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Interspecific rDNA restriction fragment length polymorphism in *Globodera* species, parasites of Solanaceous plants

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Summary – The status of the *Globodera* species that are parasites of Solanaceous is still unclear due to low morphological polymorphism. Two 21-mers oligonucleotide primers were used to amplify the Internal Transcribed Spacer (ITS). The amplified product was aproximately 1.2 Kb in the 26 *Globodera* populations examined. Thirty-one restriction enzymes were tested, of which twelve produced some fragment length polymorphism. These RFLP's were recorded, genetic distances were calculated, and a dendrogram was generated. Intraspecific polymorphism was low, but interspecific polymorphism allowed for easy species differenciation. The four revealed clusters corresponded to *G. tabacum* group, *G. rostochiensis, G. pallida,* and *G. "mexicana"*. These results confer a species status to *G. "mexicana"* whose genetic identity was unclear and are discussed with regard to previous hybridisation and to 2D electrophoresis. Further sequencing of the ITS was done to search for intraspecific polymorphism. These three *G. rostochiensis* sequences were compared to previously published sequences for five *Heterodera* populations and for *Caenorhabditis elegans.*

Résumé – ***Polymorphisme interspécifique de fragments de restriction au niveau de la région non codante des ADNr des* Globodera, *parasites des Solanacées*** – Le statut d'espèce au sein des *Globodera* parasites des Solanacées est encore mal défini dû au faible polymorphisme morphologique détectable. Deux oligomères de 21 bases sont utilisés comme amorces pour amplifier la région non codante des ADN ribosomiques : " Internal Transcribed Spacers " (ITS) par réaction de polymérisation en chaîne (PCR). Le produit d'amplification obtenu mesure 1,2 Kb dans chacune des 26 populations étudiées. Trente-et-un enzymes de restriction sont testées sur les ITS pour la longueur des fragments de restriction; douze ont révélé du polymorphisme. Ces RFLP sont analysés, les distances génétiques sont calculées et un dendrogramme comportant quatre groupes est produit. Le polymorphisme intraspécifique est pratiquement inexistant; cependant le polymorphisme interspécifique apparaît clairement et permet une différenciation aisée des espèces. Les quatre groupes révélés correspondent aux groupes *G. tabacum sensu lato, G. rostochiensis, G. pallida* et *G. « mexicana »*. Ces résultats confèrent un statut d'espèce à *G. « mexicana »* dont l'identité génétique est peu précise. Ces résultats sont comparés à ceux des hybridations et aux résultats d'électrophorèse bidimensionnelle obtenus. Le séquençage des ITS a été réalisé pour rechercher un éventuel polymorphisme intraspécifique. La comparaison des séquences des ITS de trois populations de *G. rostochiensis* et de cinq *Heterodera* est réalisée puis comparée à la séquence publiée de *Caenorhabditis elegans.*

Key-words : cyst nematodes, *Globodera,* nematodes phylogeny, molecular evolution, PCR, ITS-rDNA, sequence.

All the Solanaceous cyst nematode *Globodera* species originate from America. *G. rostochiensis* Woll. and *G. pallida* Stone come from South America and were introduced into Europe after 1850 (Evans *et al.,* 1975). They were differentiated from each other by Stone (1972). These two sibling species develop mainly on tuberous Solanaceae, especially on *Solanum tuberosum.* The species *G. tabacum* (Lownsbery & Lownsbery) and its subspecies *G. t. solanacearum* (Miller & Gray) and *G. t. virginiae* (Miller & Gray) originate from the USA. Two populations of *G. tabacum s. lat.* were recently found in France. The *G. tabacum* group does not develop on *S. tuberosum* but it does on non tuberous wild Solanaceae and on *Nicotiana tabacum.* In Mexico, some populations found on wild Solanaceae do not develop on *Solanum tuberosum.* One of these populations was re-ported as *G. "mexicana"** by Campos-Vela (1967).

The specific status of those entities is still unclear. Morphological polymorphism is low between the six *Globodera* species mentioned above, except *G. rostochiensis* (Stone, 1983; Motta & Eisenback, 1993 *a, b, c*). Hybrids have been produced between the six *Globodera* species and their viability and fecundity were studied to identify genetic clusters (Mugniéry, 1979; Mugniéry *et al.,* 1992). A dendrogram was revealed by two-dimensional gel electrophoresis (2-DGE) of total proteins using one population of each of the six species (Bossis & Mugniéry, 1993). The *G. tabacum* group and the *G. rostochiensis* group where clearly separated while the *G. pallida* and *G. "mexicana"* groups were closely related.

