Fundam. appl. Nematol., 1993, **16** (1), 47-56

Specific status of six *Globodera* parasites of solanaceous plants studied by means of two-dimensional gel electrophoresis with a comparison of gel patterns by a computed system

Michel BOSSIS and Didier MUGNIÉRY

Laboratoire de Recherches de la Chaire de Zoologie, INRA, Domaine de la Motte-au-Vicomte, B.P. 29, 35650 Le Rheu, France.

Accepted for publication 30 March 1992.

Summary – Two-dimensionnal gel electrophoresis is applied to the study of the specific status of six *Globodera* parasites of solanaceous plants. A modified O'Farrell's technique, mainly according to Hochstrasser *et al.* (1988*a, b*), is used with mini protean II apparatus (Bio-Rad). The gels (84 × 58 × 0.75 mm) are stained with an ammoniacal silver solution (Oakley *et al.*, 1980). The digitized gels are matched with the Kepler's software on a VAX station. After a digital filtering, about 150 spots of main polypeptides are taken into account in the specific comparison which is made automatically. Genetic distances (D) are calculated and the clusters are built with the UPMGA method. The shared spots of each specific combination are given. The biochemical results are discussed with regard to morphological and biological features and with regard to hybridizations obtained by Mugniéry (1979) and Mugniéry *et al.* (1992). Results confirmed that *G. rostochiensis* is far from *G. pallida* and pointed out the closeness of *G. pallida* and *G. " mexicana "*, though differences in the host range are quite large. The group *G. virginiae, G. solanacearum* and *G. tabacum* is considered as only one species, and called *G. tabacum sensu lato* from which pathotypes would be defined. The great interest of a digitized analysis and of the constitution of a database is pointed out. Further biological and biochemical studies on *G. " mexicana "* are needed to clarify the status of this entity.

Résumé – ***Statut spécifique de six* Globodera *parasites des Solanacées étudié par électrophorèse bidimensionnelle, avec comparaison des gels par analyse d'images.*** – L'électrophorèse bidimensionnelle est appliquée à l'étude du statut spécifique des *Globodera* parasites des Solanacées. La technique d'O'Farrell (1975) modifiée principalement selon Hochstrasser *et al.* (1988*a, b*) est utilisée avec le système « mini protean II » (Bio Rad). Les gels (84 × 58 × 0,75 mm) sont colorés au nitrate d'argent selon une technique dérivée de celle d'Oackley *et al.* (1980). Après digitalisation, les images obtenues sont comparées avec le logiciel Kepler sur une station VAX. L'utilisation d'un seuil intégrant la surface et l'intensité des spots élimine les protéines qui sont trop faiblement révélées. Environ 150 protéines principales sont retenues pour les comparaisons d'espèces. Les distances génétiques sont calculées et les dendrogrammes construits selon la méthode UPGMA. Les spots communs à chacune des quinze combinaisons spécifiques sont donnés. Les résultats biochimiques sont discutés par rapport aux données morphologiques et biologiques connues et par rapport aux résultats des hybridations obtenues par Mugniéry (1979) et Mugniéry *et al.* (1992). L'éloignement de *G. rostochiensis* par rapport à *G. pallida* est confirmé. La proximité de *G. pallida* et de *G. " mexicana "* est mise en évidence, bien que des différences au niveau de la gamme d'hôtes soient importantes. Le groupe *G. virginiae, G. solanacearum* et *G. tabacum* est considéré comme appartenant à la même espèce, *G. tabacum sensu lato*, à l'intérieur de laquelle des pathotypes pourraient être définis. Le grand intérêt du traitement des résultats par l'analyse d'images et de la constitution d'une base de données est souligné. Des études biologiques et biochimiques complémentaires sont nécessaires pour clarifier le statut spécifique de *G. " mexicana "*.

Key-words : Nematodes, *Globodera*, solanaceae, electrophoresis, genetic distance.

Two-dimensional gel electrophoresis (2-DGE) of total proteins is a useful tool in the study of closely related species. Using this technique Aquadro and Avise (1981) identified six closely related species of wild mice. A range of biochemical techniques has been used in systematics (Hussey, 1979; Wharton *et al.*, 1983). Some nematologists applied 2-DGE to the study of phytoparasitic nematode species or pathotypes (Bakker & Gommers, 1982), despite the difficulties associated with the biological material, particularly small amounts of proteins.

At an interspecific level, Bakker and Gommers (1982) differentiated potato cyst nematode *Globodera rostochiensis* (Woll.) from *G. pallida* (Stone). Premachandran *et al.* (1984) observed that there were many more spots in *Meloidogyne incognita* Chitwood than in other *Meloidogyne* species. On cereal cyst nematodes, Ferris *et al.* (1989) separated two groups, one as strict *Heterodera avenae* Woll. and another as the Gotland strain of *H. avenae*. They pointed out greater differences between these two groups than were expected on the basis of the morphological data and pathogenicity tests, but they were unable to differentiate the pathotypes of *H. avenae* found in Sweden.

