

Molecular approaches to the taxonomic position of Peruvian potato cyst nematodes and gene pool similarities in indigenous and imported populations of *Globodera*

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Peruvian potato cyst nematode populations were analysed to assess both their inter- and intraspecific similarities. ITS-RFLP and two satellite DNA sequences were used as taxonomic tools. Both techniques have confirmed that the Peruvian populations have as their closest relatives the European *Globodera pallida*, despite the detection of clear differences that prevents us from assigning these South American populations unambiguously to any *Globodera* species. A more precise study of the variability of these Peruvian populations was investigated and they were compared with the imported European populations using protein (2-DGE) and DNA (RAPD) datasets. The clear distinction between the Peruvian and the European populations was confirmed and, inside each group, no correlation was found between the pathotype classification and the observed clustering of the populations. Surprisingly, while RAPDs revealed a higher variability in the Peruvian group than in the European one, some characteristic proteins were found by 2-DGE in some European populations, whereas it was impossible to find any in the Peruvian populations. It is concluded that the primary founders of the European populations may have an origin other than that of the Peruvian populations involved in this study.

Keywords: DNA fingerprinting, *Globodera* spp., imported populations, protein electrophoresis, species determination, tandem repeat sequence.

Introduction

The potato cyst nematodes (PCN) *Globodera rostochiensis* and *G. pallida* are thought to have originated in the Andean region of South America (Evans & Stone, 1977) and to have been introduced into Europe after 1850 (Evans *et al.*, 1975). They are major pests of potato, causing significant economic losses. Plant resistance is an effective means to control PCN, but its wide application is hampered by the occurrence of various virulent nematode populations. Determining whether there has been more than one introduction has practical importance for the management of these pests, since the genetic basis of virulence may differ.

PCNs are classified into pathotypes on the basis of their ability to multiply on different potato clones. However, the status of their pathotypes is still a matter of debate (Phillips & Trudgill, 1983; Mugniéry *et al.*,

1989). The International Pathotype Scheme introduced by Kort *et al.* (1977) recognizes, among the European populations, five pathotypes within *G. rostochiensis* and three within *G. pallida*, while the Latin American Scheme (Canto & Scurrah, 1977) recognizes among the South American populations of these two species four and seven pathotypes, respectively. As outlined by Bakker *et al.* (1993), these schemes are not able to reflect all of the genetic diversity introduced into Europe.

Biochemical and molecular characterizations of PCN populations have been conducted to relate the genetic diversity to the virulence and to find markers that readily distinguish the pathotypes (Folkertsma *et al.*, 1996b; Blok *et al.*, 1997; Thiéry *et al.*, 1997). Except to a certain degree for *G. rostochiensis*, no clear correlation has been found between the groupings obtained using various molecular techniques and the virulence. Moreover, some South American populations of *G. pallida* tend to form a

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distinct group, separate from the European *G. pallida* (Blok *et al.*, 1997; Bendezu *et al.*, 1998).

The objectives of this research were to look first at the taxonomic relationships among *Globodera* species. Following the work of Thiéry & Mugniéry (1996), a complementary study of the polymorphism of the rDNA Internal Transcribed Spacer (ITS) was conducted on the Peruvian populations. Highly repetitive satellite DNAs were also isolated and used as species-specific markers. Then, two-dimensional gel electrophoresis (2-DGE) of proteins and Random Amplified Polymorphic DNA (RAPD) techniques were used to produce

two independent but complementary datasets for the assessment of the intraspecific genetic variation among both indigenous (Peruvian) and imported (European) populations identified as *G. pallida*.

Materials and methods

Nematode populations, genomic DNA and total soluble protein preparations

All the populations (Table 1) came from our laboratory collection and were reared on their common host. DNA

Species	Origin of populations	Pathotype	Code
<i>G. artemisiae</i>		Germany	
<i>G. rostochiensis</i>	Ecosse	Great Britain	Ro1
<i>G. t. tabacum</i>	Agen	France	Age
<i>G. t. virginiae</i>		USA	GV1
<i>G. t. solanacearum</i>		USA	GS1
<i>G. mexicana</i>	Santa Ana	Mexico	Ana
<i>G. pallida</i>		Germany	Pa2/3
<i>G. pallida</i>		Germany	Pa2/3
<i>G. pallida</i>	Noirmoutier	France	Pa2/3
<i>G. pallida</i>	Lanslebourg	France	Pa2/3
<i>G. pallida</i>	Audierne	France	Pa2/3
<i>G. pallida</i>	Perpignan	France	Pa2/3
<i>G. pallida</i>	Pas de Calais	France	Pa2/3
<i>G. pallida</i>	Guiclan	France	Pa2/3
<i>G. pallida</i>	Saint Malo	France	Pa2/3
<i>G. pallida</i>	Lufness	Great Britain	Pa2/3
<i>G. pallida</i>	Bedale	Great Britain	Pa2/3
<i>G. pallida</i>	Duddingston	Great Britain	Pa1
<i>G. pallida</i>	Chavornay	Switzerland	Pa2/3
<i>G. pallida</i>	Mase	Switzerland	Pa2/3
<i>G. pallida</i>	Pukekoe	New Zealand	Pa2
<i>G. pallida</i>		Peru	P4A
<i>G. pallida</i>		Peru	P4A
<i>G. pallida</i>		Peru	P4A
<i>G. pallida</i>		Peru	P4A
<i>G. pallida</i>		Peru	P4A
<i>G. pallida</i>		Peru	P4A
<i>G. pallida</i>		Peru	P4A
<i>G. pallida</i>		Peru	P4A
<i>G. pallida</i>		Peru	P4A
<i>G. pallida</i>	Chocon	Peru	P4A
<i>G. pallida</i>		Peru	P5A
<i>G. pallida</i>		Peru	P5A
<i>G. pallida</i>		Peru	P5A
<i>G. pallida</i>		Peru	P5A
<i>G. pallida</i>		Peru	P5A
<i>G. pallida</i>		Peru	P5A
<i>G. pallida</i>	Otuzco	Peru	P5A
<i>G. pallida</i>		Peru	P6A
<i>G. pallida</i>		Peru	P6A
<i>G. pallida</i>		Peru	P6A
<i>G. pallida</i>		Peru	P6A
<i>G. pallida</i>		Peru	P6A
<i>G. pallida</i>		Peru	P6A
<i>G. pallida</i>		Peru	P6A
<i>G. pallida</i>		Peru	P6A
<i>G. pallida</i>	Huamacucho	Peru	P6A

Table 1 Names and related codes of the *Globodera* populations tested. The origins and the pathotype classification of each population are indicated when known. Pathotype determination was usually made by the institute which had sent us the population (i.e. CIP Lima, SCRI Dundee and BBA Münster)

was extracted from frozen females ground in liquid nitrogen by a phenol/chloroform procedure followed by ethanol precipitation as described in Thiéry & Mugniéry (1996). The proteins were extracted as described in Bossis & Mugniéry (1993) and stored at -70°C . DNA and protein extractions were carried out, respectively, on 2500 and 100 females per sample and the average yields were 10 μg of DNA and 150 μg of protein per sample.