RFLP and other direct DNA analytical methods are of proven usefulness in *Drosophila* (Williams *et al.,* 1985) and in helminths (Qu *et al.,* 1986). In nematodes, the small amount of DNA per individual is a limiting factor. The polymerase chain reaction (PCR) is more appropriate for nematode DNA study. Hillis and Dixon (1991) noted that so far spacer regions had been infrequently used for phylogenetic studies, and they emphasised a need to investigate the usefulness of compara-

* *G. "mexicana"* having been produced in a " non published " thesis the name of that species cannot be considered as valid following the International Code of Zoological Nomenclature.

tive studies of rDNA spacers for closely related species and populations. Vrain *et al.* (1992) characterised the rDNA restriction fragment length polymorphism in some species of the *Xiphinema americanum* group. They amplified the internal transcribed spacers (ITS) region with primers originating from a *Xiphinema* genomic library screened with the *Caenorhabditis elegans* rDNA cistron. The same approach was done on the genus *Heterodera* (Ferris *et al.*, 1993). The ITS polymorphism has been exploited by others authors on animals (McLain & Collins, 1988; Fontenille *et al.*, 1993; Vogler & DeSalle, 1994) on plants (Santoni & Bervillé, 1991; Sun *et al.*, 1994) on bacteria (Lu *et al.*, 1994) and on fungi (Ogorman *et al.*, 1994). We have used the same approach, with the primers used by Vrain *et al.* (1992), to verify the earlier results (Mugniéry *et al.*, 1992; Bossis & Mugniéry, 1993) on *Globodera* species cluster. Here, we have used populations from species of *G. rostochiensis, G. pallida, G. virginiae, G. solanacearum, G. tabacum,* and few Mexican populations called *G. "mexicana".*

Materials and methods

Populations

The origins of five *G. rostochiensis,* six *G. pallida,* six *G. "mexicana",* and nine *G. tabacum sensu lato* populations used in this study are listed in Table 1. Populations of *G. rostochiensis* and *G. pallida* were reared on *Solanum t. tuberosum* cv. Desiree, and the other populations were reared on *Lycopersicon esculentum* cv. St Pierre or *Solanum dulcamara,* in a greenhouse.

DNA extraction

Young white or yellow females were extracted from pots by the Kort elutriator, and centrifugated in magnesium sulphate (density 1.23). Females were hand picked under a dissecting microscope, washed, and aliquots of 250 mg were stored at – 70 °C. Genomic DNA was isolated according to Folkertsma *et al.* (1994). An extraction with 6M NaCl solution was done to precipitate the majority of proteins. RNA was removed by incubation with 20 µg of RNase A at 37 °C for 30 min. The DNA concentration was estimated on agarose gels. At this stage, the DNA was pure enough for amplification experiments.

PCR

Two oligonucleotide sequences isolated by Vrain *et al.* (1992) were used to amplify the ITS region. These sequences positioned respectively on the 18S and the 26S genes allowed amplification of ITS1, ITS2, and 5.8s gene (Fig. 1). The amplification reaction mixture contained 10 mM TrisHCl pH 9, 50 mM KCl, 2 mM $MgCl_2$, 0.1 % Triton X100, 0.02 % gelatine, 100 µM of each dNTPs, 0.5 µM of each primer, approximately 50 ng of *Globodera* DNA, 0.5 unit of Taq DNA polymerase (Cat. No : 120181, Appligene, Illkirch-France), and deionized water to a final volume of 50 µl. A negative control without DNA was included. Amplification was performed in a Perkin Elmer Cetus DNA Thermal Cycler 480 programmed for 30 cycles of denaturation step at 94 °C for 1 min, annealing at 60 °C for 50 s, and polymerisation at 72 °C for 1 min.

Table 1. *Origin and host range of the 26 populations of* Globodera.

Species	Location	Principal host and (pathotype)
G. rostochiensis	Scotland (GB)	potato (Ro1)
	Noirmoutier (F)	potato (Ro1)
	Castellane (F)	potato (Ro1)
	Sedan (F)	potato (Ro1)
	Mierenbos (NL)	potato (Ro1)
G. pallida	Guiclan (F)	potato (Pa2/3)
	Saint-Malo (F)	potato (Pa2/3)
	Chavornay (CH)	potato (Pa2/3)
	Pas-de-Calais (F)	potato (Pa2/3)
	HPL1 (NL)	potato (Pa2)
	Duddingston (GB)	potato (Pa1)
G. "mexicana"	Tlaxcala (ME)	wild solanum
	Santa-Ana (ME)	wild solanum
	GM3 (ME)	wild solanum
	GM4, Huamantla (ME)	wild solanum
	GM5, Huamantla (ME)	wild solanum
	GM6, Santa-Ana (ME)	wild solanum
G. tabacum	Aiguillon (F)	tobacco
	Agen (F)	tobacco
	Connecticut (USA)	tobacco
G. virginiae	GV1 (USA)	tobacco
	GV2 (USA)	tobacco
	GV3, Crutchlow (USA)	tobacco
G. solanacearum	GS1, Watkins (USA)	tobacco
	GS2 (USA)	tobacco
	GS3 (USA)	tobacco

Populations GM3, GM4, GM5, GM6, GV1, GV2, GV3, GS1, GS2, GS3 and GT Connecticut are from L. Miller collection (University of Virginia, USA). GB : Great Britain; F : France; CH : Switzerland; NL : Netherlands; ME : Mexico; USA : United States of America.