At an intraspecific level, Bakker and Gommers (1982) differentiate populations representing two pathotypes of *G. rostochiensis*. Ohms and Heinicke (1985), with a micro 2-DGE, were able to separate populations of the five

pathotypes of *G. rostochiensis* with specific spots from single cysts. Ferris *et al.* (1985, 1986) separated *Heterodera glycines* Ichinohe populations from northern and southern Indiana and easily identified one Japanese population out of six U.S. isolates. Nevertheless, Bakker and Bouwman-Smits (1988*a*) found protein differences within *G. pallida* pathotypes which were sometimes greater than those observed between so-called pathotypes. Bakker (1987) found spatial variations in *G. rostochiensis* proteins coinciding with the colonization of Europe by potato cyst nematodes. He argued that variations between populations within these two species were the result of three processes : the genetic structure of the primary founders, the influence of random genetic drift which is dependent on the number of secondary founding events, and gene flow which is resulting from mixing of populations also dependent on passive spread. Therefore, interpopulation variations in virulence and proteins would be determined firstly by the origin of populations in South America and secondly by their history in Europe.

The high degree of resolution of 2-DGE makes it possible to identify a large number of gene products and therefore this technique can be used as a tool to estimate the genetic distance within species and even within pathotypes (Bakker, 1987). Hybrids have been produced between *Globodera* species parasitic on solanaceous plants and their progenies have been studied for viability and fecundity (Mugniéry, 1979; Mugniéry *et al.*, 1992). However, the specific status of *Globodera* parasites of solanaceous plants is still unclear. In an attempt to define the evolutionary relationships inside this group, we have used 2-DGE.

Material and methods

Populations were obtained from different sources. *G. "mexicana"* (so-called by Campos-Vela, 1967), *G. virginiae* (Miller & Gray), *G. solanacearum* (Miller & Gray) and *G. tabacum* (Lownsbery & Lownsbery) were supplied by L. I. Miller, *G. rostochiensis* RO1 Scotland and *G. pallida* 2/3 Chavornay by W. M. Hominick and R. Vallotton, respectively. The others were French RO1 or Pa 2/3 populations (Tab. 1).

All populations were reared in Petri dishes (Mugniéry & Person, 1976) on susceptible potatoes cv. Désirée for *G. rostochiensis* and *G. pallida,* tomatoes cv. St Pierre for *G. "mexicana", G. virginiae* and *G. solanacearum,* and on egg-plants cv. Violette Longue de Barbentane for *G. tabacum.*

White females were hand-picked from the roots. Necrotic root tissues and nematode secretions attached to the neck were carefully removed with a needle. They were washed three times with a 10 mM Tris HCl, pH 7.4 solution. For each population, three samples of 100 females were crushed in an ice bath, using a ground glass homogenizer in 10 µl Tris HCl (same concentration as above) and 30 µl of a homogenizing solution with 9 M urea, 5 % (v/v) β-mercaptoethanol and 2 % ampholytes, pH range 3-10, 6-8 and 5-7 (Biolyte, Bio-Rad).

Table 1. Locations and collection codes of populations of *Globodera.*

Species	Locations	Codes
G. rostochiensis	Scotland (UK)	
	Ile de Ré (F)	
	Noirmoutier (F)	
G. pallida	Guiclan (F)	
	Saint-Malo (F)	
	Chavornay (CH)	
G. "mexicana"	Huamantla (ME)	75-122-1
	Santa Ana (ME)	75-140-1
G. virginiae	Crutchlow (USA)	Standart WHT
	Crutchlow (USA)	Hort.
G. solanacearum	Watkins (USA)	52 ADL
	Watkins (USA)	Fish. not.
G. tabacum	Connecticut (USA)	

The homogenates were centrifuged at 10 000 *g* for 10 min and supernatants frozen at – 70 °C. Just before use the samples were thawed and protein concentrations estimated according to Bradford (1976) by measuring the shift of extinction of Coomassie Brillant Blue G 250 at 595 nm. Concentration was standardized at 2 µg/µl by the addition of the required volume of the homogenizing solution, so that standards of 4 µg in an equal volume were available for electrophoresis.

2-DGE was carried out on three gels or more for each sample, essentially as described by O'Farrell (1975) with some modifications as listed in Table 2. The main differences were the use of piperazine diacrylamide (PDA) and of ammonium thiosulfate to decrease background staining (Hochstrasser & Merril, 1988; Hochstrasser *et al.*, 1988).

Staining was carried out with ammoniacal silver solution (Oakley *et al.*, 1980), modified by de Boer *et al.* (1992) with slight changes : the ammoniacal silver solution was prepared with 1.1 ml NaOH (1N), 0.05 ml NH_4OH (25 % NH_3), 98 mg $AgNO_3$, water to 50 ml/gel and staining solution with 0.25 ml of citric acid, 0.05 ml of formaldehyde (37 % w/v), water to 100 ml/gel. The staining was stopped with a 0.1 % aqueous acetic acid solution.