Cloning and sequencing of the satellite DNAs

Two micrograms of genomic DNA from *G. pallida* Guiclan and P6A populations were digested to completion with the *Sma*I and *Alu*I restriction enzymes (Boehringer Mannheim), respectively. The smallest fragments (≈ 200 bp) visualized following agarose gel electrophoresis were first recovered onto DEAE paper (Sambrook *et al.*, 1989) and then cloned into pUC 18 plasmid (Pharmacia). The ligation mixture was used to transform the competent *Escherichia coli* strain JM109 (Promega). The sequencing of the inserts selected after cloning was performed on an ABI Prism 310 automated sequencer (PE Applied Biosystems). The consensus sequences appear in the EMBL and GenBank databases under the accession numbers AF250033 and AF250034 for the *Sma*I and *Alu*I satellite DNAs, respectively.

Dot blot procedure

Dot blots were prepared by denaturing 100 ng of genomic DNA of each population with 2 M NaOH for 10 min, and neutralizing it with ammonium acetate 4 M. Whole samples were then dotted by vacuum onto nitrocellulose membranes (Amersham) in a dot blotting apparatus (Bio-Rad).

ITS amplification and restriction procedures

ITS-RFLP analyses were performed as described by Thiéry & Mugniéry (1996). Primers were the same as those described in Vrain *et al.* (1992) and PCR conditions were 30 cycles of a denaturation step at 94°C for 1 min, annealing at 60°C for 50 s and extension at 72°C for 1 min. A final extension was performed at the end for 5 min at 72°C . The amplified products were digested to completion with a set of 16 restriction enzymes (Table 2). Digestion products were separated by electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining.

RAPD procedure

PCR reactions were performed as described by Thiéry *et al.* (1997) in a final volume of 12.5 μL , using eight

random primers from the kits G and L of Operon Technologies (USA). Genomic DNA (15 ng per reaction) was first denatured by heating at 94°C for 5 min and then processed through 40 cycles, each consisting of 1.0 min at 94°C , 1.0 min at 35°C and 1.0 min at 72°C . A final extension was performed at the end for 5 min at 72°C . PCR products were visualized by ethidium bromide staining on agarose gels after electrophoresis. Three replicates of each amplification were performed independently and a negative control was always included as a test for contamination. A population marker was chosen if it was present without ambiguity in the three repeated amplification reactions. Differences in band intensity were not a consideration.

Two-dimensional gel electrophoresis

2-DGE analyses were carried out with the mini Protean II equipment (Bio-Rad) mainly following O'Farrell's procedure (O'Farrell, 1975). Modifications included the use of PDA as crosslinker and of ammonium thiosulphate as a reducer of the background (Hochstrasser *et al.*, 1988). Staining was obtained with ammoniacal silver solution (Oakley *et al.*, 1980) with slight modifications (Bossis & Mugniéry, 1993). Molecular weights and isoelectric points were determined with the 2D SDS-PAGE standards (Bio-Rad), consisting of six reduced denatured proteins: hen egg white conalbumin, bovine serum albumin, bovine muscle actin, bovine carbonic anhydrase, soybean trypsin inhibitor and equine myoglobin.

Analysis of protein patterns

Images of gels were compared on a Vax station 4000-60 computer (Digital Equipment Corporation) with the Kepler software (Large Scale Biology Corporation, Rockville, USA). Gels were digitized (2048×2048 elements) with a scanner (Eikonix EC78/99, Kodak) at a resolution of 100 $\mu\text{m}/\text{pixel}$. From each image file a fitted spotfile was generated and used as the basic data in all the analysis. This step was run with the procville process (Zivy, 1990). Each protein spot encoded by a master number (MSN) was characterized by its coordinates (x, y), its width (s_x, s_y) and its amplitude (amp). The amplitude is dependent on the optical density of the elements of each spot. The volume (vol) of each modelled spot was calculated according to $\text{vol} = 2 \cdot \pi \cdot s_x \cdot s_y \cdot \text{amp}$.

Triplicates of protein samples were prepared from each population. Whatever its volume, a spot was considered to be present in a population when it was detected in at least two of the three gels of this population. A synthetic pattern called the master was built including the spots analysed in all the populations. Then, the matching was initiated in an interactive approach using 40 spots

Enzyme	Number of markers revealed	Polymorphism	Digestion pattern type like that of the Peruvian
<i>Alu</i> I	5	Yes	GR, GT, GM and GP
<i>Bgl</i> II	3	No	
<i>Bsh</i> 1236 I	10	Yes	None
<i>Bsp</i> 143 I	6	Yes	GA, GR and GT
<i>Dde</i> I	4	Yes	GA, GT, GM and GP
<i>Hae</i> III	3	Yes	GR, GT, GM and GP
<i>Hha</i> I	8	Yes	GT, GM and GP (*)
<i>Hinc</i> II	2	No	
<i>Hinf</i> I	4	Yes	GM and GP
<i>Ita</i> I	7	Yes	GM and GP (*)
<i>Mae</i> II	7	Yes	GR and GM
<i>Mae</i> III	12	Yes	GM and GP (*)
<i>Nde</i> II	6	Yes	GA, GR and GT
<i>Pst</i> I	2	No	
<i>Rsa</i> I	6	Yes	GR and GT
<i>Taq</i> I	7	Yes	None

selected as the easiest to match. On this basis, automatic matching was performed and all the combinations were studied using logical operations, so that shared proteins were identified for each pair of populations.

Cluster analysis

Polymorphic markers were recorded as a binary matrix corresponding to their absence or presence. The genetic distances were estimated on the basis of the Nei similarity index (Nei, 1972) and trees were obtained with the Neighbour Joining algorithm using the PHYLIP software (Felsenstein, 1993) with the bootstrap and consensus tree options.

