

Cuticle heterogeneity as exhibited by *Pasteuria* spore attachment is not linked to the phylogeny of parthenogenetic root-knot nematodes (*Meloidogyne* spp.)

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SUMMARY

The cuticle is a major barrier prohibiting the infection of nematodes against micro-organisms. The attachment of bacterial spores of the nematode hyperparasite *Pasteuria penetrans* (PP1) to field populations of root-knot nematodes (RKN, *Meloidogyne* spp.) from Burkino Faso, Ecuador, Greece, Malawi, Senegal and Trinidad and Tobago were assayed in standard attachment tests. The attachment of spore population PP1 to different field populations of root-knot nematode showed that the rates of attachment differed between countries. Similar tests were also undertaken on *P. penetrans* spores from these countries against 2 species of RKN, *M. incognita* and *M. arenaria*. The results showed a high degree of variability in spore attachment with no clear distinction between the 2 species of nematode. It has been hypothesized that *Pasteuria* spore attachment is linked to nematode species designations and this study clearly shows that this is not the case. Further tests showed that variation in spore attachment was not linked to nematode phylogeny. The results therefore beg the question of how do parthenogenetic root-knot nematodes maintain cuticle variability in the face of such an aggressive hyperparasite.

Key words: bacteria, biological control, spore adhesion, variation.

INTRODUCTION

The nematode cuticle is a complex structure essential for locomotion, maintenance of morphology and protection against the environment. In animal-parasitic nematodes the cuticle is important in recognition by the host immune system, and it may have a similar role in resistance of plants to plant-parasitic nematodes. It is also a site of attachment for various bacteria and fungi that are parasitic on nematodes (Bird & Bird, 1991; Spiegel & McClure, 1995; Blaxter & Robertson, 1998). Interest in the pathogens of nematodes has increased because of the need for alternatives to anthelmintics and nematicides for controlling nematodes. However, relatively few of the many groups of micro-organisms which inhabit the soil have the ability to infect

nematodes and have potential as biological control agents (Stirling, 1991). Some of these have the ability to produce adhesive spores, and the initial contact between pathogen and nematode is the critical first step in infection which involves an interaction between the surface coat of the nematode and the adhesive material of the pathogen.

The *Pasteuria* group of Gram-positive endospore-forming bacteria are hyperparasites of plant-parasitic nematodes and water fleas (*Daphnia* spp). Four species of *Pasteuria* have been described which differ in their host ranges and virulence: (1) *P. penetrans* is parasitic on *Meloidogyne incognita* and probably other root-knot nematodes (Sayre & Starr, 1985), (2) *P. thornei* on *Pratylenchus brachyurus* (Sayre & Starr, 1988), (3) *P. nishizawae* which parasitizes cyst nematodes (Sayre *et al.* 1991) and (4) *P. ramosa* a parasite of water fleas *Daphnia* spp. (Sayre, Gherna & Wergin, 1983). All the economically important genera of plant-parasitic nematodes are parasitized by these bacteria (Sayre & Starr, 1988; Chen &

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Dickson, 1998), and *P. penetrans* is of particular interest as it has potential to control the root-knot nematodes (Stirling, 1991; Oostendorp, Dickson & Mitchell, 1991).

Many of the most damaging root-knot nematodes reproduce by mitotic parthenogenesis (Triantaphyllou, 1985; Evans, 1998), which effectively conserves their relatively homozygous genotype (Trudgill, 1997). This should make them highly susceptible to any bacterial parasite that can successfully infect. However, a major obstacle to the commercial development of these nematode parasites as biological control agents is their apparent specificity and host-range. Isolates of *P. penetrans* have been described which adhere only to a particular species of root-knot nematode or which adhere only to individual populations within a species (Stirling, 1985; Davies & Danks, 1992; Channer & Gowen, 1992). Most of these studies of *P. penetrans* spore attachment have been undertaken on an *ad hoc* basis using populations of nematode which were readily available. It has been suggested that attachment of spores is related to the species of nematode from which the spores were originally isolated (Davies, Kerry & Flynn, 1988) and it is claimed that the 3 major *Meloidogyne* spp. occurring in Japan can be identified using spores of *P. penetrans* stained with different reagents (Orui & Ozawa, 1999). However, this has not been established in any systematic way, thereby enabling these findings to be generalized over larger geographical areas and enabling comparisons to be made either within or between separate countries. As root-knot nematodes often occur as mixed species, and their cuticles have been shown to exhibit a high degree of heterogeneity (Davies & Danks, 1992; Davies, Redden & Pearson, 1994), simple identification techniques using *P. penetrans* spores could be particularly useful for testing the compatibility between different spore populations and the nematode populations present. Therefore, the objective of this study, which was part of a larger study investigating molecular variation in species of *Meloidogyne*, was to investigate the heterogeneity of *Meloidogyne* cuticle using spores of *P. penetrans* and to test the hypothesis that the interaction between isolates of *P. penetrans* was related to the major phylogenetic groupings of the root-knot nematodes.