Molecular weights were estimated using a calibration kit (Pharmacia) with phosphorylase b (94.0 Kd), albumin (67.0 Kd), ovalbumin (43.0 Kd), carbonic anhydrase (30.0 Kd), trypsin inhibitor (20.1 Kd) and lactalbumin (14.4 Kd).

Table 2. Modification of the O'Farrell's technique.

Material :	
	– Glass tubing (length : 75 mm, inner diameter 1.1 mm outer diameter 1.5 mm).
	– Capillary tubes filled up to 8 mm below the top, without any overlay solution.
	– Slab gels performed in mini protean II 2D. (Bio Rad).
Lysis buffer :	
	– Nonidet - P. 40 discarded.
	– 0.8 % ampholytes (Biolyte) range 5-7.
	– 0.8 % ampholytes (Biolyte) range 6-8.
	– 0.4 % ampholytes (Biolyte) range 3-10.
IEF gels :	
	– Bisacrylamide replaced by piperazine diacrylamide (PDA) at the same concentration.
	– Same ampholytes than in lysis buffer used at an equal concentration.
	– Overlaying of gel with " H " solution, " A " lysis buffer and water discarded.
First dimension run :	
	– No prerun.
	– Voltage schedule : 17 h-18 V; 1 h 30-180 V; 0 h 30-270 V; 1 h 35-500 V.
SDS gels :	
	– Size : 84 × 58 × 0.75 mm.
	– Bisacrylamide replaced by PDA at the same concentration.
– Separating gel :	
	– Acrylamide-PDA (T : 12.9 %; C : 2.7 %).
	– Sodium thiosulfate at 1.2×10^{-3} Ml^{-1}.
– Stacking gel :	
	– Acrylamide-PDA (T : 4 %; C : 2.7 %).
Second dimension run :	
	– No agarose used to keep the cylindrical isoelectric focusing in place.
	– Run at 5 mA - 20 mm and 10 mA - 2 h 10/gel.

Gels were digitized with a Eikonix 1412 scanner (Kodak) and patterns were compared using the Kepler's software (Large Scale Biology Corporation, Rockville, USA) on a 3100 VAX Station (Digital Equipment Corporation). In this process, spots were modelled as Gaussian least-squares, so that size of each pattern was reduced in a spot file. They were matched firstly by an interactive landmarking and secondly by an automatic process.

A general master pattern with all main spots detected in the six groups was generated using, as a control, co-migration gels which were obtained by the electrophoresis of a mixture of proteins from two species (fifteen combinations).

For each group, a master was generated and only spots always present in all patterns were taken into account and listed in a typical species map. These masters were compared to the general master pattern, and together. The basic zones with poorly separated proteins were discarded when the informative part of the gel was defined.

The similarity between groups was estimated according to Aquadro and Avise (1981). The protein proportion of shared spots was estimated from the equation : $F = 2Nxy/(nx + ny)$, in which nx and ny are the total number of spots scored for individuals x and y respectively, and Nxy is the number of shared spots by x and y. The genetic distance (D) was obtained according to the equation : $D = 1 - F$.

Among the methods used for inferring phylogenies (Felsenstein, 1988; Baldwin & Schouest, 1990), we chose the widely used distance matrix method. A general cluster was computed with a SAS software (SAS Institute Inc., Cary, North Carolina, USA). The matrix of genetic distance with average linkage (Sokal & Michener, 1958) was used according to the group average unweighted pair-group method using arithmetic averages (UPGMA). We tested different options including unweighted pair-groups (centroid and complete), weighted pair-groups (Mcquitty, median), nearest neighbours (single), Ward's minimum variance (Ward).

Results

A preliminary study with three populations from each of the two species *G. rostochiensis* and *G. pallida* showed that at a specific level, interpopulation differences were very small, and genetic distances were less than 0.06.

Fig. 1 A, B gives examples of protein patterns obtained for two species *(G. rostochiensis* and *G. pallida)* with molecular weight and pH scales. After the dig-