Results

Comparison of the ITS restriction fragment length polymorphisms

The amplified ITS of all the *Globodera* species and populations tested was 1200 bp. More than 90 markers were revealed after digestion of the ITS (Table 2). Only 44 were used in the cluster analysis of Fig. 1 as some were not polymorphic (*Bgl*II, *Hinc*II and *Pst*I), or others, when summed, resulted in a much higher size than the expected 1200 bp due to additional digestion of some markers. As expected, *G. artemisiae* — as representative of the Compositae *Globodera* species — is distant from the Solanaceae *Globodera* species. '*G. mexicana*' and the European *G. pallida* are extremely close, whereas *G. rostochiensis* and *G. tabacum* are more distinct. The Peruvian *G. pallida* split into three groups without clear correlation with their pathotypes. These three groups are closely related to each other and to a lesser extent to

Table 2 Restriction enzymes used in the ITS-RFLP analysis of the Peruvian populations and digestion pattern comparison of the Peruvian with different *Globodera* species. GA, GR, GT, GM and GP stand for *G. artemisiae*, *G. rostochiensis*, *G. tabacum*, '*G. mexicana*' and European *G. pallida*, respectively. Presence of an asterisk (*) means that some Peruvian populations have produced other digestion pattern types than the ones mentioned

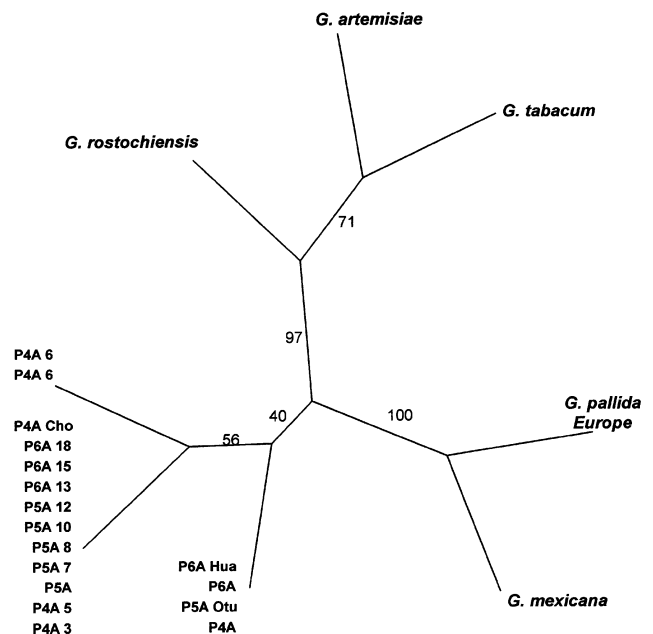


Fig. 1 Unrooted boot-strapped tree of *Globodera* species and of the Peruvian populations inferred by Neighbour Joining from the ITS-RFLP data. Bootstrap values are indicated besides each branch and were obtained after 500 iterations.

the '*G. mexicana*' and European *G. pallida* cluster. The Peruvian populations are separated from the European *G. pallida*, however, the bootstrap values are quite low.

Isolation and primary structure of two satellite DNA families

Digestion of genomic DNA from the *G. pallida* Guiclan and P6A populations has revealed intensely staining

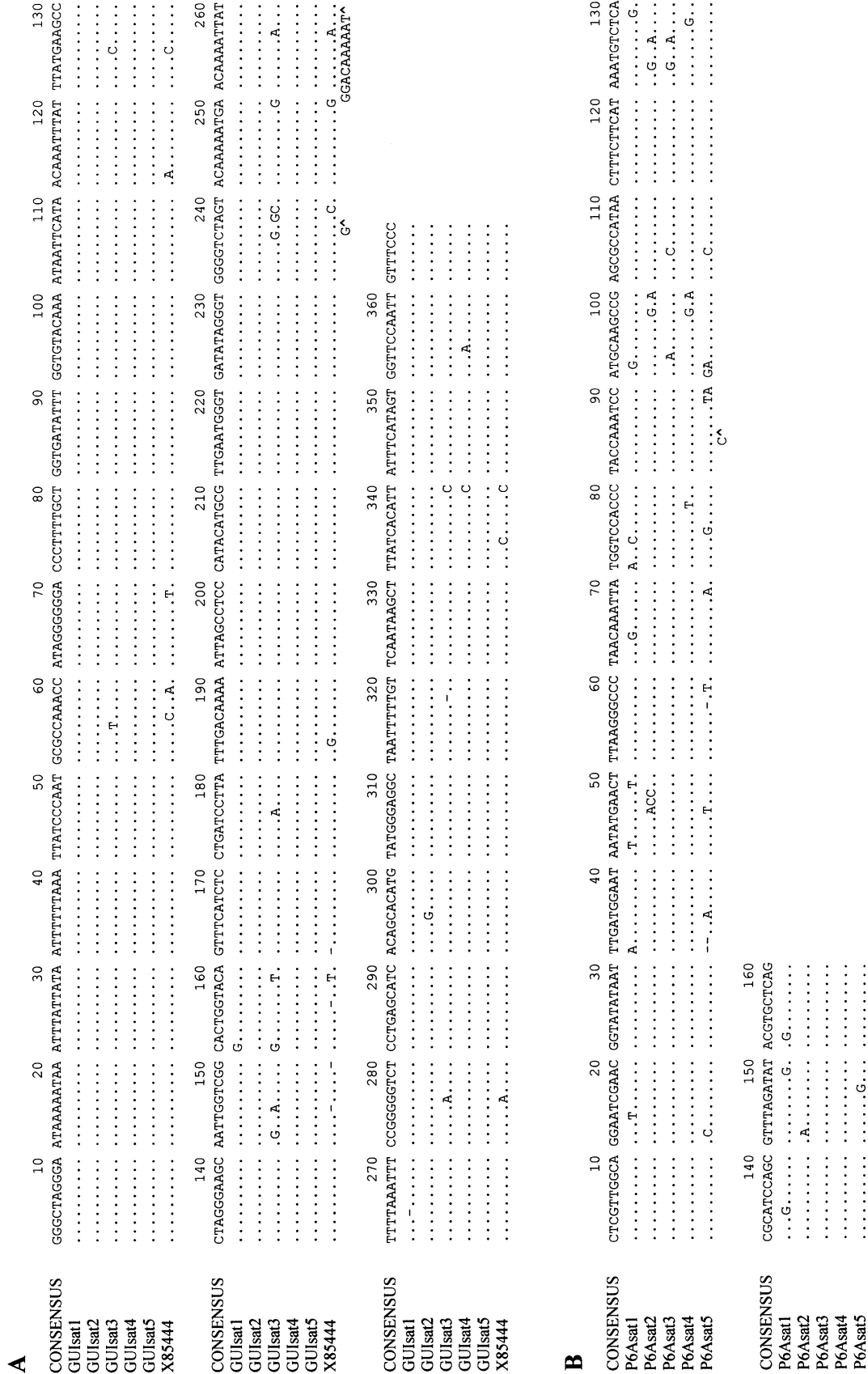


Fig. 2 Consensus sequence and monomer sequences of the *Sma*I (A) and *Aha*I (B) satellite DNAs. All the monomer repeats were aligned manually, point mutations relative to the consensus sequence are shown in each repeat, insertions are indicated by brackets and deletions by (-). Alignment with GenBank accession number X85444, corresponding to Burrows *et al.* (1995) satellite DNA, is also shown in A.