MATERIALS AND METHODS

Attachment of a single Pasteuria population on field populations of root-knot nematodes

As part of a survey of *Meloidogyne* spp. in Burkina Faso, Ecuador, Greece, Malawi, Senegal and Trinidad and Tobago, field populations of *Meloidogyne* were obtained for use in a standardized spore-binding assay using a single isolate of *P. penetrans*. The isolate, designated PP1, originated in

California (Table 1), was chosen for its previously observed ease of culture and relatively high level of spore attachment. It was cultured on *M. incognita* Race 2 using the method of Stirling & Wachtel (1980) and dry tomato root powder was despatched to each of the participating laboratories. Suspensions of spores were prepared by grinding *P. penetrans*-infested tomato root powder (0.1 g) in tap water with a pestle and mortar to release the spores that were made up in a suspension of 25 ml. The populations of *Meloidogyne* were reproduced from a representative number of field sites in each of the participating laboratories on susceptible tomato in a glass/shade house. Samples of single females from these field populations were identified using native gel electrophoresis and staining for non-specific esterases (Fargette, 1987). Second-stage juveniles were hatched from 10 egg masses by placing them in tap water on a small tray at room temperature (Hooper, 1986). Samples (0.5 ml) of the spore suspension were then placed in a 24-well tissue culture dish and freshly hatched (2–3 days old) root-knot nematode juveniles (*ca* 100) were added to each well. After overnight incubation the nematodes were removed and 40 juveniles were assessed for spore attachment either using one (see Table 2) or the following indices: 1 = 0 spores per J2; 2 = 1–9 spores per J2; 3 = > 10 spores per J2 (spores adhering concave surface to the nematode cuticle). Data from these tests was used to construct a similarity matrix and a hierarchical, single linkage cluster analysis was computed (Digby & Kempton, 1987; Clark *et al.* 1992) using GENSTAT™ (1997). A limited number of females from each of these populations which were tested for the attachment of PP1 were identified using non-specific esterases. Having established the species composition of each population a similarity matrix of the populations was then also calculated. Principal co-ordinate analyses on each matrix was then done and the 2 sets of principal coordinates compared by Procrustes rotation to see if any relationship existed between the groupings of species of nematode and patterns of spore attachment.

Attachment of different isolates of Pasteuria on defined populations of root-knot nematodes

Experiment 1. Two populations of root-knot nematode *Meloidogyne incognita* (North Carolina State University, Race 2) and *M. arenaria* (North Carolina State University, Race 1) which are routinely cultured in the glasshouse at Rothamsted, and are known to have very different cuticles from their reaction to both *Pasteuria* and antibodies (Davies & Danks, 1992) were tested for their compatibility to field populations of *Pasteuria*, which were collected by each collaborator and sent to Rothamsted for testing. The *Pasteuria* populations were obtained

Table 1. Source and origin of *Pasteuria penetrans* populations

<i>Pasteuria</i> (designation)	Geographical origin	Nematode	Source
PP1	California, USA	<i>M. incognita</i>	S. R. Gowen, University of Reading
PP2 (ORS-92-Aus)	Australia	<i>Meloidogyne</i> spp.	G. Germani, IRD
PP3 (ORS-Bra)	Brazil	<i>Meloidogyne</i> spp.	G. Germani, IRD
PP4 (ORS-21414)	Senegal	<i>M. javanica</i>	M. T. Diop and T. Mateille IRD
PP5 (ORS-NC1-Ncal)	New Caledonia	<i>M. javanica</i>	T. Mateille IRD
PP6 (ORS-81-Sen)	Senegal	<i>M. javanica</i>	C. Netscher ORSTOM