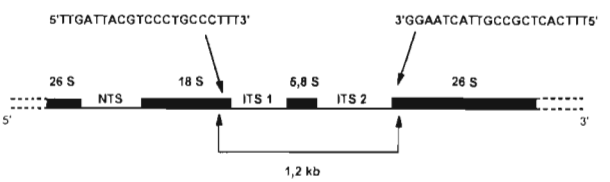


Fig. 1. *Primers localisation. (The arrows indicate the amplified region. Sequences represent the two primers. ITS = Internal Transcribed Spacers. 18 S, 5.8 S and 26 S are coding rDNA genes).*

Restriction fragment length polymorphism

The product of amplification was digested with one of 31 restriction enzymes (Boehringer, Eurogentec), overnight in the recommended buffers. The enzymes used

are listed in Table 2. The DNA fragments generated were separated by electrophoresis in a 1.5 % agarose gel (1/4 Nusieve GTG low melting temperature agarose; 3/4 agarose Ultra pure) in Tris-borate/EDTA (TBE) at 5.5 V/cm. The markers used are bacteriophage λ Hind III/EcoR I, and pBR322/Hae III (Appligene). Gels were stained with ethidium bromide (0.5 μg/ml) and photographed under UV light (312 nm). For each nematode population, the bands obtained with the cutting enzymes were recorded as a binary matrix of 0 and 1 corresponding to the absence or presence of individual bands. The matrix was given as input data to the Phylogeny Inference Package (Phylip, version 3.5 c) program. Gendist was the Philip program used to compute the genetic distances of Nei (Nei & Li, 1979). The cluster analysis by the unweighted pair group method with arithmetic mean (UPGMA) was performed by Neighbor, and finally the tree was constructed by Drawgram (Phylip programs).

Table 2. *Polymorphism and number of fragments generated by restriction enzymes on ITS, on the 26 populations of* Globodera.

Enzyme	Polymorphism	Number of fragments generated
Acc I	–	0
Alu I	+	7
Apa I	–	0
Bam HI	–	0
Bgl I	–	2
Bsh 1236I	+	8
Bsh 1285I	–	0
Dde I	+	2
Dra I	–	0
Eco RI	–	0
Eco RV	–	0
Hae III	–	5
Hha I	–	6
Hinc II	–	2
Hind III	–	0
Hinf I	+	4
Hpa II	–	0
Ita I	+	3
Mae II	+	3
Mae III	+	6
Nde II	+	6
Pst I	+	3
Pvu II	–	0
Rsa I	+	4
Sau 3 AI	+	8
ScrF I	–	4
Sfu I	–	2
Ssp I	–	2
Taq I	+	6
Vsp I	–	0
Xba I	–	0

SEQUENCING

400 bp of the two ITS extremities : the 5′ end of ITS2 and the 3′ end of ITS1 were sequenced using a Taq DyeDeoxy™Terminator Cycle Sequencing Kit developed specifically for the preparation of samples for sequence analysis on the Applied Biosystems Model 373A DNA sequencing System (automated sequencer, INRA, Jouy-en-Josas, France).

The ITS of only three *G. rostochiensis* populations (Écosse, Noirmoutier and Castellane) were sequenced. These sequences were compared with nine *Heterodera* and *C. elegans* ITS sequences according to Ferris *et al.* (1993).

Results

The yield of DNA extraction averaged 20 μg of DNA per aliquot of 250 mg (approximately 6 000 females) and DNA quality was satisfactory (Fig. 2). The RNA were situated at lower molecular weights than DNA.

The amplification of the ITS region of each *Globodera* population gave one fragment of approximately 1.2 kb. A few light bands – products of non specific amplifications – were obtained in some cases but they did not interfere in the restriction analysis (Fig. 3). No PCR

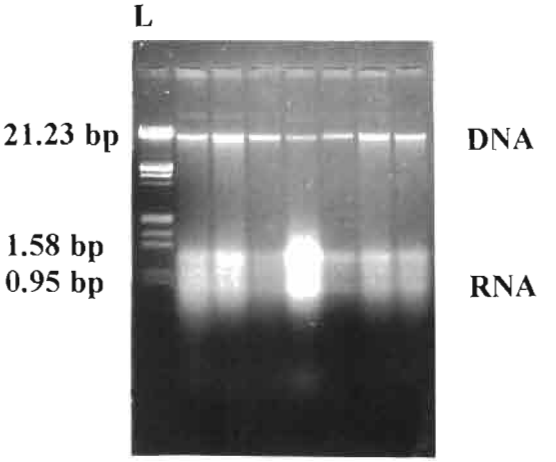


Fig. 2. *Samples of total genomic DNA from randomly chosen* Globodera *spp. populations (see text) (gel : 0.8 % TAE; migration : 10 V/cm; L : ladder, bacteriophage λ cutted by EcoRI and HindIII).*