bands of approximately 350 bp and 150 bp in, respectively, the *Sma*I digest of Guiclan and the *Alu*I digest of P6A. After cloning, 10 suitable clones displaying an insert of the expected size (five of 350 bp and five of 150 bp) were selected for further analysis. Unambiguous consensus sequences of 367 bp and 160 bp were deduced from the alignment of the *Sma*I and *Alu*I fragments, respectively (Fig. 2).

The *Sma*I and *Alu*I satellite DNA families have an adenine + thymine content of 63% and 58%, respectively. The five monomers cloned in each of these families differ mainly by single base substitutions, but two- and three-point deletions plus one insertion are found in the *Sma*I and *Alu*I satellite DNA families, respectively. Sequence variation among the cloned monomers of each family relative to the derived consensus sequence is quite low, ranging from 0 to 15 variant nucleotides per cloned repeat for the *Sma*I satellite DNA family and 4 to 16 for the *Alu*I satellite DNA family. The average divergences from the consensus sequence for these two satellite DNA families are 1.7% and 5.5%, respectively.

No significant similarity was detected between these two satellite DNA families (only 31% DNA identity). Comparison with other sequences recorded in the GenBank and EMBL databases revealed no substantial similarities in the case of the *Alu*I satellite DNA family, but 94% of DNA identity with a repetitive element of *G. pallida* (GenBank accession number X85444) in the case of the *Sma*I satellite DNA family.

Species-specificity of the isolated satellite DNA sequences

The *Sma*I and *Alu*I repeated elements were hybridized to genomic DNA of various *Globodera* populations. Figure 3A indicates that the *Sma*I satellite DNA isolated from Guiclan hybridizes strongly with the European populations of *G. pallida* but more weakly with the Peruvian ones. Hybridization was never detected with genomic DNA of *G. rostochiensis* and '*G. mexicana*' even with long exposure times, but a slight signal was consistently observed with a subset of the *G. tabacum* populations classified as *G. t. virginiae*.

The *Alu*I satellite DNA isolated from P6A hybridized equally with the European and Peruvian populations of *G. pallida* (Fig. 3B), but a signal was also clearly observed with the closely related species '*G. mexicana*'. With longer exposure times, hybridization signals were even observed with *G. rostochiensis* and *G. t. virginiae*.

Two-dimensional gel electrophoresis of proteins

On the master, 542 spots were scored including the very faint ones (vol. < 500). A mean of 286 spots per

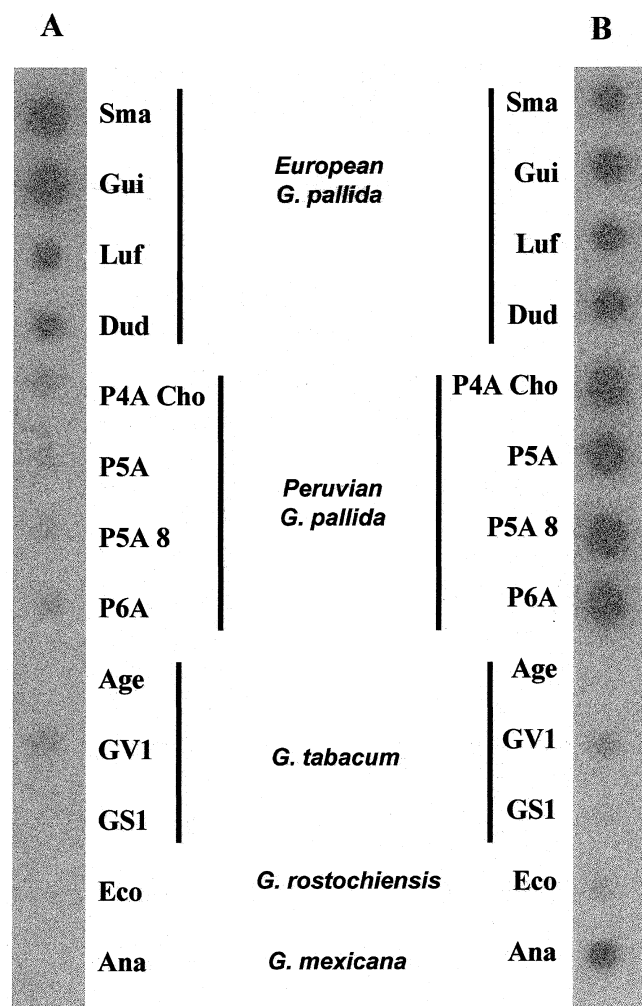


Fig. 3 Species-specificity of the isolated satellite DNA sequences. Genomic DNAs of populations of different *Globodera* species were dot blotted onto a membrane and hybridized with the *Sma*I (A) or *Alu*I (B) satellite DNAs.

population was detected. Figure 4 gives the master on which all spots are displayed and where some of them (in dark) are identified by their MSN as in Table 3.