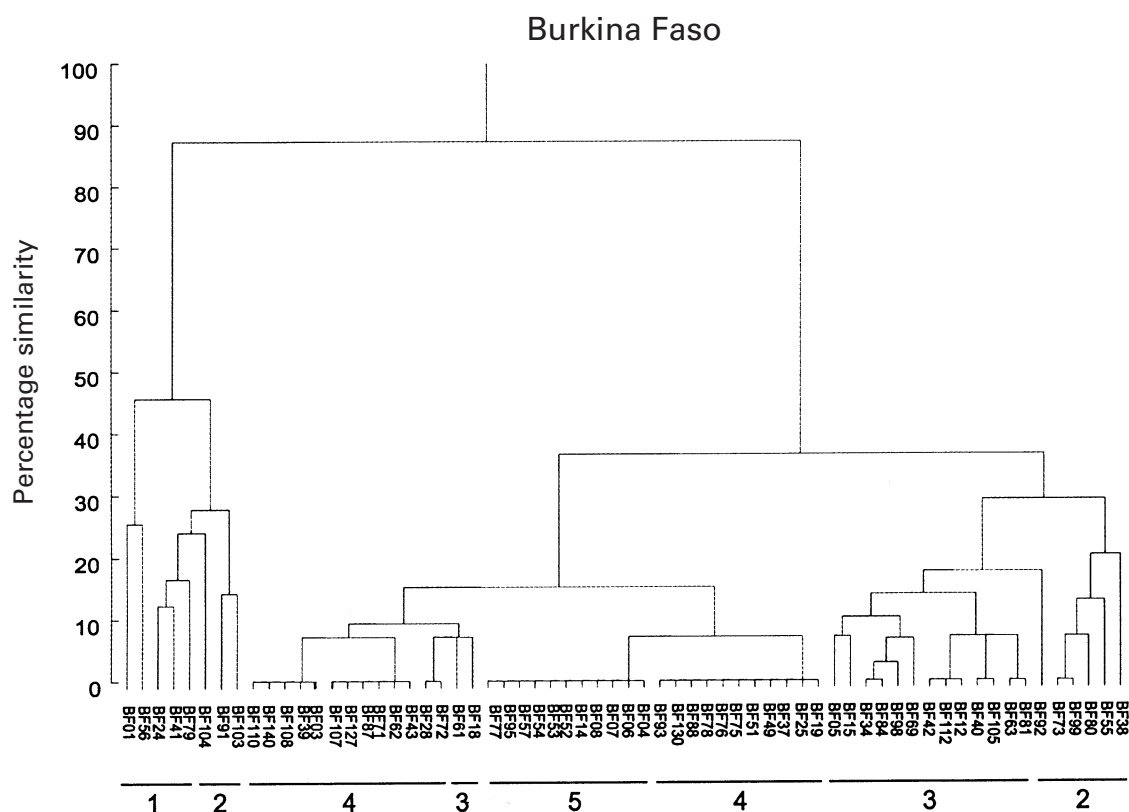


Fig. 1. Cluster analysis of categorical attachment data between 64 populations of root-knot nematode samples from Burkina Faso and spores of *Pasteuria penetrans* population PP1.

from naturally infected females of root-knot nematodes present on tomato roots that had been grown at field sites in soil containing spores of *Pasteuria*. Tomato roots from these sites were dried and milled into root-powder and samples of powder containing spores were sent to Rothamsted for testing. Attachment tests were performed with each of the root-knot nematode species against each *Pasteuria* isolate, either by following the method described above, or by placing juveniles (0.1 ml of 10^3 juveniles per ml of suspension) with 0.1 ml of 10^5 endospore suspension in Eppendorf tubes that had previously been coated with silane and spinning the samples in a centrifuge (Hermle Z 230 M) at 10000 g for a minimum of 3 min (Hewlett & Dickson, 1993).

Experiment 2. Single egg-mass isolates were set up from the root-knot nematode populations sampled in the different countries and identified by their esterase

phenotypes (Fargette, 1987) in IRD Montpellier. Populations of *M. javanica* (11), *M. incognita* (12), *M. arenaria* (6), *M. mayagensis* (3), *M. hispanica* (3) and *M. hapla* (1) were tested against 6 populations of *Pasteuria* (Table 1) as described above. Spore attachment was assessed using a high power microscope ($\times 400$) and attachment levels calculated on the basis of (a) mean number of spores per juvenile, assessed on all juvenile nematodes observed and (b) mean number of spores per juvenile, calculated using only those juveniles to which spores attached.

RESULTS

Attachment of a single Pasteuria population on field populations of root-knot nematodes

Cluster analysis of the attachment between *Pasteuria* population PP1 and a range of field populations of

