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Variations in ribosomal DNA sequences and phylogeny of *Globodera* parasitising solanaceous plants

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Summary – The D3 expansion region of the 28S gene and the ITS1-5.8S-ITS2 region of rDNA sequences from *Globodera rostochiensis*, *G. pallida*, *G. tabacum tabacum*, *G. tabacum virginiae* and *G. tabacum solanacearum* have been aligned and compared. There are no nucleotide differences in the D3 region sequences between *G. rostochiensis* and *G. pallida*. Sequence analysis and RFLPs of ITS-PCR products showed that several haplotypes are present in the genomes of *G. rostochiensis* and *G. pallida* populations. Restriction patterns of PCR products for eight enzymes for differentiation of these two species are given. Phylogenetic analysis of 41 ITS region sequences obtained from populations and species of the subfamily Punctoderinae revealed four distinct main clades within *Globodera* parasitising solanaceous plants: *G. rostochiensis*, *G. tabacum*, *G. pallida* and an undescribed *Globodera* sp. from South America. The utility of RFLP profiles and sequences of the rDNA are discussed for diagnostics and phylogeny of *Globodera*.

Résumé – ***Variabilité dans les séquences de l'ADN ribosomal et phylogénie des* Globodera *parasitant les Solanacées –*** La région d'expansion D3 du gène 28S et la région ITS1-5.8S-ITS2 des séquences d'ADNr de *Globodera rostochiensis*, *G. pallida*, *G. tabacum tabacum*, *G. tabacum virginiae* et *G. tabacum solanacearum* ont été alignées et comparées. Il n'y a pas de différence dans les nucléotides de la séquence de la région D3 entre *G. rostochiensis* et *G. pallida*. L'analyse séquentielle et les RFLP des produits du ITS-PCR montrent que plusieurs haplotypes sont présents dans les génomes des populations de *G. rostochiensis* et *G. pallida*. Les profils de restriction des produits du PCR de huit enzymes choisies pour la différenciation de ces deux espèces sont donnés. L'analyse phylogénique séquentielle de 41 regions ITS de populations et espèces appartenant à la sous-famille des Punctonerinae a révélé l'existence de quatre clades chez les *Globodera* parasitant les Solanacées: *G. rostochiensis*, *G. tabacum*, *G. pallida* et une espèce non décrite provenant d'Amérique du Sud. L'utilité pour le diagnostic et la phylogénie des *Globodera* des profils de RFLP et des séquences d'ADNr est discutée.

Keywords – *G. pallida*, *G. rostochiensis*, *Globodera* sp., *Globodera tabacum*, 5.8S gene, 28S gene, heterogeneity, ITS regions, phylogeny, RFLP.

The potato cyst nematodes (PCN), *Globodera rostochiensis* and *G. pallida*, are considered the most economically important nematode pests of potatoes and are the subject of strict quarantine regulations in many countries. Subspecies of the *G. tabacum* complex may cause significant losses of yield in tobacco plantations in Mexico and USA (Baldwin & Mundo-Ocampo, 1991; Brodie *et al.*, 1993). Traditional diagnosis of *Globodera* species based on examination of morphology and morphometrics can be time-consuming, even for an expert. Development of diagnostic tests based on monoclonal antibodies and differences in proteins and DNA were considered to have potential for identification (Fleming & Powers, 1998). Comparative analysis of coding and non-coding regions of ribosomal DNA (rDNA) is becoming a popular tool for species and subspecies identification and to evaluate relationships between plant-parasitic nematodes from many genera. Amplification and analysis of rDNA genes have many advantages. Profiles are obtained rapidly from a few cysts and the clarity of the results enables species to be identified very easily without being affected by environmental and developmental variation (Vrain *et al.*, 1992; Wendt *et al.*, 1993; Zijlstra *et al.*, 1995; Powers & Fleming, 1998). RFLP analysis of the internal transcribed spacers (ITS) were useful for identification of *Globodera* species parasitising solanaceous plants (Flem-

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ing & Mowat, 1993; Thiéry & Mugniéry, 1996; Szalanski *et al.*, 1996; Subbotin *et al.*, 1999). PCR-rDNA-RFLP techniques, used for routine identification of PCN and closely related species, are based on a RFLP catalogue and sequence information from different populations and species. However, as recent investigations showed (Bulman & Marshall, 1997; Blok *et al.*, 1998), some intraspecific sequence variations in the ITS regions within PCN populations exist. Biochemical and rDNA PCN diagnostics have been reviewed extensively by Fleming and Powers (1998).

Studies of phylogenetic relationships within nematodes are not only a basis for stable and predictive taxonomy, but also make possible a more complete understanding of the biology of nematodes as agricultural pests. Several studies have demonstrated that sequences of ITS region are very useful for phylogenetic analyses of relationships of some species within the genera *Heterodera* (Ferris *et al.*, 1993), *Globodera* (Ferris *et al.*, 1995), *Bursaphelenchus* (Beckenbach *et al.*, 1999) and *Meloidogyne* (Hugall *et al.*, 1999).

This work examines the intra- and interspecific variations in the ITS regions, including the 5.8S rDNA genes plus flanking areas of the 18S and 28S genes and also the D3 expansion region of 28S, between Russian populations of *G. rostochiensis* and other *Globodera* species. We also present the results of phylogenetic analysis of the *Globodera* species using maximum parsimony and minimum evolution, based on original and published sequences of the ITS regions.

Materials and methods

Nematodes

Nine populations of *Globodera rostochiensis* and one population each of *G. pallida*, *G. tabacum tabacum*, *G. tabacum virginiae* and *G. tabacum solanacearum* were used in this work (Table 1). Prior to the study, air-dried cysts were stored at room temperature in plastic tubes. Sequences of *Globodera* species were obtained from published articles and GenBank (Table 1). Sequence of *Cactodera estonica* obtained from an unpublished study (Subbotin *et al.*) was taken as an outgroup taxon for phylogenetic and sequence analysis.

DNA extraction

For each extraction, four to six cysts were crushed with a microhomogeniser in a sterile Eppendorf tube containing 18 μl of worm lysis buffer (125 mM KCl, 25 mM Tris-HCl pH 8.3, 3.75 mM $MgCl_2$, 2.5 mM DTT, 1.125% Tween 20, 0.025% gelatin), and 2 μl of proteinase K (600 μg/ml). The tubes were incubated at 65°C for 1 h and then at 95°C for 10 min. The samples were stored at – 40°C.