Some of these discriminating spots were characteristic of only one population and could be used as markers for these populations: MSNs 537 for Audierne, 565 for Luffness, 582 for Pukekoe and 502 for P6A. The absence of one spot can also be used in the identification of five populations: MSNs 42 and 409 for Luffness, 62 for Duddingston, 218 for Perpignan, 254 for Chavornay and 322 for Pukekoe. Of the 18 spots strongly represented in the European populations, three (MSNs 531, 535 and 541) were never observed in the Peruvians. But, among the 19 spots strongly represented in the Peruvian populations, none were absent from the Europeans (Table 3). However, MSN 453, which was never detected

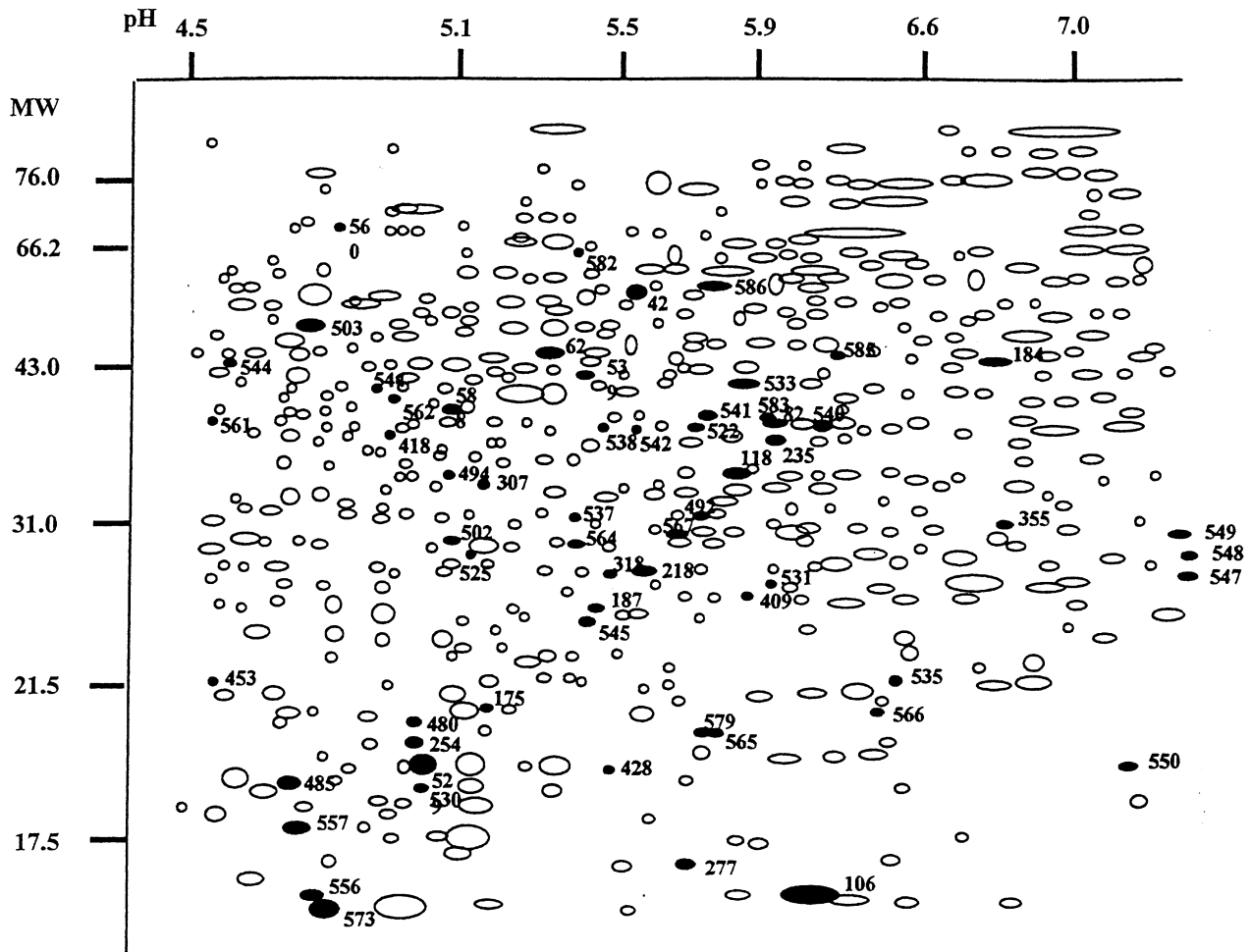


Fig. 4 Master image obtained after 2-DGE analysis of the European and Peruvian *Globodera pallida* populations. Black spots represent spots listed in Table 3 with their master numbers.

ted in the European populations, was present in two populations of each Peruvian pathotype.

The mean genetic distance in the Peruvian populations was 0.39 [0.23–0.50] whereas the mean genetic distance in the European populations was 0.45 [0.29–0.61]. The mean genetic distance between the two groups was 0.53 [0.42–0.59] (Table 4(a)). The consensus tree (Fig. 5A) derived from the genetic distances shows that the European and Peruvian populations can clearly be separated and that this clustering is supported by strong bootstrap values. In the Peruvian group, two pairs of populations were observed with bootstrap values above 96 (P4A 6 and P4A 1, P6A 14 and P6A 15). In the European group, two pairs of populations were also found: (Saint Malo, Pas de Calais) and (Perpignan, BB1), with respective bootstrap values of 84 and 79.

RAPD fingerprinting

An average of 25 markers per primer combination was observed in a size range between 225 and 3000 bp, and 180 polymorphic markers were retained for the analysis. Clearly the Peruvian populations are closer to themselves than to those from Europe (Table 4(b)). The mean genetic distance in the Peruvian populations was 0.41 [0.12–0.57] whereas the mean genetic distance in the European populations was 0.25 [0.10–0.40]. The mean genetic distance between the two groups was 0.57 [0.33–0.58]. The P5A 8 population seems intermediate between the Peruvian and European populations. If this population is removed from the analysis, the genetic distance between the two groups increases from 0.57 to 0.59. Similarly the genetic distance within the Peruvian group drops from 0.41 to 0.38. With this set of populations

Table 3 Presence or absence of some 2-DGE spots characteristic of some populations or group of populations. The characteristic 2-DGE spots are identified by their master number, MSN and their presence in each population is indicated by X. Population-specific spots and their absence in the different populations is indicated by an asterisk. For easier reading, all the European populations were grouped on the left side of the table and all the Peruvians on the right side

MSN	Populations																						
	Aud	BB1	Bed	Cha	Dud	Luf	Noi	Pdc	Per	Puk	Sma	P4A 1	P4A 2	P4A 3	P4A 4	P4A 6	P5A 7	P5A 8	P5A 12	P6A 14	P6A 15	P6A	
42	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
62	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
82	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
106		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
118		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
175	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
184	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
187		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
218	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
235	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
254	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
277	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
307		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
318		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
322	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
355	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
409	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
418	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
428	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
453		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
480																							
485																							
492	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
494	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
502	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
503	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
525		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
529	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
530	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
531	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
533	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
535	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
537	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
538	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
539	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
540	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
541	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
542	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
544																							
545																							
547	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
548	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

				X	X	*	X		*
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	X			X	*		X		*
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					*				*
					*				*
	X	X	X		*	X		X	*
			X	X	*		X	X	X
		X	X	X	*		X	X	X
	X	X		X	X	*	X	X	X
		X	X		*	X	*		X
			X		*	X	X		X
				X	X	X	*		X
		X	X	X	X	*			*
	X	X	X		*				X
	X	X	X		*		X	*	X
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there are two groups with a higher diversity within the Peruvian group than within the European one.

