Globodera artemisiae* and three foreign *G. artemisiae* populations.

Life cycle stage	Character	Sweden (n=20-100)	Russia, from Eroshenko and Kazachenko (1972)	China, from Cheng *et al.* (1984)	Germany (n=32), provided by Sturhan (unpublished)
Eggs	Length (µm)	105.2±4.3 (94.7-115.9)	99.0 (69.0-114.0)	-	-
	Width (µm)	43.6±1.8 (39.9-48.6)	45.0 (33.0-60.0)	-	-
Second-stage juveniles	Length (µm)	461.8±23.5 (417.0-520.1)	413.0 (355.0-419.0)	476.5	458,2±21.2 (397-495)
	a	27.8±1.2 (24.9-30.7)	23.3 (21.2-26.8)	23.7	-
	c	9.94±0.81 (7.4-12.0)	9.6 (6.1-14.0)	-	-
	Tail length (µm)	46.7±3.57 (41.2-62.4)	40.0 (33.0-64.0)	47.2	49.5±3.15 (43.1-53.4)
	Hyaline tail length (µm)	23.1±1.5 (20.0-26.0)	23.0 (18.0-28.0)	25.4	25.7±2.3 (21.5-29.5)
	Stylet length(µm)	22.3±1.0 (20.4-23.6)	22.6 (18.0-29.0)	23.9	23.7±0.7 (21.8-24.6)
Mature cysts	Vulval basin diameter (µm)	22.9±1.8 (17.6-26.4)	23.0 (14.0-28.0)	25.0	-
	Distance from anus to vulval basin (µm)	28.8±4.1 (24.2-37.4)	26.0 (19.0-47.0)	33.5	-
	Granek's ratio	1.3±0.2 (1.0-1.7)	1.0 (0.8-1.7)	1.3	-
	No. of cuticular ridges between anus and vulval basin	7.8±2.0 (5.0-16.0)	-	-	-

Table 2. Distance matrix, indicating differences, from five *Globodera* populations: uncorrected "p" (lower triangular) and HKY85 (upper triangular). Original of populations as in Fig. 1.

	Population				
Population	Sösdala	Lühe	China	*G.r.* Lincoln	*G.p.* Lincoln
Sösdala	-	0.00240	0.00358	0.07388	0.07807
Lühe	0.00240	-	0.00119	0.07380	0.07401
Bejing	0.00357	0.00119	-	0.07357	0.07377
G.r. Lincoln	0.07007	0.07006	0.06987	-	0.03334
G.p. Lincoln	0.07372	0.07010	0.06990	0.03253	-

Sequence analysis. The DNA sequences of the Swedish and the German populations are presented in Fig. 1, where they are compared with the sequences from a known Chinese *G. artemisiae* population and sequences from one *G. rostochiensis* and one *G. pallida* population. Genetic distances between populations are presented in Table 2. Sequencing of the Sösdala and Lühe populations resulted in 846 and 847 bp long sequences, respectively. The sequences of Sösdala and Chinese populations differed from each other by two transitions (64 and 182 bp), one transvertion (82 bp) and one deletion/insertion in the beginning of the sequence (5 bp). There were also two sites where identification of nucleotides was unsuccessful (167 bp and 512 bp). Apart from the

same deletion as in Sösdala, the Lühe population differed from the Chinese population, only in two sites where identification failed (64 bp and 512 bp). The differences between the Swedish and the German populations were, thus, larger than between the German and the Chinese, also reflected in the difference matrices, where the differences between the Swedish and the German was 0.24% units, between the Swedish and the Chinese 0.36% units and between the German and the Chinese 0.12% units. The two PCN populations were distinctly different from the other populations.

DISCUSSION

The non-morphometric characters mentioned above were similar in our population and those in the original *Globodera artemisiae* description, given by Eroshenko and Kazachenko (1972). In Fig.1 of that paper oval-shaped females are shown. The authors state that cysts are "more round than the females", which suggests that they are usually not quite round. The shape of juvenile knobs is described as "oval".