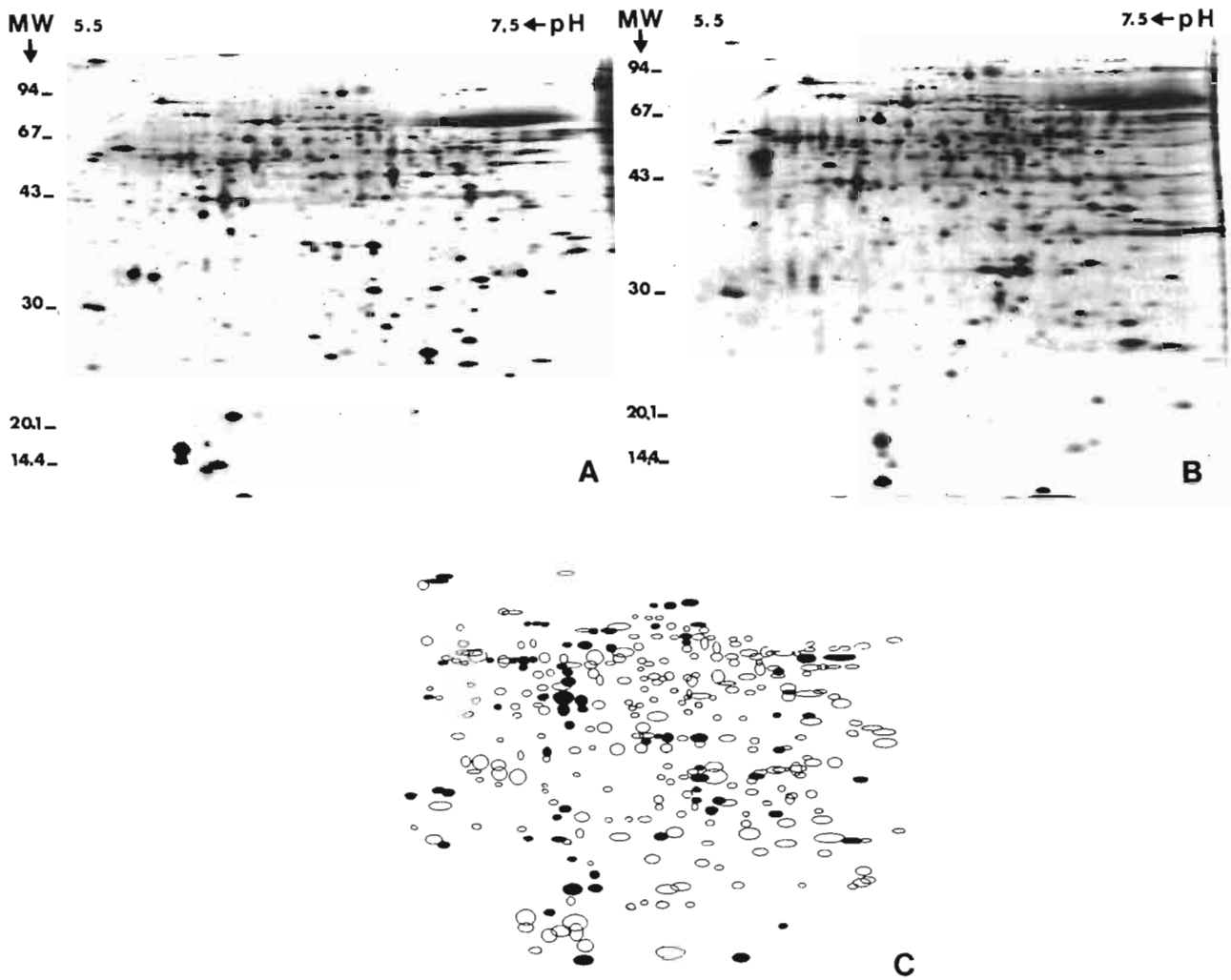

Fig. 1. 2D protein patterns of white females of *Globodera* – A : *G. rostochiensis;* B : *G. pallida;* C : Common proteins (black ellipses) from *G. rostochiensis* and *G. pallida* and main other proteins from all the group (white ellipses).

itization and the filtering of all the pictures of gels, a master for each species has been built, and by comparing these masters two by two, common spots have been highlighted. As an example of these results, Fig. 1 C gives the comparison of the *G. rostochiensis* and *G. pallida* masters for which the black ellipses are the common proteins from these two species and the white ellipses are the proteins included in all the six masters and not shared in these two species. Fig. 2 A-F, Fig. 3 A-F, and Fig. 4 A, B represent the common spots for the fourteen other combinations, with black and white ellipses, as in Fig. 1 C. From the data obtained on these figures shared spots have been scored in the Table 3 which gives the number of major spots taken into account for each species (between 141 and 159 spots), the number of shared spots (above the diagonal) used to estimate the similarity coefficient (F) and (below the diagonal) the genetic distance (D).

Although the distances vary according to the procedure used with SAS software, the clusters obtained are very similar, so only UGPMA results are given. The clusters obtained with this analysis (Fig. 5) show that *G. virginiae* and *G. solanacearum* are closely related (cluster 5) and together with *G. tabacum* (cluster 3) they form one entity. *G. pallida* and *G. "mexicana"* are also closely related (cluster 4) whereas *G. rostochiensis* is quite distinct from all the others (cluster 2). The values of the normalized root-mean-square distances are given. UPGMA analysis assigns the branch lengths so that the sum of squares of differences between observed and expected distances is minimized. Nevertheless only a few mutations could give too much weight when calcu-