The tree shown in Fig. 5B clearly separates the Peruvian and European populations. In the Peruvian populations, the clusters are in relative good accordance with the pathotype scheme of Canto & Scurrah (1977) who separated the P4A pathotype from the others. In contrast, the European populations cluster independently of the pathotype scheme. The Pa1 and Pa2 pathotypes cluster within a group of Pa2/3 pathotypes. The Luffness population, which is supposed to be different (Phillips *et al.*, 1992; Phillips & Trudgill, 1998), is not clearly distinct from other European populations.

Discussion

Two repeated DNA sequences, representing two different satellite DNA families from European and Peruvian *G. pallida*, were isolated. While no substantial similarities were detected between the *AluI* sequences and any other sequence recorded in the GenBank and EMBL database, the *SmaI* sequences belong undoubtedly to the same satellite DNA family as that isolated by Burrows *et al.* (1995) using the *HindIII* enzyme. The average divergence from the consensus sequence is estimated at only 1.7% for the *SmaI* family and 5.5% for the *AluI* family. Compared with other satellite DNAs, the *SmaI* family seems to be extremely conserved. Such a high degree of sequence similarity was shown for satellite DNAs of meiotic parthenogenetic nematodes like *Meloidogyne fallax* (2.7%) (Castagnone *et al.*, 1998) and *M. hapla* (3%) (Piotte *et al.*, 1994). Two explanations can be provided: (i) the *SmaI* satellite DNA family is younger than the *AluI* family; and (ii) the impact of the homogenizing process is stronger in the *SmaI* family. It has been suggested that during the evolution of satellite DNAs, C to T and G to A transitions are more frequent than the reverse transitions, which results in A + T enrichment of these sequences (Ugarkovic *et al.*, 1989). Under this hypothesis, the low A + T content observed within the *AluI* satellite DNA family is not in accordance with the first explanation while the observation of a higher number of multivariant positions substituted with the same nucleotide in the *SmaI* family is in accordance with the second explanation.

Satellite DNAs that evolve in a concerted manner are often described as species-specific sequences but some may be restricted to a specific group of related species (Mantovani *et al.*, 1997; Mestrovic *et al.*, 1998). This seems to be the case with the isolated *AluI* satellite DNA, which recognizes both *G. pallida* and '*G. mexicana*', two closely related species that cluster together in our ITS analysis. Though not intense, the consistent

Table 4 Genetic distances obtained from 2-DGE (a) and RAPD (b) data. Genetic distances were calculated on the basis of the Nei similarity index (Nei, 1972)

(a)																							
	Aud	BB1	Bed	Cha	Dud	Luf	Noi	Pdc	Per	Puk	Sma	P4 A1	P4 A2	P4 A3	P4 A4	P4 A6	P4 A7	P5 A8	P5 A12	P6 A14	P6 A15	P6 A	
Aud	0.00																						
BB1	0.43	0.00																					
Bed	0.39	0.37	0.00																				
Cha	0.39	0.51	0.38	0.00																			
Dud	0.50	0.48	0.50	0.58	0.00																		
Luf	0.48	0.49	0.45	0.46	0.52	0.00																	
Noi	0.59	0.47	0.54	0.61	0.50	0.51	0.00																
Pdc	0.48	0.41	0.39	0.48	0.38	0.54	0.36	0.00															
Per	0.43	0.33	0.38	0.44	0.53	0.43	0.50	0.42	0.00														
Puk	0.50	0.43	0.46	0.52	0.39	0.52	0.50	0.38	0.46	0.00													
Sma	0.46	0.40	0.41	0.50	0.39	0.50	0.38	0.29	0.40	0.33	0.00												
P4A1	0.62	0.48	0.56	0.60	0.45	0.56	0.49	0.50	0.59	0.49	0.44	0.00											
P4A2	0.55	0.54	0.60	0.58	0.56	0.55	0.49	0.48	0.59	0.55	0.46	0.34	0.00										
P4A3	0.57	0.48	0.56	0.57	0.56	0.49	0.44	0.46	0.51	0.48	0.44	0.39	0.37	0.00									
P4A4	0.52	0.42	0.54	0.54	0.49	0.56	0.55	0.47	0.49	0.48	0.48	0.30	0.43	0.42	0.00								
P4A6	0.62	0.49	0.62	0.69	0.51	0.63	0.52	0.52	0.65	0.51	0.48	0.23	0.39	0.44	0.37	0.00							
P5A7	0.52	0.54	0.52	0.54	0.51	0.53	0.47	0.52	0.55	0.49	0.47	0.31	0.42	0.36	0.40	0.38	0.00						
P5A8	0.52	0.53	0.48	0.48	0.52	0.49	0.49	0.47	0.51	0.50	0.47	0.41	0.41	0.35	0.41	0.43	0.36	0.00					
P5A12	0.60	0.58	0.58	0.66	0.55	0.58	0.55	0.53	0.58	0.59	0.53	0.39	0.40	0.40	0.43	0.41	0.37	0.46	0.00				
P6A14	0.50	0.50	0.55	0.56	0.55	0.49	0.51	0.51	0.57	0.52	0.47	0.37	0.38	0.41	0.48	0.46	0.43	0.42	0.43	0.00			
P6A15	0.53	0.52	0.54	0.57	0.55	0.50	0.46	0.47	0.61	0.53	0.51	0.38	0.37	0.40	0.50	0.44	0.40	0.43	0.37	0.26	0.00		
P6A	0.55	0.47	0.55	0.57	0.46	0.54	0.49	0.45	0.55	0.49	0.44	0.31	0.36	0.41	0.33	0.33	0.36	0.44	0.37	0.35	0.31	0.00	