As already pointed out, the morphometric data of the investigated stages of the Sösdala population agreed reasonably well in most characters with those of the original description. However, the evidently highly statistically significant 49 µm difference in second- stage juvenile length is worth noticing. Wouts and Weischer (1977), investigating juveniles from 47 populations of fifteen Heteroderidae species, considered this character to be important, suggesting that the difference in mean length within a species is normally not more than 20-30 µm. Further, they found juvenile stylet length to be the most stable character. In light of this, the differences between the stylet length means of the Swedish and Russian populations on one hand, and the German and the Chinese populations on the other, are relatively large. As a whole, Table 1 demonstrates that there is a considerable range of variability of certain morphological characteristics both within populations and between populations. Since the populations in our study are allopatric, the differences found might result from an adaptive phenotypic plasticity. Such variations have been reported in *Steinernema kraussei* by Stock *et al.* (2000).

Ribosomal DNA sequences are commonly used as genetic markers for identifying organisms. The analysis of the genetic variability of the Sösdala, Lühe and Chinese populations, using DNA sequences, revealed only microheterogeneity. Heterogeneity of the internal transcribed spacer region has been reported for other species (Szalanski *et al.*, 1997; Blok *et al.*, 1998; Subbotin *et al.*, 2000). Comparing the variability of the region assessed, the genetic distance of 0.12% units - 0.36% units is very low and illustrates a high degree of similarity between the three populations, suggesting that the populations are conspecific. The fact that the populations originate from very distinct geographical regions, support our conclusion that the differences do not stand for species level divergence, although we do not know how the sequences of the other *Globodera* species, having Asteraceae as hosts, would look like.

From the fact that our population was found in association with *Artemisia vulgaris*, which appeared to be an excellent host, the similarity in morphology with the original description of *G. artemisae*, and the close genetic resemblance with other populations, claimed to belong to this species, we conclude that the Sösdala population belongs to *G. artemisiae*. This is the first record of the species in Sweden.

Because of possible confusions at PCN identification, caused by subtle morphological and morphometric differences, there has been increasing interest in characterising European round cyst non-PCN *Globodera* populations, not least in the EPPO organisation (Anon., 2004, unpublished). However, so far little work has been done and available records do not always coincide. The occasionally confusing morphological differences found within *G. artemisiae* in this study supports the need for international, cooperative investigations, covering all round cyst species that directly or indirectly are of importance in quarantine work. Such studies might be rewarding also from other points of view, e.g. the understanding of species diversity.

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Ga-AF274415 : AGTTACCTGCTGTCCAGTTG-GTCAATGTGGGCAAAACCATATGCCTTCGTTTGTTGTTGACGGACACATGCCCGTTGGGTTTGGGCTGGCACATTGACT
Gsp-Sösdala : ....-.....................................................A.....................A...................
Ga-Lühe     : ....-.....................................................K.....................................
Gr-Lincoln  : .CC.................A....G.........C....C......C.............................C..T..A.T..............C
Gp-Lincoln  : .CC.................A....G.........C....C......C.............................C.AT..................TC

Ga-AF274415 : AACAAGTTGAACGGACAGCGCCCTGTGGGCACAAGAGTTTTGGGGTGTTACCGATGTTGGTGGCCCAATGGGTGAGCCGACGATTGCTGTCGTCGGCGGC
Gsp-Sösdala : ..........................................................K.............A.....................
Ga-Lühe     : ..........................................................................................
Gr-Lincoln  : .....--..T.......................T....G.........A.....................T.....................CT....T...G
Gp_Lincoln  : .....--..T.T..G.................T....G.........A.............................CT............CT....T...G
Ga-AF274415 : TCACTGCGCCAACAGAGGTAGTACGCCCGCAGGGCACCCTAACGGCTGTGCTGGCGTCTGTGCGTCGTTGAGCGGTTGTTGCGCCTTGCGCTGATATGCT

Gsp-Sösdala : ..........................................................................................
Ga-Lühe    : ..........................................................................................
Gr-Lincoln  : ..G..........G....A..T......A..........G.....................................................A.........
Gp_Lincoln  : ..G....A.....G.....G.T......A.....................................................G.........

Ga-AF274415 : GACATGGAGTGTGTAGGCTTCTATTCCATGTCGTACGTACCGTACCTGGCGGCATGTCGGCGTTTGTGTGCTACGTCCGTGGCCGTGATGAGACGACGTG
Gsp-Sösdala : ..........................................................................................
Ga-Lühe    : ..........................................................................................
Gr-Lincoln  : A...........--.....G...C.......G......G........T..........T...C.....................................
Gp_Lincoln  : ............--.....G...........G......G.......CA.........T...C.....................................