DNA amplification

After centrifugation of the sample (1 min; 16000 *g*), 3 μl of the supernatant were added to each PCR reaction containing: 10 mM Tris-HCl pH 9.0, 1.5 mM $MgCl_2$, dNTP-mixture 20 mM each (Life Technologies Ltd, Rockville, MD, USA), 1 μM of each primer (synthesised by MWG-Biotech Ltd, Milton Keynes, UK), 1U *Taq* Polymerase (Promega Corporation, Madison, WI, USA) and double distilled water. PCR fragments for the cloning procedure were obtained using *Pfu* DNA Polymerase (1U) with its buffer (Stratagene, Cambridge, UK), which included $MgSO_4$. The final volume was made up to 50 μl with sterile distilled water. Amplification from each DNA sample was carried out using two separate reactions. The first used primers rDNA1 and rDNA2 (Vrain *et al.*, 1992), which amplify the ITS region, and the second reaction used primers D3A and D3B (Al-Banna *et al.*, 1997), which amplify the D3 expansion region of the large subunit ribosomal gene (28S rDNA) (Table 2). Amplification was carried out in a HyBaid OmniGene DNA thermal cycler (Hybaid Ltd, Teddington, UK). A control without nematode DNA was included. PCR conditions for ITS region amplification were: denaturation at 94°C for 2 min, followed by 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min, repeated for 35 cycles and followed by an incubation period of 5 min at 72°C. PCR conditions for the D3 expansion region amplification were denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min, and an additional 5 min at 72°C. After DNA amplification, 5 μl of the products were analysed on 1% agarose gel and visualized following staining with ethidium bromide.

RFLP

The amplified ITS region products were purified with the QIAquick PCR Purification Kit and the QIAquick Gel Extraction Kit (Qiagen Ltd, Crawley, UK). One μl of each PCR-product were digested separately with each of the following restriction enzymes: *Alu*I, *Cfo*I, *Rsa*I, *Sau*3A, *Sph*I (Boehringer Mannheim Ltd, Lewes, East Sussex, UK), *Nde*I, *Bsh*1236I (MBI Fermentas Inc., Buf-

Table 1. Globodera *populations and species used in the present study.*

Identification based on the ITS sequence	Original identification	Population	Study[1)]	Codes	Source (GenBank accession number)
G. rostochiensis	*G. rostochiensis*	Russia, Moscow region, Egor'evskii district, Gavrilovskaja	SDI, RFLP, PA	Gr-Ga	Present study
	G. rostochiensis	Russia, Primorskii region, Khasan district, Khasan	SDI, RFLP	Gr-Kh	Present study
	G. rostochiensis	Russia, Jaroslavl region, Nekrasovskii district, Likhoobrazovo	SDI, RFLP	Gr-Ja	Present study
	G. rostochiensis	Russia, Tula region, Jasnogorskii district, Chertovoe	SDI, RFLP	Gr-Tu	Present study
	G. rostochiensis	Russia, Vladimir region, Gys'-Khurtal'nyi district, Ujakhino	SD, RFLP	Gr-Vl	Present study
	G. rostochiensis	Russia, Smolensk	SD, RFLP	Gr-Sm	Present study
	G. rostochiensis	Russia, Pskov, Pristan-2	SD, RFLP	Gr-Ps	Present study
	G. rostochiensis	Russia, Kaliningrad, Pravdinskii district, Zheleznodorozhmyi	RFLP	Gr-Ka	Present study
	G. rostochiensis	UK, Scarcliffe	SDI, RFLP, PA	Gr-Ro	Present study
	G. rostochiensis	UK, Feltwell, Cambridgeshire	PA	Gr-ROS	Ferris *et al.* (1995)
	G. rostochiensis	Canada	PA1	Gr-Can	Szalanski *et al.* (unpubl.)(AF016875)
	G. rostochiensis	UK, Falkland Islands	PA1	Gr-FI	Szalanski *et al.* (unpubl.)(AF016876)
	G. rostochiensis	Mexico, Cuapiaxtla	PA1	Gr-Mex	Szalanski *et al.* (unpubl.)(AF016877)
	G. rostochiensis	USA, New York	PA1	Gr-NY	Szalanski *et al.* (unpubl.)(AF016878)
	G. rostochiensis	Peru, Allpachaka	PA1	Gr-Per1	Szalanski *et al.* (unpubl.)(AF016872)
	G. rostochiensis	Peru	PA1	Gr-Per2	Szalanski *et al.* (unpubl.)(AF016873)
	G. rostochiensis	Peru, Anta	PA1	Gr-Per3	Szalanski *et al.* (unpubl.)(AF016874)
G. pallida	*G. pallida*	UK, Risby	SDI, RFLP, PA	Gp-Pa	Present study
	G. pallida	UK, Cadishead, Lancashire	PA	Gp-PAL	Ferris *et al.* (1995)
	G. pallida	UK, Halton	PA	Gp-Hal	Blok *et al.* (1998)
	G. pallida	New Zealand, Lincoln	PA	Gp-Lin	Bulman & Marshall (1997)
	G. pallida	UK, Scotland, Luffness	PA	Gp-Luf	Blok *et al.* (1998)
	G. pallida	UK, Scotland	PA	Gp-Pa1	Blok *et al.* (1998)
	G. pallida	South America	PA	Gp-P4A	Blok *et al.* (1998)
	G. pallida	Peru, Pilayo	PA1	Gp-Per	Szalanski *et al.* (unpubl.)(AF016865)
	G. pallida	Northern Ireland	PA1	Gp-NI	Szalanski *et al.* (unpubl.)(AF016869)
	G. pallida	Romania	PA1	Gp-Rom	Szalanski *et al.* (unpubl.)(AF016870)
	G. pallida	Spain	PA1	Gp-Sp	Szalanski *et al.* (unpubl.)(AF016871)
G. tabacum	*G. tabacum tabacum*	USA, Culture from IACR-Rothamsted, originated from L. Miller	SDI, PA	Gt-Tab	Present study
	G. tabacum virginiae	USA, Culture from IACR-Rothamsted, originated from L. Miller	SDI, PA	Gt-Vir	Present study
	G. tabacum virginiae	USA, Virginia	PA1	Gt-VV	Szalanski *et al.* (unpubl.)(AF016881)
	G. tabacum solanacearum	USA, Culture from IACR-Rothamsted, originated from L. Miller	SDI, PA	Gt-Sol	Present study

Table 1. *(Continued).*

Identification based on the ITS sequence	Original identification	Population	Study[1)]	Codes	Source (GenBank accession number)
	G. tabacum solanacearum	USA, Virginia	PA1	Gt-SV	Szalanski *et al.* (unpubl.)(AF016880)
	Globodera sp.	Mexico, Santa Ana, Juchitepec, Estado de México	PA	Gt-X14	Ferris *et al.* (1995)
	Globodera sp.	Mexico, La Colorado, Estado de Coahuila	PA	Gt-X76	Ferris *et al.* (1995)
	Globodera sp.	Mexico	PA1	Gt-Mex	Szalanski *et al.* (unpubl.)(AF016879)
Globodera sp.	*G. pallida*	South America	PA	Gs-P5A	Blok *et al.* (1998)
	G. pallida	Peru, Bolivian border	PA1	Gs-Per1	Szalanski *et al.* (unpubl.)(AF016866)
	G. pallida	Peru, Tiabaya	PA1	Gs-Per2	Szalanski *et al.* (unpubl.) (AF016867)
	G. pallida	Peru, Santa Ana-Junin	PA1	Gs-Per3	Szalanski *et al.* (unpubl.) (AF016868)
Cactodera estonica	*Cactodera estonica*	The Netherlands	PA	Cac-Est	Subbotin *et al.* (unpubl.)
Cactodera sp.	*G. virginiae*	USA, Virginia	PA	Cac-VIR	Ferris *et al.* (1995)

[1)] SDI — sequencing of the D3 expansion region of the 28S gene and the ITS1 and ITS2 regions; SD — sequencing of the D3 expansion region; RFLP — restriction fragment length polymorphisms study; PA — phylogenetic and sequence analysis of the ITS1 and ITS2 regions; PA1 — phylogenetic and sequence analysis of the ITS1 region.