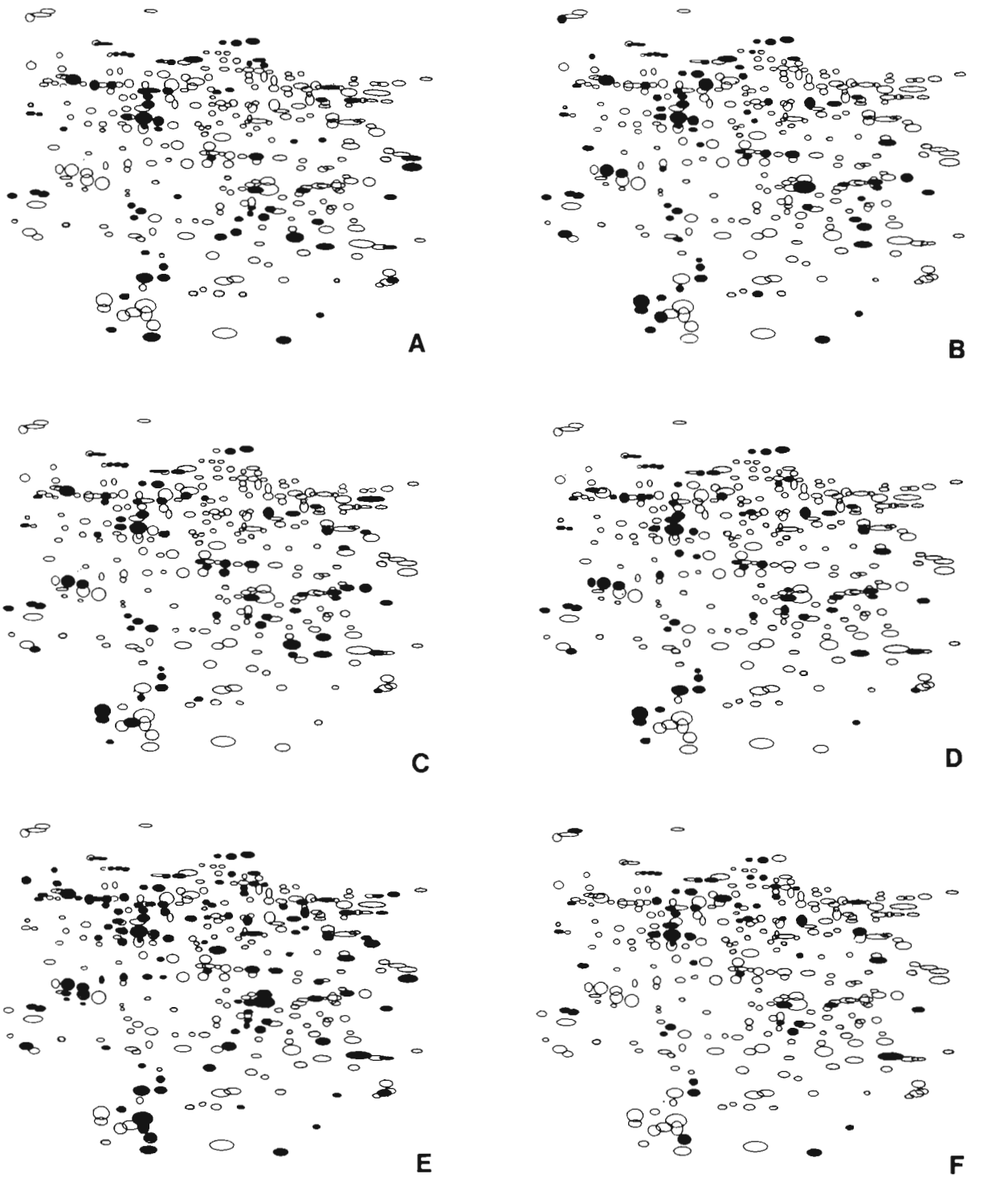

Fig. 2. Shared spots between 2D protein patterns of white females (black ellipses) and main other proteins from all the group (white ellipses) – A : *Globodera rostochiensis* and *G. "mexicana";* B : *G. rostochiensis* and *G. virginiae;* C : *G. rostochiensis* and *G. solanacearum;* D : *G. rostochiensis* and *G. tabacum;* E : *G. pallida* and *G. "mexicana";* F : *G. pallida* and *G. virginiae.*