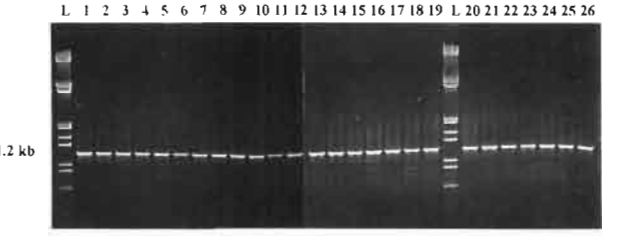


Fig. 3. *Amplified Internal Transcribed Spacers region of the 26* Globodera *populations. (Ethidium bromide stained 1 % agarose gel. L : DNA marker, λ HindIII/EcoRI; 1 to 26 populations of* Globodera *as in Table 1).*

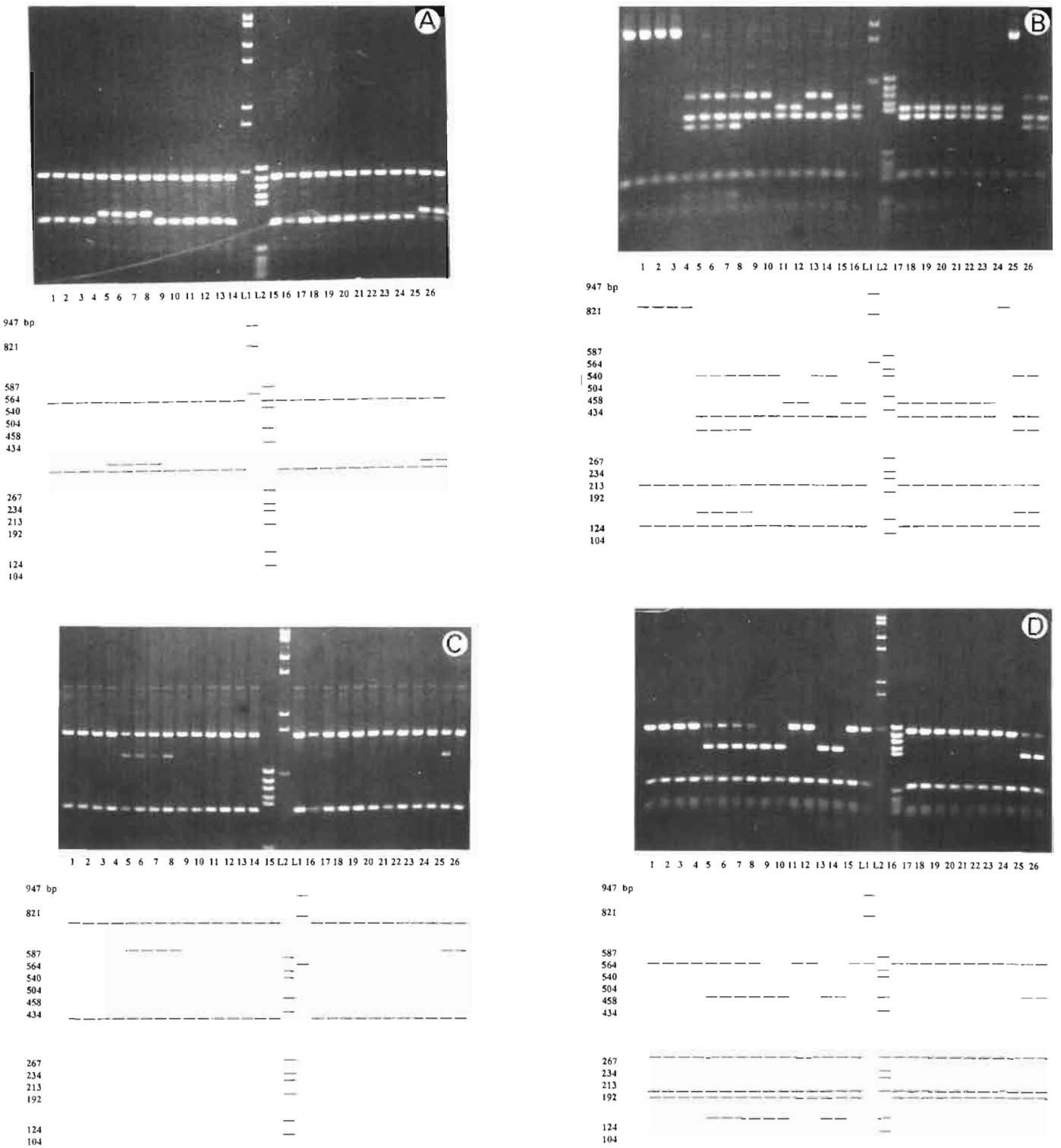
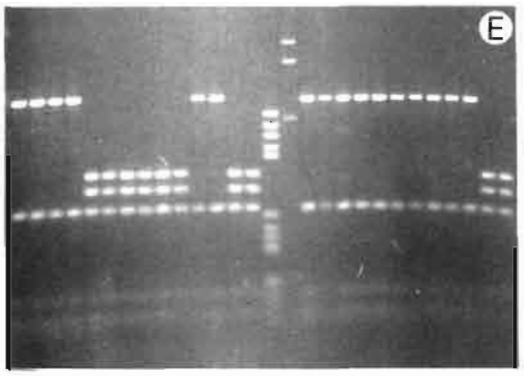
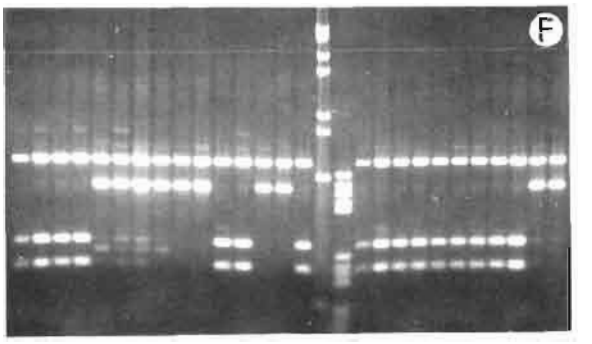
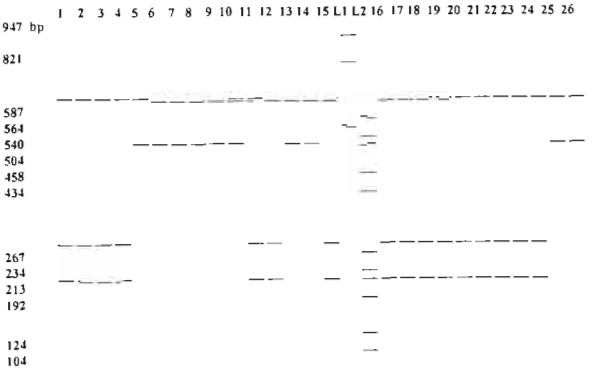
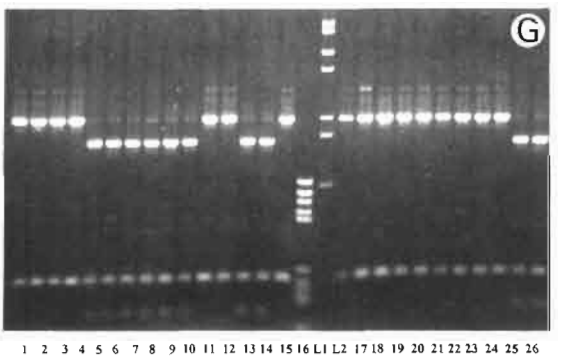