Table 2. *Primers used in this study.*

Amplified region	Primer code	Primer sequence
ITS1-5.8S-ITS2	rDNA1	5′-TTGATTACGTCCCTGCCCTTT-3′
	rDNA2	5′-TTTCACTCGCCGTTACTAAGG-3′
ITS1	rDNA1.58	5′-ACGAGCCGAGTGATCCACCG-3′
D3 of 28S	D3A	5′-GACCCCTCTTGAAACACGGA-3′
	D3B	5′-TCGGAAGGAACCAGCTACTA-3′

falo, NY, USA), *Hin*fI, *Sty*I (Life Technologies Ltd, Paisley, UK) in the corresponding buffer according to the manufacturer's instructions. Digested fragments and markers were purified using the QIAquick PCR Purification Kit (Qiagen Ltd). The resulting fragments were separated on synthetic meltable polyacrylamide OligoPrep gels in 1× OligoPrep buffer (National Diagnostics Inc., Atlanta, GA, USA) for 1 h 40 min at 100 V, stained with ethidium bromide for 20 min, visualised on a UV transilluminator and photographed using a UVP videocamera and UVP Imagerstore 7500 (Ultra Violet Products Ltd, Cambridge, UK). DNA molecular weight markers λ DNA/*Hin*dIII fragments (Life Technologies Ltd) and XIV (Boehringer Mannheim Ltd) were used as size standards. The procedures for obtaining and digestion of PCR products were repeated at least twice to determine consistency of results.

Cloning and sequencing

PCR products of the ITS region produced using *Pfu* were excised from 1% TBE buffered agarose gels using the QIAquick Gel Extraction Kit (Qiagen Ltd.), cloned into the *Eco*RV site of pBluescrip II SK- (Stratagene Ltd) and transformed into Subcloning Efficiency DH5α Competent cells (Life Technologies Ltd). Several clones of each population were isolated using blue/white selection. Plasmids were purified using the Qiagen Plasmid Mini Kit (Qiagen Ltd). ITS cloned products were cycle sequenced using ampli*Taq* DNA polymerase (PE Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. The rDNA1, rDNA2, rDNA1.58S primers and the M13 forward and reverse vector primers were used to confirm the sequences in 18S and 28S genes. PCR products of the D3 expansion region were purified using a QIAquick PCR Purification Kit (Qiagen Ltd). Sixty ng of this DNA fragment were sequenced in both directions with D3A and D3B primers using ampli*Taq* DNA polymerase. The programme used for all sequencing reactions was: 94°C for 30 s, 50°C for 30 s, and 60°C for 3 min, 30 s, repeated for 25 cycles. The resulting products were purified using a Centriflex Gel Filtration Cartridge (Edge BioSystems Inc., Gaithersburg, MD, USA). Sequences were run on a 373 DNA sequencer (PE Applied Biosystems).

Sequence alignment

Sequences were edited using the Chromas (version 1.45) program (Copyright©1996-1998 Conor McCarthy), aligned using the Clustal X program with default options

(Thompson *et al.*, 1997) and edited using GeneDOC (version 2.5.0, Nicholas & Nicholas, 1997). The boundaries of ITS1 and ITS2 were determined by comparing the aligned sequences with previously published sequences of *G. pallida* and *G. rostochiensis* (Bulman & Marshall, 1997; Blok *et al.*, 1998).

The following alignments were created: the D3 regions of the 28S genes of original sequences of *Globodera*; the ITS regions, including the 5.8S rDNA genes plus flanking areas of the 18S and 28S genes of original sequences for comparison of sequence and RFLP data (Fig. 1); the entire ITS regions and the ITS1 region of original and published sequences for sequence and phylogenetic analyses; two separated alignments containing the ITS2 region or 5.8S gene sequences for sequence analyses. Multiple alignments have not been included in this paper but are available on request from the senior author. Original sequences of the D3 expansion region of 28S genes are submitted at the GenBank.

Sequence and phylogenetic analysis

Unweighted maximum parsimony analysis was performed using PAUP (4.0 beta version) (Swofford, 1998) using heuristic search (TBR branch swapping, collapse yes, multrees yes, steepest descent no, gaps were treated as missing data). Bootstrap (Felsenstein, 1985) analysis with 100 replicates was conducted to assess the degree of support for each branch on the tree. Different measures of homoplasy, such as consistency index (CI) (Kluge & Farris, 1969), retention index (RI) and rescaled consistency index (RC) (Farris, 1989) and also $g1$ statistic, a measure of skewness of tree-length distribution (Hillis & Huelsenbeck, 1992), were computed to estimate the amount of phylogenetic information in the parsimony analysis. The $g1$ statistic was computed by generating 10 000 random parsimonious trees using the Random tree option in PAUP. Pairwise divergence between taxa as the absolute distance values and the percent mean distance values based on the ITS1, ITS2, 5.8S and the entire ITS alignment and adjusted for missing data were computed.

Distance analyses were performed using PAUP (4.0 beta version) with LogDet method (Lockhart *et al.*, 1994), the distance trees were constructed by the neighbour-joining method (Saitou & Nei, 1987). Bootstrap values based on 1000 resampling were estimated. Trees were examined with TREEVIEW program (Page, 1996).

Results

Sequence analysis

The amplification by PCR of the D3 expansion region of the large subunit 28S rDNA gene for each population of *Globodera* yielded a single PCR product with a length of approximately 340 bp. Identical sequences were obtained from populations of *G. rostochiensis* and *G. pallida*. There was no variation between the eight populations (Gr-Ga, Gr-Kh, Gr-Ja, Gr-Tu, Gr-Vl, Gr-Sm, Gr-Ps and Gr-Ro) of *G. rostochiensis* that were analysed. There were also no sequence variations in this region between *G. tabacum tabacum*, *G. tabacum solanacearum* and *G. tabacum virginiae*, and the difference between sequences of the PCN populations and species of the *G. tabacum* complex was in only one nucleotide replacement.

The rDNA1 and rDNA2 primers gave ITS-PCR products of *ca* 1190 bp from all species and populations. Other weak bands, probably from non-specific amplification, were excluded from the further analysis. Three ITS clones from the Gr-Ga and Gr-Ro populations, two clones from the Gr-Kh, Gr-Ja and Gr-Tu populations, five clones from *G. pallida* and two clones from each of *G. tabacum tabacum*, *G. tabacum virginiae* and *G. tabacum solanacearum* were sequenced. The PCR product from the Gr-Tu population of *G. rostochiensis* was also directly sequenced.