(b)																							
	P4 A6	P4 A5	P6 A18	P6 A18	P4 A5	P5 A8	P4 A8	P4 A	P4 A4	P5 A4	P5 A4	P5 A4	P6 A5	P6 A5	P6 A5	P6 A5	P6 A5	P6 A5	P6 A5	P6 A5	P6 A5	P6 A5	P6 A5
P4A6	0.00																						
P4A5	0.49	0.00																					
P6A18	0.49	0.40	0.00																				
P4Acho	0.39	0.49	0.40	0.00																			
P5A8	0.51	0.51	0.47	0.44	0.00																		
P4A	0.36	0.54	0.45	0.33	0.50	0.00																	
P4A4	0.44	0.55	0.42	0.34	0.56	0.43	0.00																
P5Aotu	0.52	0.40	0.23	0.35	0.46	0.40	0.46	0.00															
P6Ahua	0.50	0.50	0.19	0.45	0.57	0.47	0.48	0.14	0.00														
P5A	0.53	0.44	0.22	0.35	0.55	0.41	0.47	0.19	0.12	0.00													
P6A	0.53	0.42	0.21	0.35	0.55	0.45	0.45	0.22	0.18	0.12	0.00												

Pdc	0.50	0.52	0.54	0.50	0.40	0.58	0.52	0.51	0.55	0.57	0.66	0.00														
Luf	0.52	0.57	0.54	0.50	0.40	0.58	0.46	0.51	0.53	0.59	0.63	0.17	0.00													
Gui	0.57	0.57	0.54	0.55	0.39	0.62	0.48	0.54	0.58	0.54	0.61	0.16	0.19	0.00												
Sma	0.56	0.55	0.49	0.53	0.40	0.61	0.45	0.48	0.52	0.49	0.55	0.15	0.23	0.06	0.00											
Per	0.56	0.60	0.55	0.56	0.51	0.59	0.45	0.59	0.57	0.56	0.58	0.33	0.36	0.23	0.24	0.00										
Noi	0.62	0.56	0.62	0.58	0.51	0.66	0.55	0.61	0.63	0.60	0.60	0.29	0.32	0.21	0.21	0.13	0.00									
Dud	0.61	0.54	0.59	0.61	0.54	0.67	0.54	0.58	0.58	0.61	0.61	0.37	0.40	0.34	0.30	0.30	0.23	0.00								
Lan	0.61	0.63	0.63	0.63	0.48	0.67	0.50	0.67	0.67	0.66	0.66	0.34	0.29	0.24	0.23	0.23	0.15	0.22	0.00							
Puk	0.57	0.57	0.63	0.59	0.45	0.60	0.54	0.65	0.65	0.63	0.66	0.26	0.28	0.22	0.24	0.26	0.18	0.24	0.12	0.00						
Mas	0.66	0.61	0.66	0.70	0.46	0.64	0.55	0.64	0.67	0.61	0.66	0.34	0.32	0.22	0.24	0.20	0.16	0.25	0.14	0.16	0.00					
BB1	0.56	0.60	0.62	0.60	0.44	0.63	0.49	0.61	0.68	0.67	0.71	0.32	0.24	0.23	0.26	0.24	0.24	0.32	0.20	0.23	0.16	0.00				
Bed	0.59	0.57	0.59	0.63	0.43	0.60	0.55	0.65	0.60	0.54	0.57	0.32	0.32	0.19	0.24	0.22	0.22	0.28	0.17	0.18	0.10	0.18	0.00			
BB2	0.56	0.56	0.60	0.56	0.47	0.66	0.54	0.66	0.66	0.62	0.62	0.30	0.29	0.21	0.26	0.22	0.18	0.29	0.17	0.16	0.17	0.24	0.16	0.00		
Cha	0.62	0.60	0.60	0.62	0.37	0.68	0.60	0.59	0.59	0.55	0.56	0.33	0.33	0.26	0.25	0.28	0.28	0.38	0.30	0.29	0.21	0.31	0.19	0.26	0.00	
Aud	0.63	0.55	0.54	0.52	0.33	0.60	0.55	0.53	0.55	0.54	0.50	0.34	0.37	0.28	0.24	0.30	0.30	0.35	0.31	0.31	0.26	0.36	0.22	0.30	0.12	0.0

hybridization of both probes to the same subset of *G. tabacum* populations, all classified as *G. t. virginiae*, was confirmed in a more extensive dot blot (data not shown). This result gives some support to the subspecies grouping adopted inside the *G. tabacum* group but raises questions about the possible relationships which exist between some *G. tabacum* populations and the *G. pallida* and '*G. mexicana*' species.

The Peruvian populations are more complex. Their ITS heterogeneity is shown to be greater than that observed between populations inside each of the other groups (Thiéry & Mugniéry, 1996). Blok *et al.* (1998) showed little sequence variation between repeat units of the rDNA of a P5A population compared to a P4A population and one can notice that this last pathotype appears also more variable in our ITS analysis. Moreover, it is tempting to speculate that some of the Peruvian populations are mixtures of individuals with different types of rDNA or even that different types of repeat units occur in the rDNA of an individual, as it was shown to be the case in some imported populations of *G. pallida* (Blok *et al.*, 1998). ITS-RFLP and satellite DNA sequences are used as taxonomic markers for nematodes (Grenier *et al.*, 1997; Powers *et al.*, 1997). However, the expected clarification of the taxonomic status of the Peruvian populations is still far from being obtained. Although the *Sma*I satellite DNA groups the Peruvian and the European *G. pallida*, the Peruvian populations appear to be distinct from all the other species and especially from the European *G. pallida* in the ITS-RFLP analysis. On one hand, we know that satellite DNA can be unable to distinguish closely related species (Castagnone *et al.*, 1998) but, on the other hand, because of the low number of unambiguous markers revealed by the ITS-RFLP analysis, the robustness of the tree inferred from these data is not well supported. In fact, only 44 markers were found in this study, but this number would have dropped drastically to 26 or even to 22 if, respectively, *G. artemisiae* or both of the non PCN species (*G. artemisiae* and *G. tabacum*) had not been included. If the ITS region is a good candidate for species determination, then including the Peruvian populations in *G. pallida* would be questionable. Our results emphasize the need to augment these molecular data with biological and morphological studies before addressing the exact taxonomic position of these Peruvian populations.