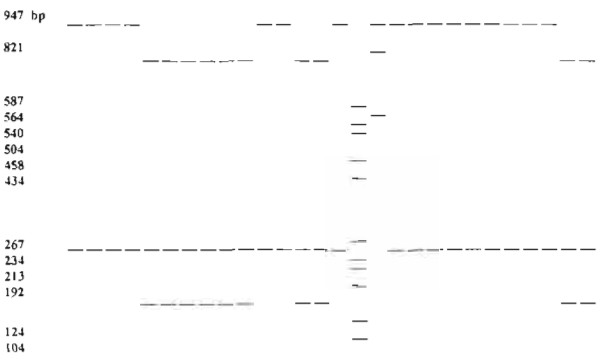
Fig. 4. *Restriction fragments of amplified Internal Transcribed Spacers of* Globodera *species. A : MaeII; B : Bsh1236I; C : PstI; D : NdeII; Tlaxcala, Santa-Ana, GM3, GM4, GM5, GM6, Aiguillon, Agen, Connecticut, GV1, GV2, GV3, GS1, GS2, GS3, Mierenbos, HPL1, [NuSieve GTG : 50 %] gel. Non informative top gels were not represented in the diagrams).*











E : TaqI; F : RsaI; G : HinfI. Lanes 1 to 26. Ecosse, Noirmoutier, Castellane, Sedan, Guiclan, Saint-Malo, Chavornay, Pas-de-Calais, Duddingston. L1 : DNA marker, λ HindIII/EcoRI; L2 : DNA marker, pBR322/HaeIII (Ethidium bromide stained 1.5 % agarose