Ga-AF274415 : TTAGGACCCATGCCTGGCATTGGCGTGTGGTTTAAGACTTGATGAGTGCCCGGAGGCACCGCCAGCGTTTTTCTCATTTTTATTTATTTTTTCAATGCAA
Gsp-Sösdala : ..........................................................................................
Ga-Lühe    : ..........................................................................................
Gr-Lincoln  : .........G.............AC.........................C.............-......C.................-.......
Gp_Lincoln  : .........G.............A..C.....................C.............-.............AA.......-.......

Ga-AF274415 : TTTGATTGCACKAAATATTCTAGCCTTATCGGTGGATCACTCGGCTCGTGGATCGATGAAGAACGCAGCCAACTGCGATAATTAGTGTGAACTGCAGAAA
Gsp-Sösdala : ..........G...............................................................................
Ga-Lühe    : ..........G...............................................................................
Gr-Lincoln  : ..C......-TA...........T...................................................................
Gp_Lincoln  : .........-TA...........T...................................................................

Ga-AF274415 : CCTTGAACACAGAACTTTCGAATGCACATTGCGCCATTGGAGTAACATCCATTGGCACGCCTGGTTCAGGGTCGTAACCAAAAAATGCACTGCATGTGCG
Gsp-Sösdala : ..........................................................................................
Ga-Lühe    : ..........................................................................................
Gr-Lincoln  : ..........................................G.......................................................
Gp_Lincoln  : ..........................................G.......................................................

Ga-AF274415 : TGTTTTATTTGCTAAGATCACGACGCTTCGTCGTGTTCTTGCATAAAAGTGGAATGCTACGCTGTGTGGCGGTTGGGTGTGCTGGCGCGAAAATATGCTT
Gsp-Sösdala : ..........................................................................................
Ga-Lühe    : ..........................................................................................
Gr-Lincoln  : ............A.........---.....G.............CT.T..-.............A...-....AC.................G..T.G
Gp_Lincoln  : ......................---.....G.............CC.T..-.............A.T.-....AC.................G..T.G

Ga-AF274415 : TCTTTCGCGCTTTACAGACCGTAATTTAGGCACGCCCTTCGG
Gsp-Sösdala : ...................................
Ga-Lühe     : ..A................................
Gr-Lincoln  : ...................................
Gp_Lincoln  : ...................................
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Fig. 1. CLUSTAL W (1.81) multiple sequence alignment of the 5.8S rDNA with flanking ITS1 and ITS2 regions (842 alignment positions) for the Swedish Sösdala round-cyst population, the German *Globodera artemisiae* Lühe population, a Chinese *G. artemisiae* population (from Subbotin *et al.*, 2001), and *G. rostochiensis* and *G. pallida* populations (from Bulman and Marshall, 1997).

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Manduric S., Andersson S. Таксономическая принадлежность шведской популяции *Globodera* (Nematoda, Heteroderidae) и сравнение с известными популяциями *G. artemisiae* (Eroshenko et Kazachenko, 1972) Behrens, 1975.

Резюме. Морфологические (морфометрические) признаки и секвенирование ITS-участка рибосомального гена были использованы для видового определения популяции *Globodera* их Сесдала (Sösdala), на южной оконечности Швеции. Данные по цистам и личинкам полностью соответствовали сведениям первоописания вида *G. artemisiae* (Eroshenko & Kasachenko, 1972) Behrens, 1975, за исключением лишь длины тела личинок. По отношению к немецкой и китайской популяциям *G. artemisiae,* популяция из Швеции продемонстрировала 0.24 и 0.36 % различий по 842 нуклеотидам ITS-участка. Сходство по выбору растения-хозяина, морфологии и последовательностям ДНК подтверждают отнесение популяции из Сесдала к виду *G. artemisiae*, что представляет собой первое сообщение из Швеции. Необходимо международное сотрудничество по выяснению таксономического статуса популяций *G. artemisae* из разных стран, поскольку между ними наблюдаются определенные различия, и не выяснены их филетические отношения с другими видами *Globodera* обитающими на Asteraceae.