Two independent data sets were used to determine the intraspecific variation among European and South American *G. pallida* populations: both 2-DGE and RAPD analysis confirm the clear distinction between the Peruvian and the European populations. With RAPD the P5A 8 population is the only one to be somewhat intermediate, although it belongs clearly to the Peruvian group when analysed by ITS-RFLP. The same type of

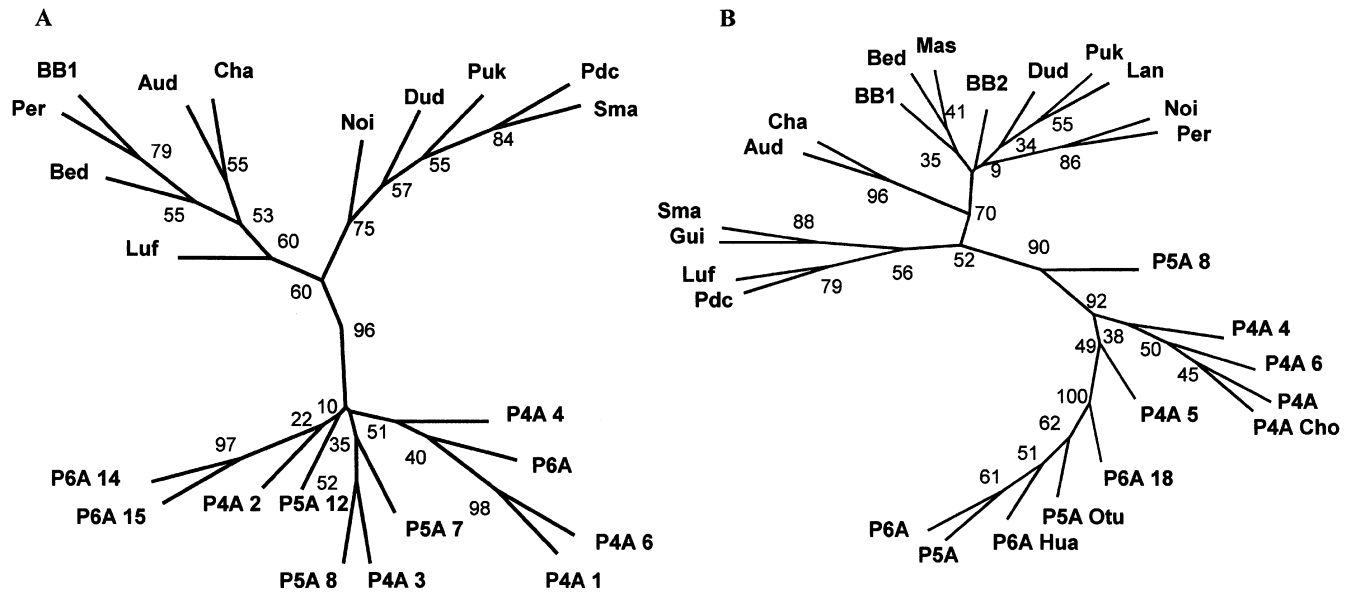


Fig. 5 Unrooted boot-strapped trees of the European and Peruvian *Globodera pallida* populations estimated by Neighbour Joining from the 2-DGE (A) and RAPD (B) genetic distances presented in Table 4. Bootstrap values obtained after 500 iterations are indicated beside each branch.

South American/European clustering was observed by Blok *et al.* (1997) who found, using RAPDs with a set of two Peruvian and 17 British *G. pallida* populations, an average similarity of 51.8% between these two groups. Our mean RAPD and 2-DGE genetic distances can also be compared to those reported in Folkertsma *et al.* (1996a) on a set of 36 European *G. pallida* field populations. While RAPD genetic distances are similar, our 2-DGE distances are higher. We have scored a large number of faint spots which undoubtedly have tended to increase the protein variability. Our 2-DGE genetic distances are also probably overestimated due to some artefacts of the technique, particularly the failure to detect very faint spots on some gels. It would have been possible to eliminate these faint spots, but we have preferred to keep all the detected and modelled spots in the patterns because some of them were scored in all the populations. In addition to and in contrast to the 2-DGE data of Folkertsma *et al.* (1996a), we chose to consider all the proteins as independent markers, although some are probably produced by alleles at polymorphic loci.

Inside each group, no clear correlation between pathotype is evident. The Pa1 pathotype (the Duddingston population) clusters within the Pa2 and Pa2/3 pathotypes. While 2-DGE associates the Peruvian populations in a way that does not reflect the virulence groups, the RAPD data show that a P4A and a P5A/P6A groupings tend to be observed. This difference is expected, as, biologically, the P4A pathotype differs

strongly from the P5A and the P6A pathotypes by its inability to develop on *Solanum vernei* (VTⁿ)² 62.33.3 (Canto & Scurrell, 1977; Phillips & Trudgill, 1998). However, as the Pa1 pathotype also differs from the Pa2/3 pathotype by its inability to develop on the *H2* gene from *S. multidissectum* P55/7 (Kort *et al.*, 1977) the same type of difference would also be expected for the European Duddingston population, but was not found. Some pairs of populations appear to be closely related. These populations might have the same origin, with the same founders, but more probably are the result of secondary founding events. The slight differences observed between each member of a pair could be explained by the reduction of the diversity associated to the founder effect and also by occurrence of subsequent random genetic drift.

Apart from the Peruvian/European distinction, considerable discrepancies are observed between the trees obtained by 2-DGE and RAPD. Folkertsma *et al.* (1996a) did a comparison of the two techniques with a same set of *G. pallida* populations; they reported such inconsistencies and concluded that when clusters of populations are not well differentiated from each other, different types of data set may result in clusters with differing compositions. In our case, the results obtained are surprising in that if one assumes that the European populations were imported from the Andean region, it would be expected that the variability in the South American populations would be greater than the variability in the European populations. RAPD data reveal,

in the Peruvian group, high genetic distances and a completely unresolved branching, consistent with this great variability. However, 2-DGE data reveal high genetic distances in the European populations and several characteristic proteins are observed in these populations. If we assume that the European populations were introduced from other South American populations that were not investigated in this study, then the characteristic proteins observed can be explained. Why RAPD and 2-DGE display opposite results concerning the most variable group remains to be further investigated. But, the codominant nature of 2-DGE markers, in contrast to the usual dominant nature of RAPD markers, can be part of the explanation. Assuming that very few nematodes were at the origin of the primary founders of the European populations and that the genetic drift has resulted in the fixing of different alleles in these imported populations, it is possible that our inability to identify products of alleles at polymorphic loci has led to an overestimation of the genetic distances by 2-DGE in the European group.

The European populations seem therefore to have come from a different part of the Peruvian area than that investigated in this study. The PCN samples in this study may not be representative of the whole of Peru. Further samples, both of Peruvian PCN populations and of other South American populations such as Bolivian and Chilean populations, may reveal the origin of European PCN.

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