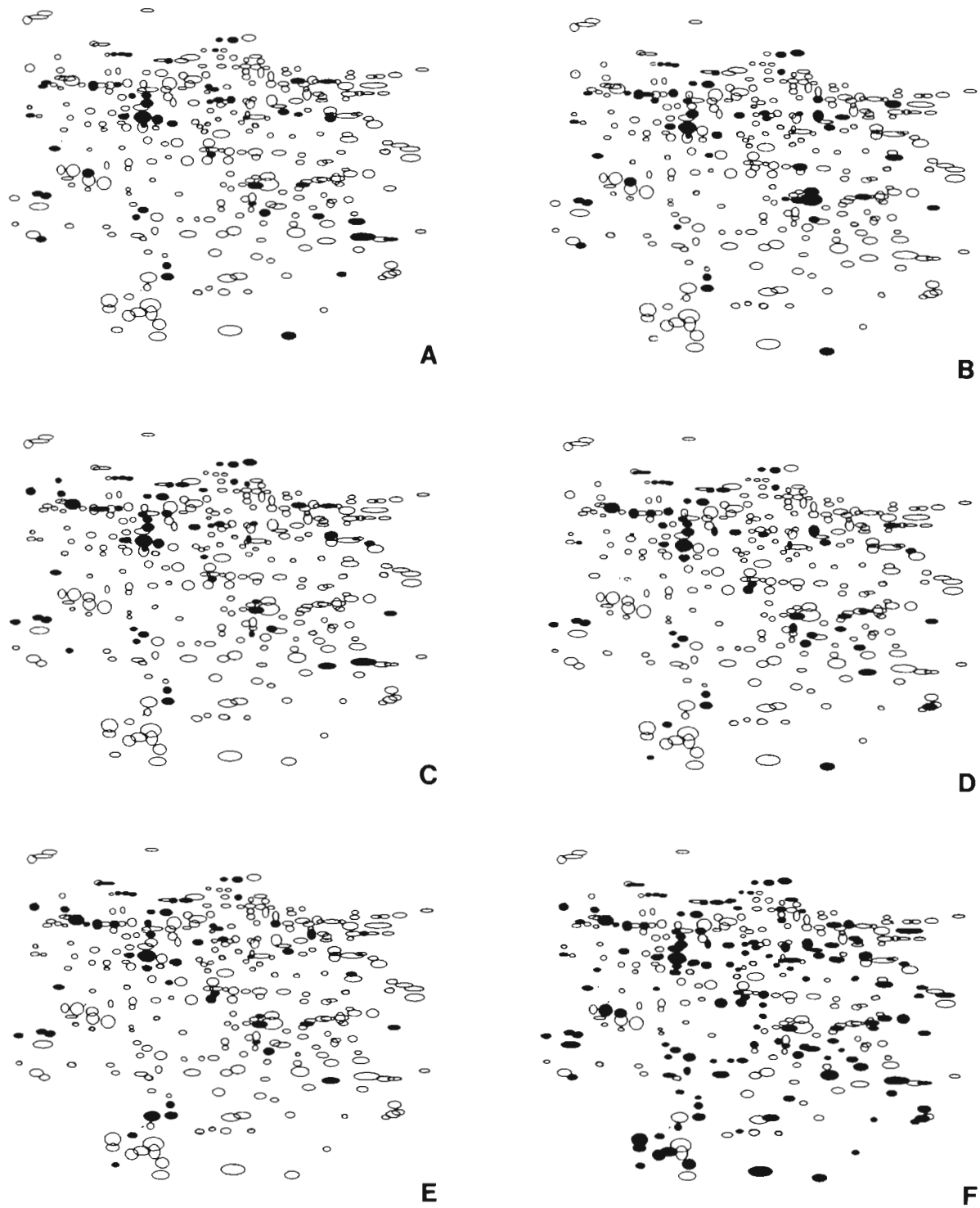

Fig. 3. Shared spots between 2D protein patterns of white females (black ellipses) and main other proteins from all the group (white ellipses) – A : *Globodera pallida* and *G. solanacearum;* B : *G. pallida* and *G. tabacum;* C : *G. "mexicana"* and *G. virginiae;* D : *G. "mexicana"* and *G. solanacearum;* E : *G. "mexicana"* and *G. tabacum;* F : *G. virginiae* and *G. solanacearum.*

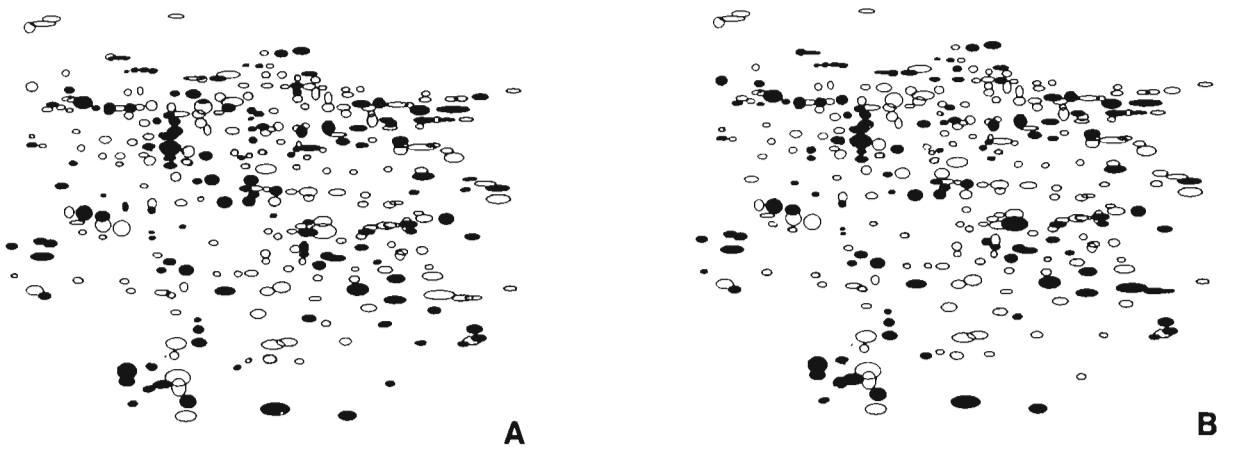

Fig. 4. Shared spots between 2D protein patterns of white females (black ellipses) and main other proteins from all the group (white ellipses) – A : *G. virginiae* and *G. tabacum;* B : *G. solanacearum* and *G. tabacum.*