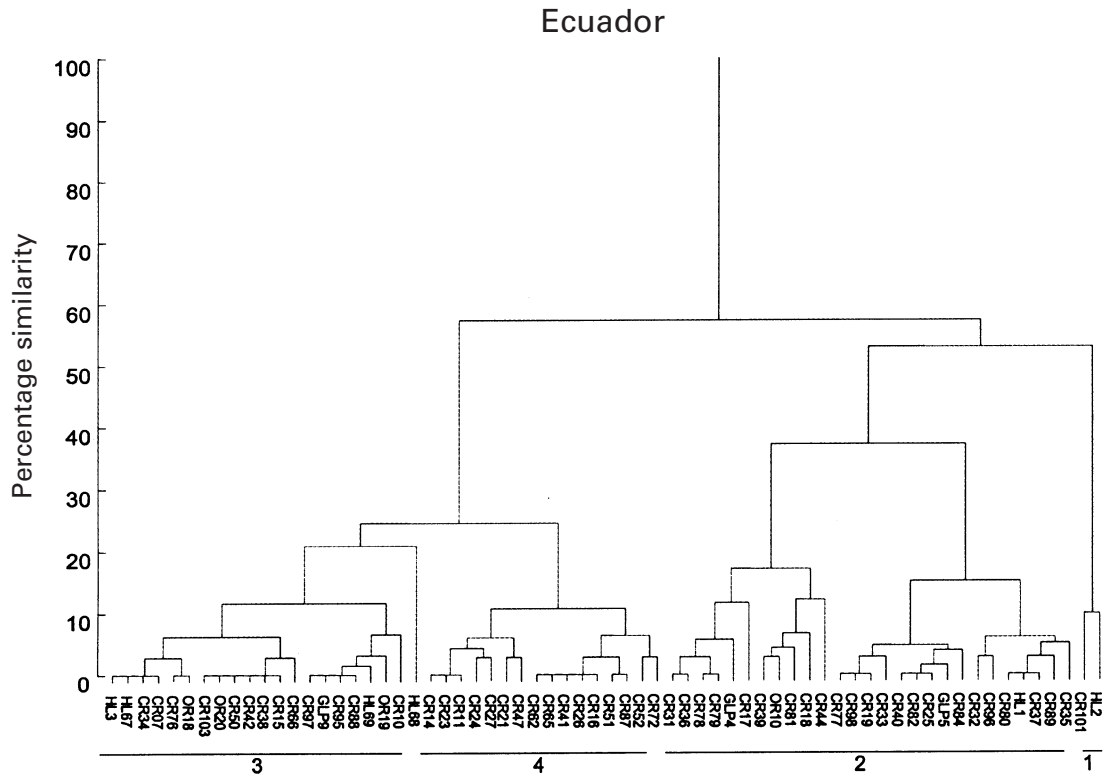


Fig. 2. Cluster analysis of categorical attachment data between 64 populations of root-knot nematode samples from Ecuador and spores of *Pasteuria penetrans* population PP1.

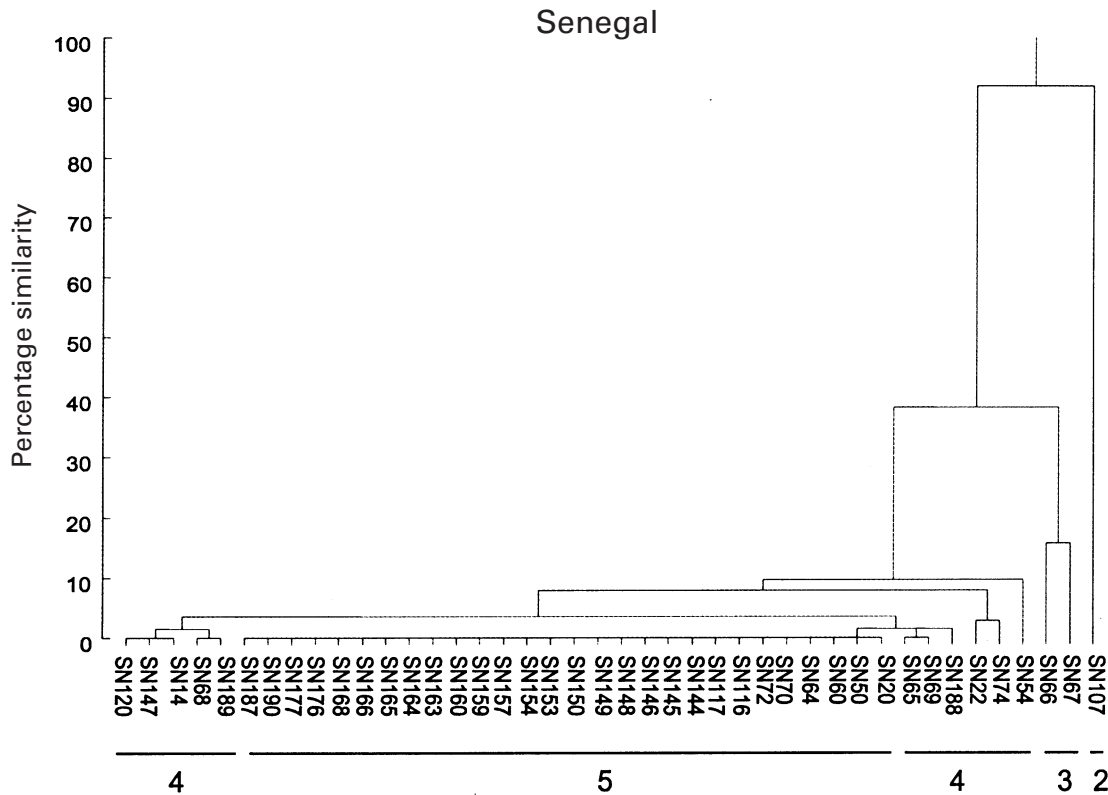


Fig. 3. Cluster analysis of categorical attachment data between 42 populations of root-knot nematode samples from Senegal and spores of *Pasteuria penetrans* population PP1.