The aligned original sequences from clones of *G. rostochiensis* (Gr-Ja1), *G. pallida* (Gp-Pa1), *G. tabacum tabacum* (Gt-Tab1), *G. tabacum virginiae* (Gt-Vir1) and *G. tabacum solanacearum* (Gt-Sol1) are shown in Fig. 1. Sequence analysis showed that 11 *G. rostochiensis* clones had sequence lengths of 1191 bp including primers, and one clone (Gr-Ro2) had a sequence length of 1190 bp. Twelve point mutations in the amplified region were observed between 12 clones. Five of the point mutations were situated in the ITS1 region, one in the 5.8S gene and six in the ITS2 region. These nucleotide differences corresponded to three types of ITS1 (A, B and C) and three types of ITS2 regions (a, b and c) (Table 3). Types Aa and Cc were found three times, Bb five times and Cb only once among the clones studied; the last type contained two base pair deletions. Population Gr-Ga contained clones with Aa, Bb and Cc, Gr-Kh contained Bb and Cc, Gr-Ja contained Bb, Gr-Tu contained Aa and Cc and Gr-Ro contained Aa, Bb and Cb types of ITS regions (Table 3). Direct sequencing of the Gr-Tu ITS regions revealed the same ambiguous or heterogeneous nucleotide positions at varying intensities.

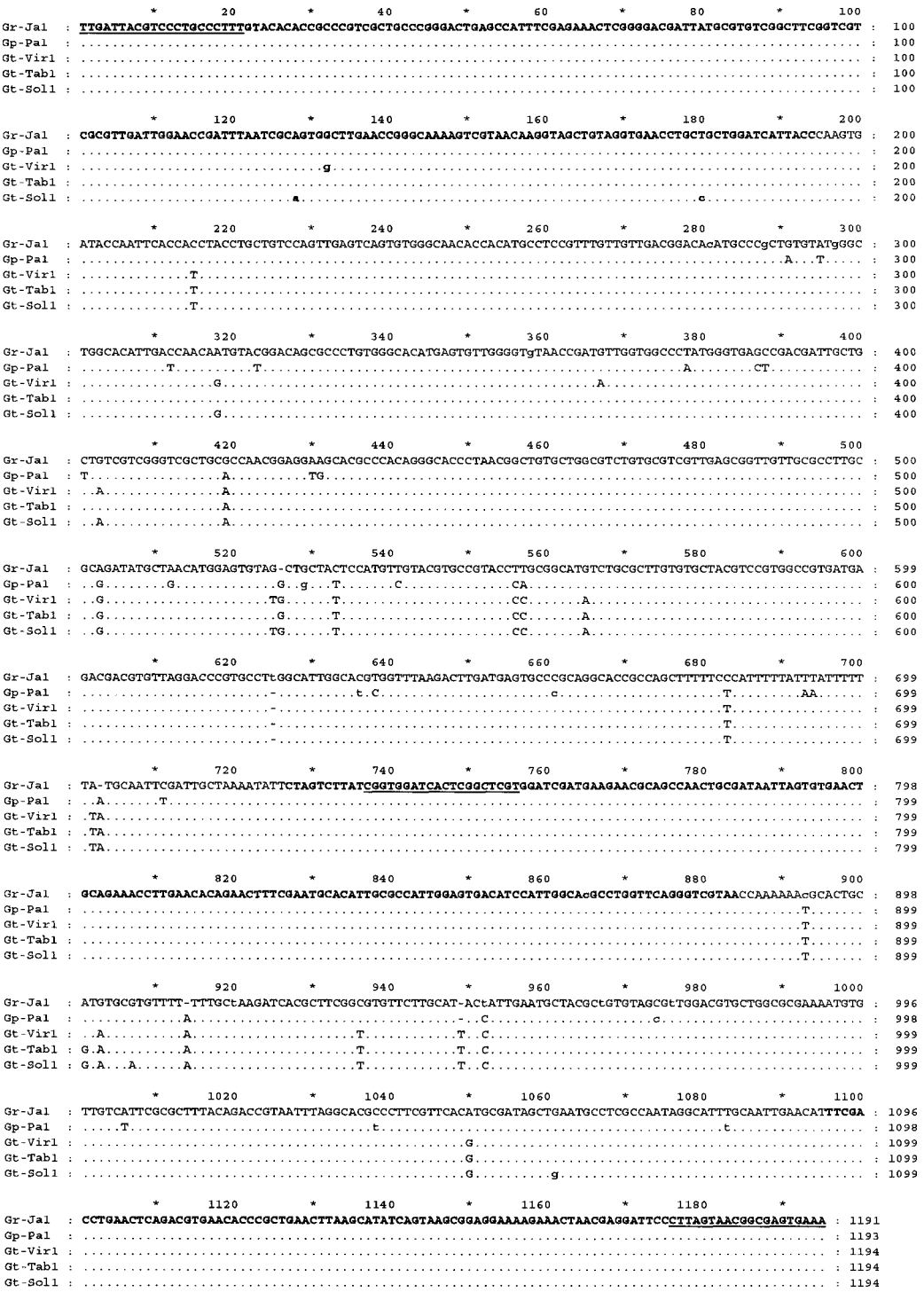

Fig. 1. *Sequences of ITS1, 5.8S and ITS2 and partial sequences of 18S and 28S gene regions from* Globodera rostochiensis *(Gr-Ja1),* G. pallida *(Gp-Pa1),* G. tabacum virginiae *(Gt-Vir1),* G. tabacum tabacum *(Gt-Tab1) and* G. tabacum solanacearum *(Gt-Sol1) (the 18S, 5.8S and 28S rDNA gene sequences are marked in bold and the primer sequences are underlined; heterogeneous nucleotide positions between clones of each species are represented with lower case lettering; individual and alignment sequence lengths are shown).*

Table 3. *Sequence differences between clones from studied* Globodera rostochiensis *(Gr) populations from Russia and UK.*

ITS1 and ITS2 types	Clones	ITS1					5.8S	ITS2					
		281	288	297	358	624	863	891	912	918	949	964	973
Aa	Ga1, Tu1, Ro1	C	G	T	G	–	C	T	A	A	T	T	T
Bb	Ga2, Kh1, Ja1, Ja2, Ro3	C	G	G	G	T	C	C	–	T	T	T	T
Cc	Ga3, Kh2, Tu2	T	A	G	A	–	T	T	A	T	C	G	G
Cb	Ro2	T	A	G	A	–	C	C	–	T	T	T	T

Original sequences of *G. pallida* and three subspecies of *G. tabacum* had lengths of 1193 and 1194 bp, respectively. Six point mutations, three in ITS1 and three in ITS2, were observed between the five clones of the *G. pallida* population, enabling recognition of three types of ITS regions (Aa, Bb and Cb) (Table 4). One point mutation in the 18S gene (132 position — G/A) between two clones of *G. tabacum virginiae* and three point mutations: two in the 18S gene (128 position — A/T, 180 position C/T) and one in the ITS2 (1060 position — G/A) between two clones of *G. tabacum solanacearum* were found, respectively (Fig. 2). There were no differences between the two clones of *G. tabacum tabacum.*

Sequence characteristics of the ITS regions and the 5.8S gene for *Globodera* species based on analyses of original and published data are given in Table 5. Sequence divergence within *Globodera* species ranged from 0 to 4.47% (Table 5), and that between *C. estonica* and *Cactodera* sp. was 4.06%. The ITS1 region of *Globodera* was less divergent than ITS2 and had relatively higher G + C contents.