Table 3. The number of shared proteins (above diagonal) and the genetic distances (below diagonal).

Species	Main Protein spots	*G. rostochiensis*	*G. pallida*	*G. " mexicana "*	*G. virginiae*	*G. solanacearum*	*G. tabacum*
G. rostochiensis	159	–	73	71	78	71	68
G. pallida	141	0.51	–	121	55	51	50
G. " mexicana "	150	0.54	0.17	–	55	55	52
G. virginiae	155	0.50	0.63	0.64	–	142	128
G. solanacearum	144	0.53	0.64	0.63	0.05	–	123
G. tabacum	153	0.56	0.66	0.66	0.17	0.17	–

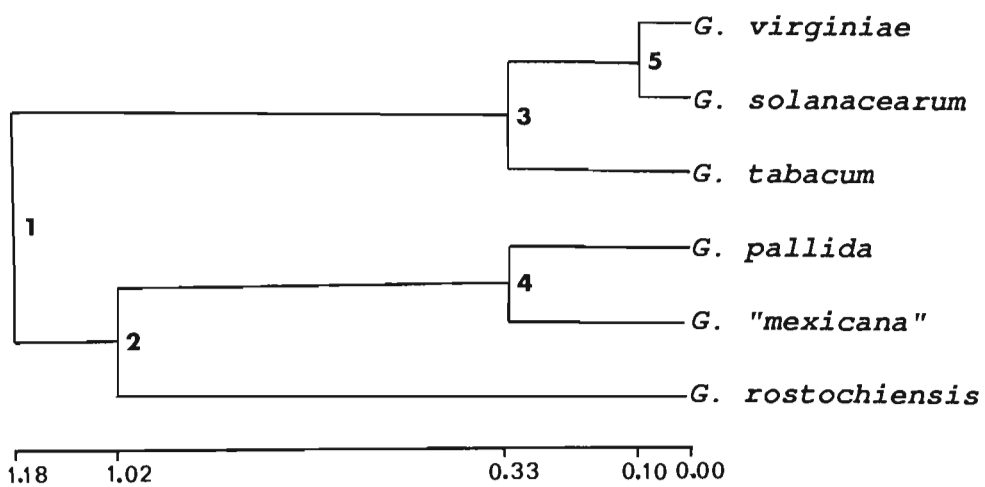

Fig. 5. Similarity clusters of *Globodera* parasites of solanaceous plants built by average linkage with normalized root-mean-square genetic distances.

lating genetic distances (e.g. a single change in a regulatory gene could modify the occurence of many proteins).

Discussion

Technique

Our modifications of the O'Farrell's technique, mainly the use of PDA as cross-linker and the slight adaptation of Oackley *et al.*'s staining technique, give protein patterns without any background on which the polypeptides are easily detected by scanning.

Comparison of protein patterns has been made easy by the use of the LSB's Kepler. Gels are computed in two steps : firstly, in an interactive landmarking which aligns the digitized pictures and identifies typical spots for which there is no ambiguity and, secondly, in an automatic general comparison. Moreover, this system provides a database which will be readily accessible for further experiments on this group of nematodes or for large comparison involving other genera.

Genetic distances

Spots taken into account are less numerous than spots scored by Bakker and Bouwman-Smits (1988*b*) in an intergeneric comparison carried out with a similar electrophoretic analysis. This difference is due to the fact that in the zone of interest selected for automatic processing some of the basic proteins were eliminated and to the fact that the faint spots were discarded by the filtering process.

Genetic distance is always estimated as a relative value. For example with the two species *G. rostochiensis* and *G. pallida,* the most studied species of this group, it was estimated as 0.70 by Bakker (1987) based on total proteins or as 0.87 by De Jong *et al.* (1989) based on RFLP and bands obtained with a β-4-tubulin probe. The value obtained in our study (0.51) confirms their conclusions that there are substantial differences between these two valid species.

In contrast, the variability between populations within a species was very small. We found genetic distances of less than 0.06, i.e. similar to those calculated by Bakker and Bouwman-Smits (1988*b*) based on total proteins of D = 0.04 for *G. rostochiensis* and D = 0.06 for *G. pallida.* Consequently in the constitution of the representative master pattern for each species, the assumption that this distance is zero is almost satisfied and the small deviations from zero will have had a negligible effect on the comparison between species.