root-knot nematodes from Burkina Faso, Ecuador, Greece, Malawi, Senegal and Trinidad and Tobago showed that within each country there was a

continuous range in the levels of attachment (Figs 1, 2 and 3; Greece, Malawi and Trinidad and Tobago data not shown as the number of sites tested were

Table 2. The percentage of root-knot nematode population samples from Burkina Faso (BF), Ecuador (EC), Greece (GC), Malawi (ML), Senegal (SN), Trinidad and Tobago (TT) occurring in each of 5 categories of levels of spore attachment of *Pasteuria penetrans* (PP1)

Attachment*	BF	EC	GC	ML	SN	TT
High (5)	7	3	0	0	0	0
Good (4)	14	41	0	100	5	7
Average (3)	23	32	40	0	5	23
Poor (2)	38	24	60	0	26	63
None (1)	18	0	0	0	64	7
<i>n</i>	64	64	15	9	42	13

* Attachment indices: High, < 25% juveniles with no spores; up to 30% juveniles with 1–9 spores and > 20% juveniles with > 10 spores; Good, < 60% juveniles with no spores; > 40% juveniles with 1–9 spores; 0 juveniles with > 10 spores; Average, 40–80% juveniles with no spores, < 60% with 1–9 spores; 0 with > 10 spores; Poor, 80–99% with no spores; < 20% with 1–9 spores; 0 with > 10 spores; None, no spores.

Table 3. Table of mean levels of attachment of 6 populations of *Pasteuria penetrans* when tested against 39 populations of root-knot nematode (*Meloidogyne* spp.)

Spores	PP1	PP2	PP3	PP4	PP5	PP6
Mean spores/J2*	6.3	0.4	2.1	3.3	5.6	7.4
(SE)	(9.6)	(0.6)	(2.5)	(4.3)	(8.1)	(10.8)
Mean spores/J2†	4.7	0.1	1.7	2.9	5.1	7.2
(SE)	(9.8)	(0.2)	(2.6)	(4.4)	(7.9)	(10.9)
% J2 no spores	26	64	36	33	28	28

* Mean calculated using only those J2 with spores.

† Mean calculated using all J2.

< 16). It was possible to distinguish 5 broad categories of attachment: (5) high attachment, where less than 10% juveniles have no spores, up to 30% juveniles had between 1 and 9 spores, and at least 60% juveniles had greater than 10 spores; (4) good attachment, where less than 60% juveniles had no spores, more than 40% juveniles had between 1 and 9 spores no individual juveniles had over 10 spores; (3) attachment, between 40 and 80% of juveniles have no spores, less than 60% had between 1 and 9 spores, and no juveniles had greater than 10 spores; (2) poor attachment, between 80 and 99% of juveniles had no spores, less than 20% had between 1 and 9 spores and no juveniles had greater than 10 spores, (1) no attachment, no juveniles had any spores. Each root-knot population was placed in 1 of these 5 categories. The proportion of RKN nematodes occurring in each category for each of the respective countries can be seen in Table 2. Spores attached to all populations of RKN tested from

Ecuador, Greece and Malawi; however, the number of RKN populations tested for the latter 2 countries were only 15 and 9 respectively. High levels of spore attachment were observed on RKN in Burkina Faso (Fig. 1) and Ecuador (Fig. 2) where many more RKN were tested. The level of PP1 spore attachment to nematodes from Senegal (Fig. 3) was generally lower than in any of the other countries and no spores at all were observed on 64% of the RKN tested. All RKN populations from Malawi were equally susceptible to PP1 spore attachment, although only 9 RKN populations were tested (Table 2); in attachment tests using PP1 spores from the remaining countries less than 2% of the sites showed all the nematodes from those sites to be susceptible to spore attachment (Table 2). It should be noted that overall 78% of second-stage juveniles tested were not observed to be susceptible to the attachment of PP1 spores, and 15% of the sites, across all countries, contained populations of juveniles where no spores attached to any of the second-stage juveniles (Table 2). Principal co-ordinate analysis of attachment data, using data where the species composition was known, followed by Procrustes rotation did not reveal any correlation between attachment of PP1 spores and species composition.