RFLP ANALYSIS OF THE ITS REGION

All enzymes cut the PCR product obtained from population Gr-Ga (Fig. 2A). The total length of the fragment sizes produced by *Nde*I and *Sph*I was greater than the size of the amplified PCR product, indicating a heterogeneity of ITS regions present in the genome of this population. Repeated digestions with extended periods suggest that this heterogeneity is not the result of partial digestion. Subsequently, six restriction enzymes, *Sph*I (Fig. 3A), *Sty*I (Fig. 3B), *Bsh*1236I, *Cfo*I, *Sau*3A, *Nde*I (data not shown) were used to test for RFLPs in the PCR product from the other eight *G. rostochiensis* populations. Enzymes *Bsh*1236I, *Cfo*I and *Sau*3A did not show any differences in patterns between populations or any heterogeneity of the ITS regions.

Analysis of digested PCR products separated on high resolution synthetic meltable OligoPrep gels showed that all products from the *G. rostochiensis* populations were partially digested by *Nde*I, *Sph*I and *Sty*I and that heterogeneity in the ITS regions probably occurred in all populations. Positions 281, 624 and 863 lay within *Nde*I, *Sty*I and *Sph*I recognition sites, respectively, which meant that these enzymes would cleave only ITS1 type C, ITS1 type B and ITS2 type c, respectively. However, *Sty*I showed different levels of digestion intensity and this may indicate polymorphism between populations. No restricted fragments were observed in populations Gr-Ka and Gr-Vl and the intensity of digested fragments was much less for Gr-Tu, Gr-Ga and Gr-Ro, than for Gr-Sm, Gr-Ja, Gr-Ps and Kh (Fig. 3B). Thus, RFLP data showed that, in the mixture of different ITS types for the populations of *G. rostochiensis*, at least ITS1 types C and A or B and ITS2 types c and a or b were present.

Table 4. *Sequence differences between clones from a* Globodera pallida *(Gp) population from UK.*

ITS1 and ITS2 types	Clones	ITS1			ITS2		
		529	635	660	972	1036	1081
Aa	Pa1	G	T	C	C	T	T
Bb	Pa2, Pa3	G	C	T	T	C	C
Cb	Pa4, Pa5	A	C	T	T	C	C

Eight restriction enzymes (*Alu*I, *Bsh*1236I, *Cfo*I, *Hin*fI, *Nde*I, *Rsa*I, *Sau*3A and *Sph*I) were used to study the PCR product from the UK population of *G. pallida.* All the enzymes, except for *Nde*I and *Sph*I, cut the product (Fig. 2B). Each of these eight enzymes generated patterns which clearly distinguished *G. rostochiensis* from *G. pallida.* Enzymes *Bsh*1236I and *Rsa*I produced additional weak bands, indicating heterogeneity of the ITS regions. Repeated digestions with extended periods suggest that this heterogeneity is not the result of partial digestion.

PHYLOGENETIC ANALYSIS

The *g*1 statistics calculated from 10 000 random trees for the ITS1 and entire ITS alignments indicated that the

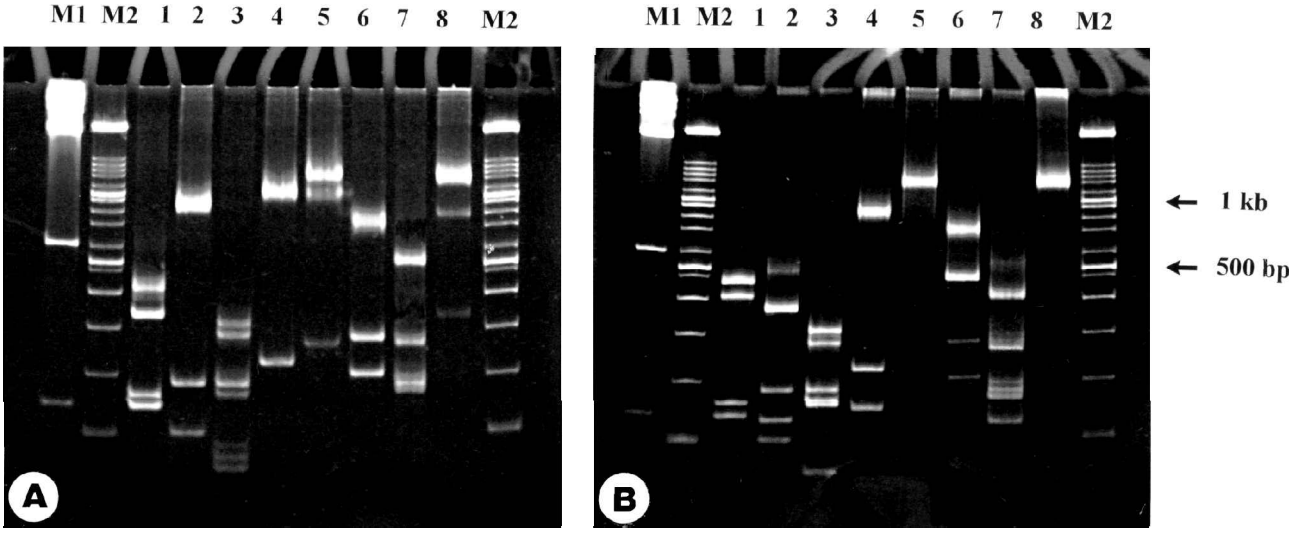

Fig. 2. *Restriction fragments of amplified ITS regions digested by eight restriction enzymes for PCN. A:* Globodera rostochiensis *(Gr-Ga); B:* G. pallida *(Gp-Pa). (M1 and M2: markers; 1:* Alu*I; 2:* Bsh*1236I; 3:* Cfo*I; 4:* Hinf*I; 5:* Nde*I; 6:* Rsa*I; 7:* Sau*3A; 8:* Sph*I).*