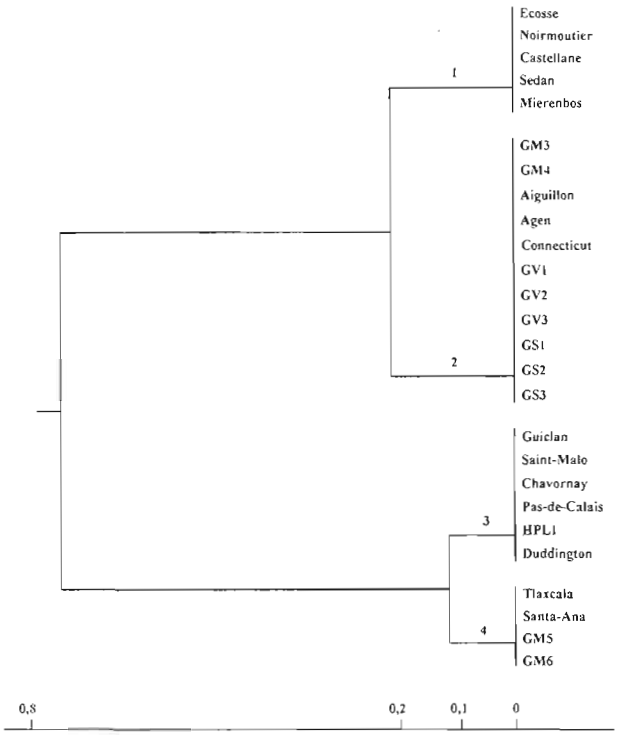


Fig. 5. *Similarity clusters of* Globodera *parasites of Solanaceous plants built by average linkage using the unweighted pair group method with arithmetic mean (UPGMA) in the PHYLIP program (Branch length corresponds to genetic distances).*

products were obtained in the negative control lacking DNA template.

Only 12 of the 31 tested restriction enzymes used separately to digest the amplification product generated polymorphism. Seven enzymes cut the ITS fragment without generating any polymorphism and twelve did not cut the fragment (Table 2). Not all the bands obtained after fragment digestion were scored. Because of the gel nature (agarose), the faint bands with a molecular weight less than 125 bp were not taken into account. In many cases, particular restriction sites were present in all populations studied. The pattern of restriction bands obtained with Alu I, Mae III, and Dde I clearly distinguished the *G. rostochiensis* group from other groups, and Nde II distinguished the *G. "mexicana"* group from others. Pst I and Mae II distinguished the *G. pallida* group, while Bsh 1236 I separated all groups (Fig. 4 A, B, C). The 60 scored fragments, represented in the diagrams, were obtained with twelve enzymes (Fig. 4 D-G), and their presence or absence allowed the construction of a matrix. All the populations of a particular species showed the same pattern. This absence of intraspecific polymorphism allowed the clustering of the populations depending on the species to which they belong. The Phylip program allowed the grouping of the 26 populations into four clusters (Fig. 5). Cluster 1 contained the five *G. rostochiensis* populations. Cluster 2 contained the nine *G. tabacum sensu lato* and two *G. "mexicana"* populations. Cluster 3 contained the six *G. pallida* populations and cluster 4 contained four *G. "mexicana"* populations.

The ITS sequencing revealed very little intraspecific polymorphism between the 3 *G. rostochiensis* populations tested. In ITS1, five base pair differences were found in the extremities of the sequenced part (Fig. 6 A). Eight base differences were found in ITS2 : four are distal and four are positioned at the base 144, 217, 282, and 339, respectively (Fig. 6 B). It was noteworthy that the coding 3′ end of the 26 S gene is more variable than the coding 5′ end of the 18 S gene, in three *G. rostochiensis* populations, as was already found in *Globodera, Heterodera* and *Caenorhabditis elegans* sequences (Fig. 6 A, B).

Discussion

Ribosomal DNA is often present in many copies in the genome of many organisms, and represents an excellent target for amplification when only small amounts of nematodes are available. The ITS fragment size amplified averaged 1.2 kb in *Globodera* species parasites of Solanaceous plants. The same size was found in *Heterodera* (Ferris *et al.,* 1993), while it was of 1.5 kb in *Xiphinema* (Vrain *et al.,* 1992), 1.4 kb in *Caenorhabditis elegans* (Ellis *et al.,* 1986), and only 1 kb in *Heterorhabditis* (Joyce *et al.,* 1994), when the same primers were used.

If the choice of the 5.8s gene and ITS fragment for the restriction polymorphism analysis proved to be judicious to separate species and populations of the *Xiphinema americanum* group (Vrain *et al.,* 1992), it was not variable enough to distinguish intraspecific *Globodera* populations. The analysis of the band patterns obtained after *Globodera* ITS fragment digestion clearly showed no intraspecific polymorphism, which allowed grouping the populations of one species into a single Operational Taxonomic Unit (OTU). We noted the absence of polymorphism among *G. pallida* populations which, however, comprise three pathotypes : Pa_1 (Duddingston), Pa_2 (HPL1), and $Pa_{2/3}$ (others). The absence of polymorphism could be due to a too small number of restriction enzymes tested or to the nature of the sequence itself. The comparison between the *C. elegans* (Ellis *et al.,* 1986) and the obtained *Globodera* rDNA sequences showed that the amplification product included 79 nucleotides at the 5′ end of the 26s gene, and 170 nucleotides at the 3′ end of 18s gene. We sequenced 440 bp at each extremity of the amplified fragment to be sure to analyse a portion of the less conserved ITS itself, and not only the conserved coding sequences. The sequencing of the fragment among three *Globodera rostochiensis* populations : Écosse, Noirmoutier and Castellane revealed only two significant differences in the ITS-2. The