Attachment of different isolates of Pasteuria on defined populations of root-knot nematodes

Initial assessments of levels of spore attachment calculated as (a) mean number of spores per juvenile, based on all juvenile nematodes observed and (b) mean number of spores per juvenile, calculated for only those juveniles to which spores attached showed that there was very little difference in the means calculated irrespective of the method used (Table 3). In the experiment where the attachment of 6 populations of *Pasteuria* to 35 populations of RKN, representing 6 separate species, was tested, based on the mean number of spores adhering, the groupings of the nematode species were independent of levels of spore attachment (Fig. 4).

In further tests, 25 populations of *Pasteuria* spores were assessed for their attachment to second-stage juveniles of a single egg-mass population of *M. arenaria* and a single egg-mass population of *M. incognita*. The level of spore attachment for each *Pasteuria* population was assessed on 20 juveniles of each nematode species as 0 spores, 1–9 spores or greater than 10 spores. As only 2 spore populations produced levels of attachment with juveniles having greater than 10 spores the data were analysed as 2 categories, those with spores and those without spores. The percentage of individuals with no spore attachment was then plotted for each of the 2 species of root-knot nematode (Fig. 5). Generally, levels of spore encumbrance were greater for *M. incognita*

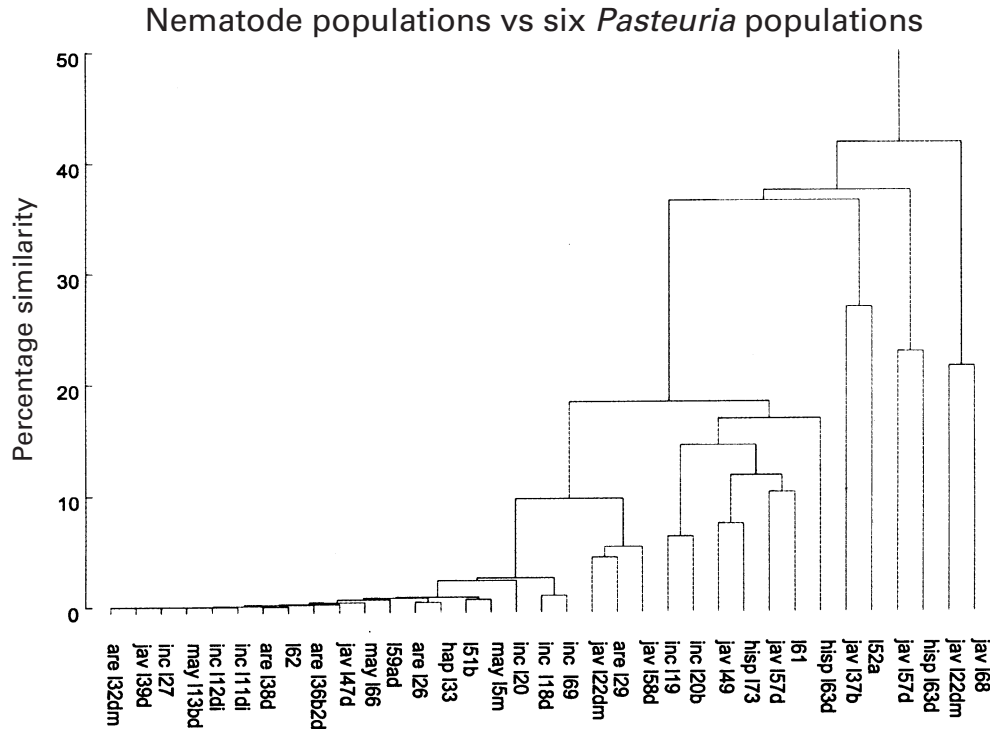


Fig. 4. Cluster analysis of mean spore attachment between 35 populations of root-knot nematode, representing 6 major species, *Meloidogyne arenaria* (are), *M. incognita* (inc), *M. javanica* (jav), *M. mayaguensis* (may), *M. hapla* (hap) and *M. hispanica* (hisp) and 6 populations of *Pasteuria penetrans*.