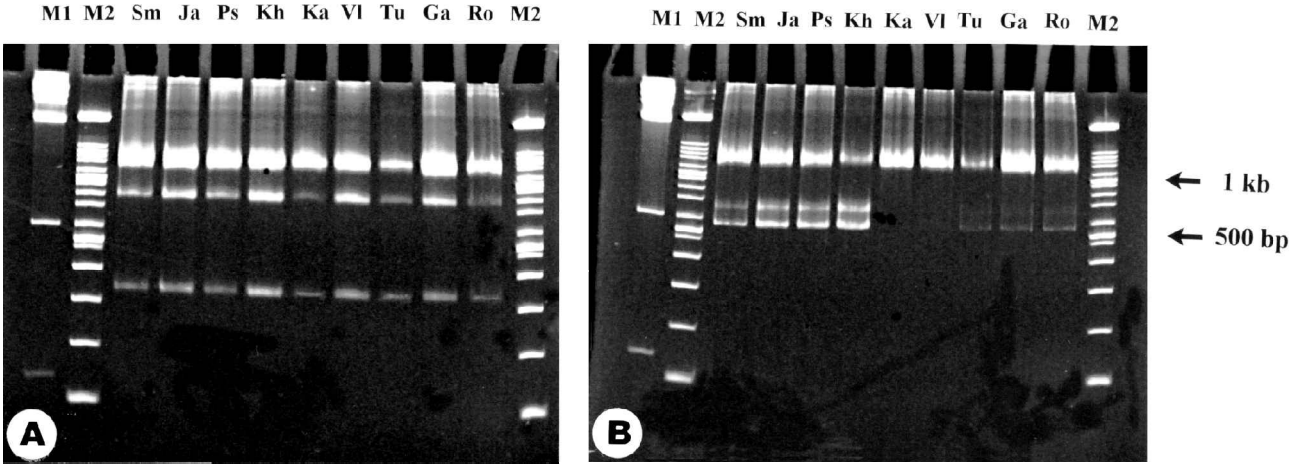

Fig. 3. *Amplified PCR products from nine* Globodera rostochiensis *populations digested by two enzymes. A:* Sph*I; B:* Sty*I. (M1 and M2: markers; see Table 1 for population codes).*

tree length distribution skewed to the left (negative value), suggesting that there is a statistically significant amount of phylogenetic signals in these data sets (Hills & Huelsenbeck, 1992). Four moderate and highly bootstrap supported main clades: *G. rostochiensis*, *G. tabacum*, *G. pallida* and *Globodera* sp., were observed in trees obtained from maximum parsimony and distance analyses of the entire ITS region and ITS1 alignments (Figs 4, 5). Sequence of the population originally named as *G. virginiae* (Cac-VIR) was clustered with high support with *C. estonica* indicating that it is an unidentified species of the genus *Cactodera.* Relationships between populations of *G. pallida* were poorly resolved, however; four populations (Gs-Per2, Gs-P5A, Gs-Per3 and Gs-Per1), originally identified as *G. pallida*, formed a separate cluster with high bootstrap support (Fig. 5) and are considered here to belong to a representative of an undescribed *Globodera* species. In some parsimonious trees, Gp-Hal, Gp-PAL and Gp-P4A formed a cluster outside the other *G. pallida* or with *Globodera* sp. clade with low bootstrap support. Several populations (Gt-X14, Gt-X16, Gt-Mex) previously named as *Globodera* sp. clustered with moderate and high

Table 5. *Sequence characteristics of the ITS region and 5.8S gene alignments of* Globodera *and an outgroup taxon.*

Parameter	ITS1 region*	5.8S gene**	ITS2 region**	Entire sequence**
Length, bp	530 ± 2.9 (521-533)	158	210 ± 0.8 (209-213)	897 ± 3.8 (888-901)
Aligned length, bp	540	158	214	911
G + C content, %	54.3 ± 0.5 (52.8-56.2)	49.2 ± 0.5 (47.4-49.3)	47 ± 0.6 (46.0-49.0)	51.6 ± 0.4 (50.7-52.9)
Sequence divergence within *Globodera* species, %	3.13 ± 1.68 (0.0-5.85)	0.059 ± 0.18 (0.0-0.63)	2.94 ± 1.23 (0-5.74)	2.51 ± 1.24 (0-4.37)
Sequence divergence within *G. rostochiensis* populations, %	0.72 ± 0.36 (0.0-1.54)	0.25 ± 0.33 (0.0-0.63)	1.82 ± 0.95 (0.0-3.35)	0.85 ± 0.39 (0.33-1.58)
Sequence divergence within haplotypes of a single *G. rostochiensis* population, %	0.50 ± 0.29 (0.19-0.75)	0.32 ± 0.35 (0.0-0.63)	1.27 ± 0.78 (0.0-1.91)	0.74 ± 0.36 (0.33-1.02)
Sequence divergence within *G. pallida* populations, %	1.18 ± 0.7 (0.19-2.82)	0	1.42 ± 0.90 (0.0-3.35)	1.02 ± 0.56 (0.11-1.79)
Sequence divergence within haplotypes of a single *G. pallida* population, %	0.38 ± 0.19 (0.19-0.56)	0	0.95 ± 0.82 (0.0-1.43)	0.44 ± 0.29 (0.11-0.67)
Sequence divergence within *G. tabacum* populations, %	0.39 ± 0.27 (0.0-0.95)	0 0	1.72 ± 1.07 (0-3.33)	0.67 ± 0.33 (0-1.34)
Sequence divergence within *Globodera* sp. populations, %	0.28 ± 0.19 (0-0.56)	0	–	–
Sequence divergence between *Globodera* spp. and *Cactodera estonica*, %	16.63 ± 0.32 (16.09-17.31)	2.53 ± 0.13 (2.53-3.16)	15.25 ± 1.26 (13.33-18.09)	13.72 ± 0.32 (13.03-14.32)

*analysis included 41 sequences; **analysis included 24 sequences.

level of bootstrap support with the *G. tabacum* complex and are considered here as *G. tabacum*. Relationships between subspecies of *G. tabacum* complex were poorly resolved after analysis of the entire ITS and ITS1 region sequences, except for the position of *G. tabacum tabacum* population. Genetically *G. rostochiensis* populations were closer to the *G. tabacum* complex than to *G. pallida*.

Discussion

Utility of rDNA for diagnostics and phylogeny of *Globodera* species

Nucleotide sequences of the D3 expansion region of 28S rDNA corresponding to positions 3304-3648 in *Caenorhabditis elegans* (Ellis *et al.*, 1986) are available for some nematode species. This region was successfully amplified from formalin-fixed nematodes and could be useful for nematode identification (Thomas *et al.*, 1997). Although this region is specific at the species level in the genus *Pratylenchus*, for example, and may be used for species identification (Al-Banna *et al.*, 1997; Duncan *et al.*, 1999), it is unlikely to be useful for the identification of *Globodera* species parasitising solanaceous plants as it is highly conserved.

One of the potential dangers in using the multigene families such the rDNA (ITS region) for diagnostics and phylogenetic analysis could be the presence of nonhomogenized paralogues of these genes at different loci. It is clear from the present study, that several haplotypes coexist within the genome of a *G. rostochiensis* population. The mixture of three ITS1 types and three ITS2 types could be present within the genome of a population. ITS microheterogeneity was already revealed within several clones of the Lincoln population of *G. rostochiensis* from New Zealand (Bulman & Marshall, 1997). Out of 15 point mutations observed in the clones of the Lincoln population, 11 corresponded to mutations observed in populations used in the present study. If we apply the ITS type classification used here then, according to published sequence data for 12 clones, the Lincoln population contains ITS type Cc in six clones, Ab and Cb each in one