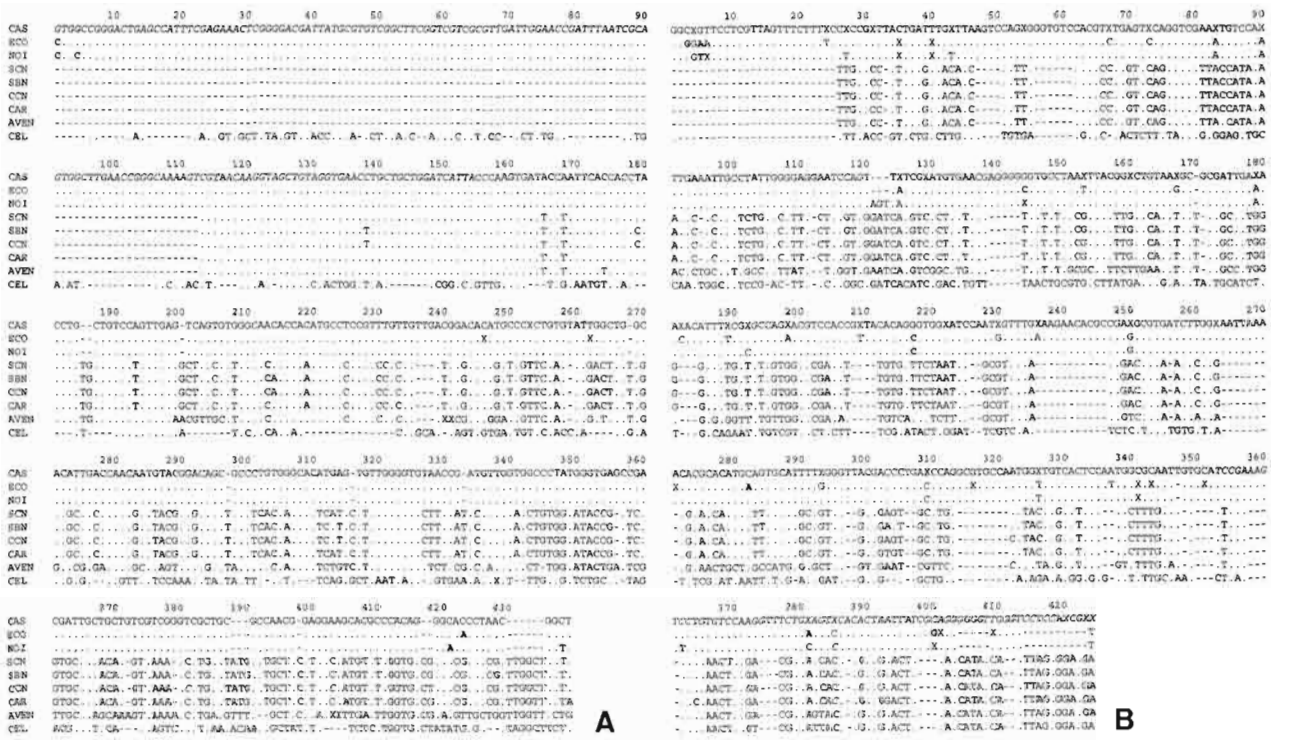


Fig. 6. *Alignment of ITS rDNA sequences. A : Alignment of 18S-ITS1 rDNA sequences; B : Alignment of 26S-ITS2 rDNA sequences. (*Globodera rostochiensis *populations used : CAS = Castellane, ECO = Ecosse, NOI = Noirmoutier;* Heterodera *species used : SCN* = H. glycines, *SBN* = H. schachtii, *CCN* = H. trifolii, *CAR* = H. carotae, *AVEN* = H. avenae. *CEL* = Caenorhabditis elegans. Globodera *sequences are longer than those of* Heterodera *and* Caenorhabditis. *(–) = absence of a base,* i.e., *deletion or shorter sequences. X = undetermined base. Italicised bases are located in the 18S or in the 26S genes. Bold bases represent differences between* Globodera *sequences).*

other differences found in both ITS are too distal or too ambiguous to be taken into account.

The three *G. rostochiensis* ITS sequences were aligned with five *Heterodera* and *C. elegans* ITS sequences. This alignment verified that the DNA fragment we amplified was really rDNA-ITS. Furthermore, it also verified the little polymorphism present among the three *G. rostochiensis* sequences. The *Heterodera* sequences (represented by SCN-Posey, SBN, CCN, and *H. carotae*) were not very polymorphic exept for *H. avenae* which is more distinct (Ferris *et al.,* 1993).

The low polymorphism found in the 3′ end coding part of 26 S gene, absent in 5′ end coding part of 18 S gene was remarked on *Rana* too by Hillis and Davis (1986). These authors found that the 1000 bp from the 3′ end of the 28 S gene of *Rana* contained polymorphism, while this is not the case in the 18 S gene.