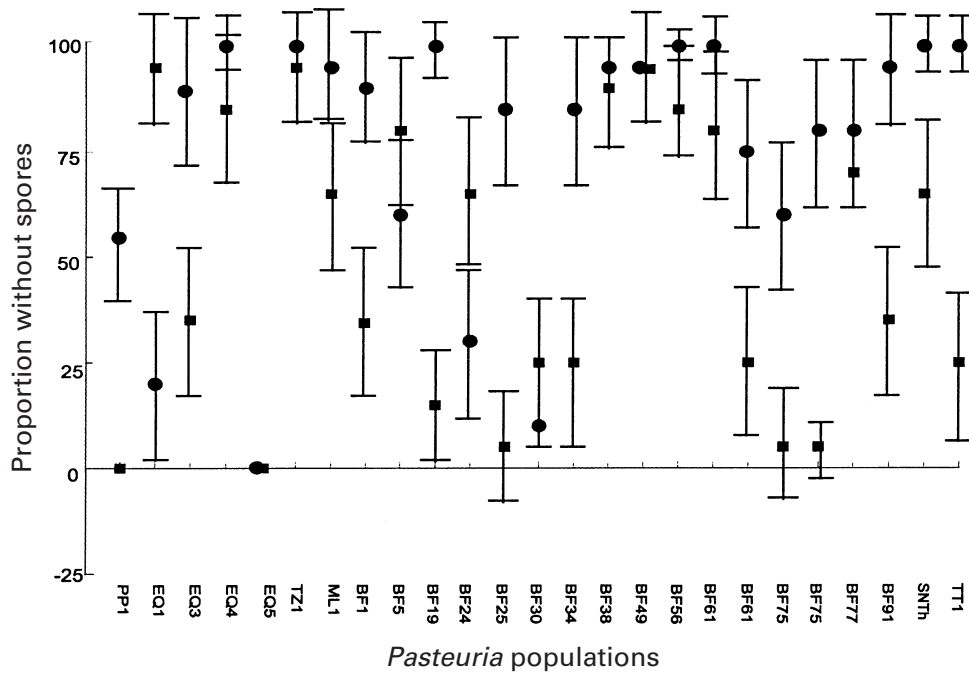


Fig. 5. Percentage of juveniles of *Meloidogyne arenaria* (●) and *M. incognita* (■) to which no spores attached in tests against 25 populations of *Pasteuria penetrans*.

than *M. arenaria*, but there was much variation between populations. Binding by 5 spore populations, EQ1, BF19, BF25, BF75 and TT1 to juveniles of the 2 species differed by > 75% in the absolute number of juveniles encumbered. Only 2 spore populations produced the same level of attachment on each species of nematode, spores of EQ5

adhered to every juvenile (100%) and spores of BF49 adhered to only 5% of both species (Fig. 5). However, populations were identified which had a high proportion of spores recognizing *M. incognita* and a low proportion recognizing *M. arenaria* (e.g. BF25) and *vice versa* (e.g. EQ1). There is also a continuum in the levels of spore attachment between