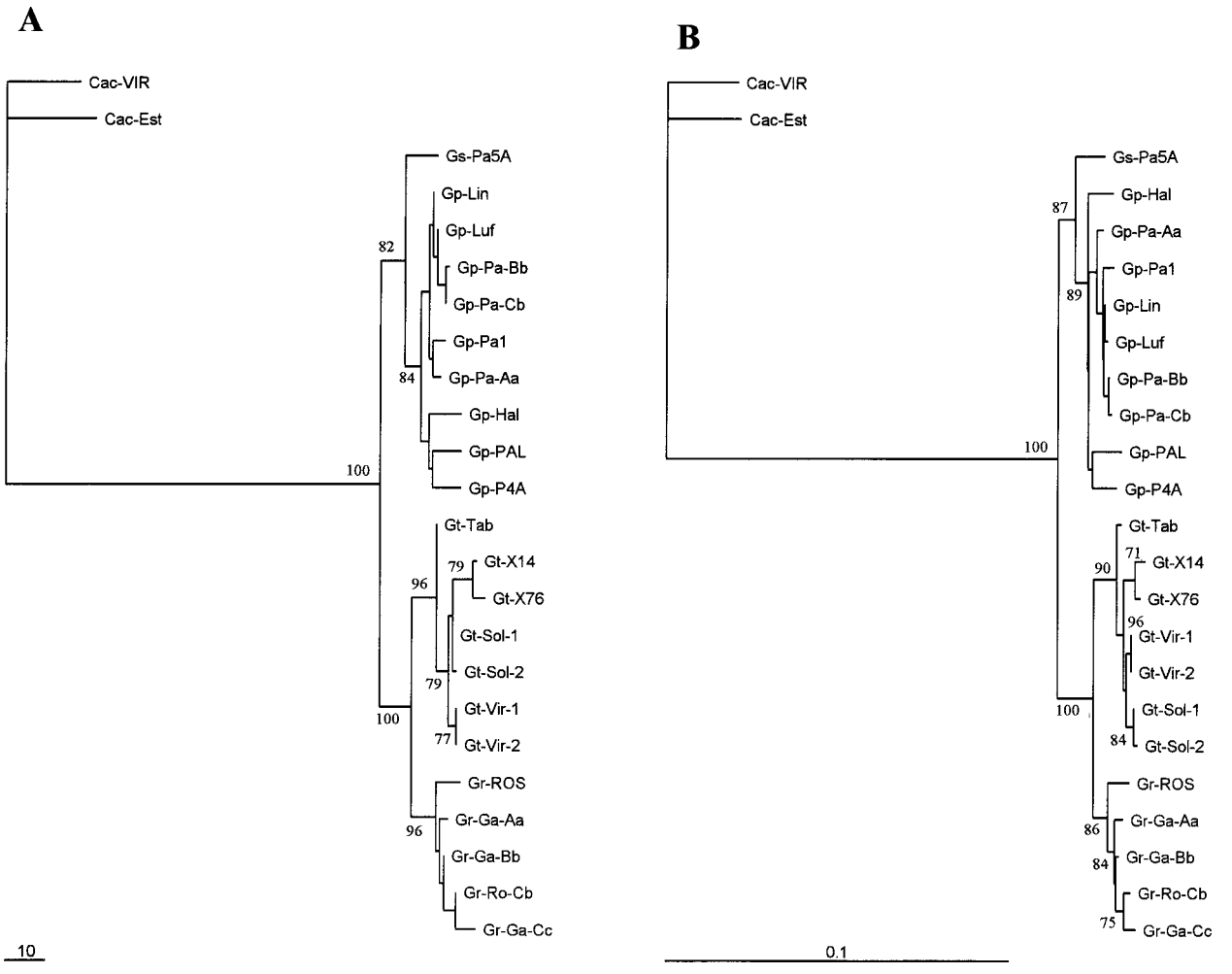

Fig. 4. *Phylogenetic analysis of the entire ITS region sequences from 22 populations of* Globodera *and two populations of* Cactodera *species. A: One of the 79 most parsimonious trees with a length of 242 (number of parsimony informative characters = 131; CI = 0.8306; HI = 0.1694; RI = 0.8930; RC = 0.7417;* g1 = − *1.2650) constructed using PAUP (4.0 beta version) program; B: Neighbour-joining tree constructed using using PAUP (4.0 beta version) program with LogDet method. Bootstrap values below 70% are not given.*

clone and four other clones contained at least one additional mutation in the ITS1 or ITS2 regions.

The *Sty*I restriction of the ITS region showed that different populations of *G. rostochiensis* may contain different ratios of ITS types. From nine possible ITS combinations, only five were recognized in this present study and the study by Bulman and Marshall (1997); two of them, ITS types Ab and Cb, were rarely found. However, it is not possible to be conclusive about the real ratio of haplotypes in this genome. PCR is considered to amplify accurately genotypic differences quantitatively but, as some investigations of ITS regions of aphids showed, PCR conditions could have an influence on the amplification of different ITS haplotypes (Fenton *et al.*, 1998).

Several haplotypes (three ITS1 types and two ITS2 types) were revealed from the genome of one *G. pallida* population and the results of the RFLPs showed that other haplotypes could be present. The positions of six point mutations obtained from five clones of *G. pallida* corresponded to position sequence differences between pairs of clones or populations of this species as published by Blok *et al.* (1998).

ITS heterogeneity has been reported within many nematode genera and species, *e.g.*, *Meloidogyne* (Zijlstra *et al.*, 1995; Hugall *et al.*, 1999); *Belonolaimus* (Cherry