The absence of intraspecific variation was found in the ITS-2 sequence of *Trichostrongylus* (Hoste *et al.,* 1993); the authors concluded that this region of rDNA is inadequate to discriminate between populations of *T. colubriformis,* but that it may prove useful for distinguishing between species. The *Heterodera avenae* rDNA ITS sequences were found to be highly conserved among all of the *H. avenae* isolates by Ferris *et al.* (1994), just as they earlier found them to be conserved among species of the *Heterodera schachtii* group (Ferris *et al.,* 1993).

Some intraspecific polymorphism seems to be revealed in *G. pallida* populations when ITS fragment is cut by Rsa I. But the polymorphic fragments, not well revealed by the technique, appeared as faint bands. Those changes that do occur may not be incorporated into a large number of repeating units. Then this low polymorphism may be intra-population and even may be present at the individual level between the repeated units of the rDNA tandem array. The same observation was made on *Caenorhabditis* species by Files and Hirsh (1981). The large differences between *C. elegans* and *C. briggsae* spacer sequences contrasted with the homogeneity of spacer sequences among the repeating units within each species. The 55 repeating units of *C. elegans* could not have each independently evolved vertically to identical sequences. They suppose there must be a mechanism of horizontal evolution driving the tandem array to homogeneity.

Globodera interspecific polymorphism appeared clearly. The dendrogram revealed four OTU : *G. ros-*

tochiensis, G. pallida, G. "mexicana", and *G. tabacum*. There were two main clusters : one with *G. pallida* and *G. "mexicana"*, and one with *G. rostochiensis* and *G. tabacum*. Surprisingly GM3 and GM4 populations were far from the other *G. "mexicana"* populations. The *G. "mexicana"* OTU was closely related to the *G. pallida* one with a genetic distance of 0.1. The genetic distance between *G. rostochiensis* and *G. tabacum* group was of 0.2, higher than the *G. pallida-G. "mexicana"* distance. When the ITS dendrogram is compared to the dendrogram obtained with the 2D electrophoresis of proteins (Bossis & Mugniéry, 1993) we can see some similarities : *G. pallida* and *G. "mexicana"* are closely related, while the *G. rostochiensis* and *G. tabacum* groups are very distant from the cluster *G. pallida - G. "mexicana"*. However there is a major difference, in that *G. rostochiensis* clusters with the *G. tabacum* group in the ITS dendrogram while this is not the case in the proteins dendrogram.

The genetic proximity of *G. pallida* and *G. "mexicana"* showed by the short genetic distance has been confirmed by *in vitro* hybridisation. Effectively these two OTU can cross and give viable and fecund progeny (Mugniéry *et al.*, 1992), even if cytoplasmic incompatibility occurs. Fertile hybrids were obtained when the male was *G. "mexicana"* GM5 and the female was *G. pallida* Guiclan. However, the crosses between *G. rostochiensis* and *G. tabacum sensu lato* were not viable (Mugniéry *et al.*, 1992). The *tabacum* group could be considered as a species with three subspecies *G. tabacum tabacum, G. t. virginiae* and *G. t. solanacearum*, which can easily cross both ways (Miller, 1983). The two populations GM3 and GM4 from Mexico, at first identified as *G. "mexicana"*, in fact belong to the *G. tabacum* group. *G. pallida* populations do not hybridise with *G. tabacum*. The first results of hybridisation between GM3 or GM4 and the *G. pallida* population Guiclan showed a lack of hybrid viability, confirming that GM3 and GM4 do not belong to *G. "mexicana"*, which can itself hybridise with *G. pallida* Guiclan.

The OTUs correspond mainly to recognized species. All the populations crossing freely both ways always figure in the same OTU. The OTU *G. tabacum* group is an exemple. Therefore, *G. "mexicana"* may be considered as a valid species even if its genetic proximity allows crossing with some populations from other species : males of the *G. "mexicana"* population GM5 hybridise with females of *G. pallida* Guiclan and *G. solanacearum* GS3 (Mugniéry *et al.*, 1992). Species inter sterile in natural conditions but inter fertile in the laboratory are called " borning species " (Blair, 1943). As an example the species of ducks *Anas platyrhynchus* and *A. acuta* belong to the same coenospecies because their first, second and third laboratory generations hybrids are fertile although they do not cross in natural conditions. However, experimental hybridisation of *Tisbe clodiensis* populations (Crustacea, Copepode) produce viable progeny in some cases, none in others cases, or intermediary results (Génermont & Lamotte, 1980). Furthermore, we must be sure when studying hybrids viability and fecundity that the host used to multiply them is appropriate (Miller, 1983).

The amplification and analysis of the ITS region has a lot of advantages : e.g. the rapidity to obtain profiles and the clarity of the results allow easy species identifications. This technique could be of use for quarantine species identification, in fact *G. pallida* and *G. rostochiensis* are classified as quarantine pests. Vrain *et al.* (1994), suggested an international cooperation to build up banks of DNA restriction patterns characteristic of each quarantine nematode species. ITS restriction patterns could be useful to follow and to control *in vitro* and *in vivo* hybridisation knowing that DNA amplification of one individual is possible (Ferris *et al.*, 1993).

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