Status of G. virginiae, G. solanacearum, *and* G. tabacum.

The genetic distance observed between *G. virginiae* and *G. solanacearum* is very small (0.05) i.e. quite similar to that observed between populations of the same species. These two allopatric entities (Virginia, USA) were described by Miller and Gray (1968, 1972) based on small morphological differences. The main physiological difference is the ability of *G. virginiae,* but not of *G. solanacearum,* to develop on *Nicotiana* × *sanderae, 50-B.* Mugniéry *et al.* (1992) have shown that the hybridizations between these two species gave viable and fertile progeny and proposed the synonymy of *G. virginiae* and *G. solanacearum.* The biochemical data confirm this point of view.

The genetic distance between *G. tabacum* and *G. solanacearum - G. virginiae* is slightly greater (0.17). *G. tabacum* is unable to develop on *N. acuminata 2-G-58,* contrary to *G. virginiae* and *G. solanacearum.* It is located in a distant geographical zone, in the Northeast of USA (New-York), Massachusetts, Connecticut). Even so, Greet (1972), in studies based on disc electrophoresis and Stone (1983) concluded that these three entities belonged to the same species. Hybridizations carried out by Mugniéry (unpubl.) produced viable and fecund hybrids between *G. tabacum* and *G. virginiae* or *G. solanacearum* whichever way the crosses were made. Although the electrophoretic results point to *G. tabacum* being a little more distant from the other two, a genetic distance of 0.17 still corresponds to intraspecific variability.

Status of G. " mexicana "

a) With regard to the G. solanacearum, G. virginiae *and* G. tabacum *group :* The genetic distances observed are very large (0.63 to 0.66) and are of a magnitude associated with the strong but not complete genetic barrier. Although the interspecific differences may be assumed to occur with genetic distance above 0.17, the morphological differences are also very small, and we were unable to distinguish *G. " mexicana "* from the other species. Very little is known about the physiology of this entity; it develops on tobacco, tomato and various solanaceous plants including *Solanum nigrum.* It is geographically separated, coming from Mexico. It will not hybridize with *G. tabacum* (Mugniéry, unpubl.) or with *G. virginiae* (Mugniéry *et al.,* 1992), but its males will produce viable hybrids when mated to females of *G. solanacearum.* The biochemical data therefore confirm the biological results.

b) With regard to G. pallida : The genetic distance is relatively low (0.17) and pointed out that *G. " mexicana "* is more closely related to *G. pallida* than to the *G. virginiae - G. solanacearum - G. tabacum* group. We observe a great morphological similarity, but there were strong differences at the physiological level as reflected by host range. *G. " mexicana "* was unable to develop on cultivated potato but able to develop on tobacco whereas *G. pallida* was unable to develop on tobacco but was able to develop on potato. During our attempts to produce hybrids, we observe cytoplasmic incompatibility as only the crosses between females *G. pallida* and males *G. " mexicana "* give viable and fertile progeny (Mugniéry *et al.,* 1992). This suggests the presence of a genetic barrier even though the genetic distance (0.17) is relatively low.

c) With regard to G. rostochiensis (see below)

Status of G. pallida

The large genetic distance between *G. pallida* and all the other species except *G. "mexicana"* confirms its status as a separate species, which is further supported by our inability to hybridize it with all, except *G. "mexicana"*. There is a strong genetic barrier which is confirmed.

Status of G. rostochiensis

We found a large genetic distance between *G. rostochiensis* and all the other species (0.51-0.56), confirming the results of Bakker (1987) with regard to *G. pallida*, even so physiologically it seems to be very closely related together. Furthermore it was the one species which could be discriminated on morphological features (Stone, 1972).

Conclusion

Our biochemical studies confirmed that *G. rostochiensis* is distant from all the other species. They confirmed too the genetical similarities of *G. tabacum, G. virginiae* and *G. solanacearum*. It must be necessary to rename them as *G. tabacum*. Consequently *G. virginiae* and *G. solanacearum* should be described as subspecies or more probably as pathotypes. Our results do not support the point of view of Golden and Ellington (1972) and Stone (1983) regarding *G. "mexicana"*. We found this entity to be close to *G. pallida* and relatively distinct from *G. tabacum sensu lato*. To affirm that *G. "mexicana"* is a valid species though not described is another problem.

Clearly, further studies on the biological and biochemical variability in the Mexican populations of *Globodera* are needed. In this way, other techniques could be applied, such as serology similar to studies with *G. rostochiensis* and *G. pallida* (Schots *et al.*, 1990) or RFLP (Burrows & Perry, 1988; De Jong *et al.*, 1989). Together with host range and morphometrical studies they provide a comprehensive range of tools for investigating species and population relationships.

Acknowledgements

Authors thank Drs. L. I. Miller, W. M. Hominick and R. Vallotton for their kindly supplies of cysts.

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