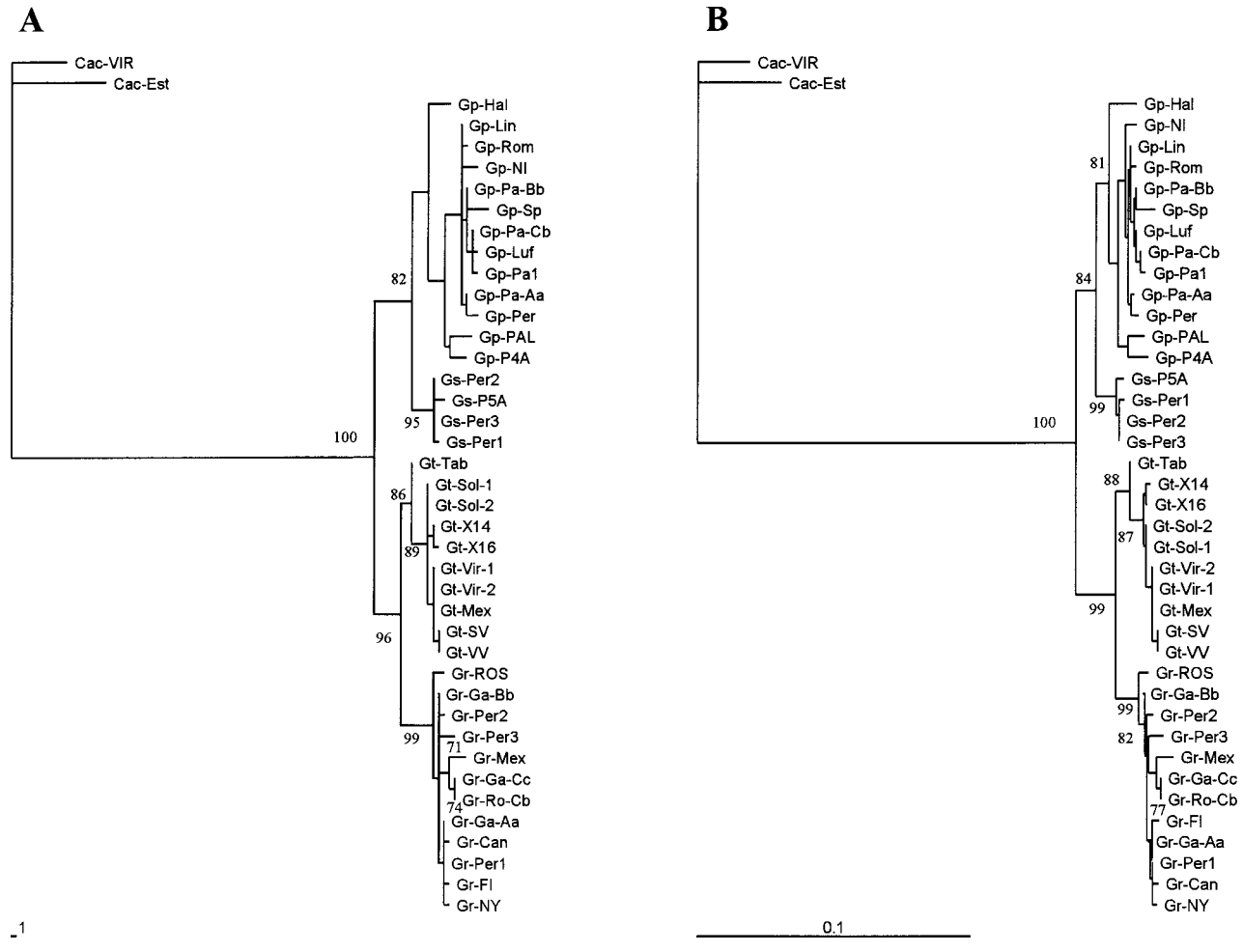

Fig. 5. *Phylogenetic analysis of the ITS1 region sequences from 39 populations of* Globodera *and two populations of* Cactodera *species. A: One of the 312 most parsimonious trees with a length of 180 (number of parsimony informative characters = 95; CI = 0.8611; HI = 0.1389; RI = 0.9483; RC = 0.8166;* g1 = – *0.4888) constructed using PAUP (4.0 beta version) program; B: Neighbour-joining tree constructed using using PAUP (4.0 beta version) program with LogDet method. Bootstrap values below 70% are not given.*

et al., 1997), *Heterodera zeae* (Szalanski *et al.*, 1997), and according to our unpublished data this heterogeneity is also observed in some populations of cyst-forming nematodes. ITS heterogeneity reflects the result of evolutionary interactions between populations and between species. The multiple copies of rDNA are considered not to evolve independently, but to be homogenized in the process of concerted evolution (Hillis & Dixon, 1991). Blok *et al.* (1998) proposed that the results of their work on the comparison of *G. pallida* sequences would indicate that in many British *G. pallida* populations the process of homogenization to a uniform repeat type is still incomplete, and small numbers of repeat types are present which could have arisen from hybridisation in their ancestry between individuals with distinct ribosomal genotypes. In principle, this explanation could also be applied to our data for *G. rostochiensis.*

Sequence variations in the ITS regions of the rDNA genes in populations of *G. rostochiensis* and *G. pallida* have been studied earlier (Ferris *et al.*, 1995; Szalanski *et al.*, 1996; Thiéry & Mugniéry, 1996; Bulman & Marshall, 1997; Blok *et al.*, 1998; Kushida *et al.*, 1998). Sequence data obtained during our study generally correspond to previously published sequence data. Some differences between sequences may be due to a natural variation within populations. However, artefacts during amplifica-

tion and cloning may cause some variation and this may also explain some dissimilarities between our and certain published data.

The present work confirms results obtained by Thiéry and Mugniéry (1996) that rDNA-RFLPs enables clear differentiation between *G. rostochiensis* and *G. pallida* as well as other *Globodera* species. Polymorphism observed within *G. rostochiensis* populations in restriction patterns produced by some enzymes was due to the presence of additional fragments only.

Thus, rDNA in the genomes of *G. rostochiensis* and *G. pallida* populations is present as a mixture of haplotypes with different sequences; the mechanism supporting such a mixture is not clear. Although our results showed rather substantial sequence diversity within haplotypes, the phylogenetic and sequence analysis revealed that their divergence did not influence the position of species in phylogenetic dendrograms, and RFLP profiles and sequences of the ITS region can be used for identification of *Globodera* species.

Systematic implications

The results of our study support the conclusion by Thiéry and Mugniéry (1996) and Thiéry *et al.* (1997) on the existence of four *Globodera* species, parasites of solanaceous plants. The evolutionary trees estimated by the maximum parsimony and distance analysis revealed four main clades within *Globodera*: *G. rostochiensis*, *G. tabacum*, *G. pallida* and *Globodera* sp. This topology was congruent with the topology of the dendrogram of putative relationships of species from this genus parasitising solanaceous plants constructed on the ITS-RFLP data (Thiéry & Mugniéry, 1996). The clade of *Globodera* sp. corresponded to *G.* "*mexicana*" clade on this dendrogram and is, perhaps, cospecific with this undescribed species. *Globodera rostochiensis* had sister relationships with *G. tabacum*, *G. pallida* was genetically close with *Globodera* sp., in very good accordance with host range grouping of these species and with the hybridisation results (Thiéry *et al.*, 1997).

The sequence and phylogenetic analysis indicated the presence of a separate group of populations (Cs-Per1, Gs-Per2, Gs-Per3, Gs-P5A) close related to *G. pallida*. We consider that these populations belong to an undescribed *Globodera* species. The Gs-P5A population has been characterised using different approaches. Study of genetic variations in PCN using the simple sequence repeat primers (Blok & Phillips, 1995), RAPDs (Blok *et al.*, 1997), the ITS-RFLPs and sequence analysis (Blok *et al.*, 1998) and virulence tests (Phillips & Trudgill, 1998) showed that Gs-P5A population stands apart from other *G. pallida* populations.

Relationships between subspecies of the *G. tabacum* complex were not well resolved in our dendrograms, except for the position of *G. tabacum tabacum*. Close sister relationships between *G. tabacum solanacearum* and *G. tabacum virginiae* are also congruent with results obtained after analysis of this species complex based on 222 RAPD markers (Thiéry *et al.*, 1997). Sequence divergence of populations belonging to the *G. tabacum* complex were less than those from *G. rostochiensis* and *G. pallida*. Thus, our data are in good agreement with results of morphological and morphometrical studies, indicating that these subspecies cannot be identified on the basis of morphology of the second stage juveniles and males, have a continuum of values for the majority of morphometric characters, and are distinguished by minor morphological differences in females and cysts only (Mota & Eisenback, 1993a, b, c).

The species originally identified as *G. virginiae* (Ferris *et al.*, 1995) clustered with *C. milleri* and *C. weissi* with high bootstrap support in a phylogenetic tree obtained using the PAUP analysis of rDNA-ITS sequence data by Ferris (1998). In our dendrograms this species formed a clade with *C. estonica* and based on these data we considered it as an unidentified species of the genus *Cactodera*.

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