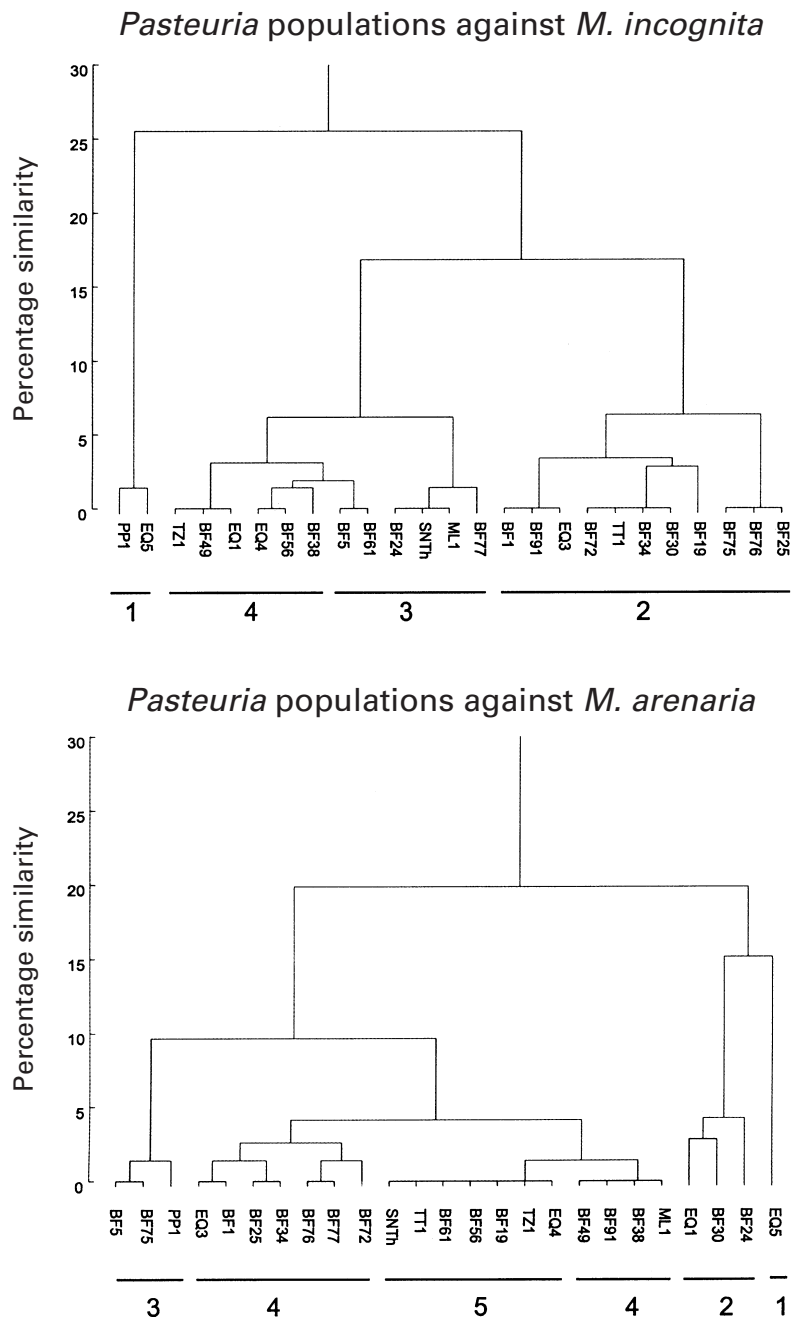


Fig. 6. Cluster analysis of categorical attachment data between 25 populations of *Pasteuria penetrans* when tested against *Meloidogyne incognita* and *M. arenaria*.

these 2 extremes (Fig. 5). Cluster analysis of the *P. penetrans* spore populations on each of the different species of nematode brings together different clustering of spores (Fig. 6). For example, when tested against *M. incognita* spore populations TZ1, BF49 and EQ1 share a similarity of 100%; however, when tested against *M. arenaria*, EQ1 shares 100% similarity with BF30, BF24 and EQ5 and therefore belongs in a very different grouping.

DISCUSSION

This paper reports 3 separate spore attachment studies; the first investigated the cuticle hetero-

geneity amongst different field populations of root-knot nematodes using 1 standardized population of *P. penetrans*, the second investigated *P. penetrans* spore heterogeneity using 2 standardized root-knot nematode populations, the cuticle of one of which is recognized by a polyclonal antibody while the other is not (Davies & Danks, 1992), and the third investigated the attachment of 6 different populations of *P. penetrans* spores against a series of single egg-mass populations of root-knot nematodes. Previous studies have shown second-stage juveniles to be encumbered with spore loads ranging from zero through to more than 90 spores per individual J2 (Stirling, 1985; Davies *et al.* 1988). However, when spore loads exceed 40 spores per J2 accurate

quantification became increasingly difficult and time consuming and hence the collection of categorical data. We used 40 second-stage juveniles in our tests and this is within the range of 25–50 individual nematodes recommended for a incubation bioassay by Chen & Dickson (1997). However, the levels of attachment were generally lower with our field populations of nematodes than those observed for some glasshouse populations, where levels of attachment of greater than 40 spores per J2 were frequently observed when high concentrations of spores and long exposure times were used (Stirling, 1985; Davies *et al.* 1988). These differences either reflect the lower concentration of spores used or, more likely, the complexity of the recognition process involved in the attachment process.

The attachment of spore populations PP1 to different field populations of root-knot nematode showed that the rates of attachment differed between the different countries. There was also a wide level of variation in the levels of spore attachment in the tests using second-stage juveniles derived from 2 single egg mass populations. If the attachment of spores followed a phylogenetic relationship one would expect that, in a cluster analysis of nematode populations using spore attachment, the nematodes would form clusters related to their phylogeny, with each species forming a discrete cluster. This was not the case and single egg mass populations of nematodes that are phylogenetically very distinct can have very similar, or conversely very dissimilar, levels of spore attachment. The data reported here clearly show that variation in spore attachment was independent of the nematode species being tested and therefore not linked to nematode phylogeny.

The results presented here do not support the suggestions of Orui & Ozawa (1999) who found that *M. arenaria*, *M. incognita* and *M. hapla* could be correctly identified by the spore binding properties of 3 different *P. penetrans* populations. For this approach to be used as a technique for the identification of root-knot nematodes in general, the assumption has to be made that spore populations can be selected whose binding properties reflect the phylogeny of the different species of *Meloidogyne*. The data presented in this paper do not suggest that this will be feasible. In an earlier paper Orui (1997) reported that one of the populations of *P. penetrans* spores (MHP) previously used in the identification of root-knot nematodes did in fact have a relatively broad host range, a finding which supports the view that predictions of host range cannot be made from knowledge of the nematode species and *vice versa*.

This study and earlier immunological studies using monoclonal antibodies (Davies & Redden, 1997) have revealed a high degree of heterogeneity both within and between different populations of *P. penetrans* spores and it has been shown in baiting experiments that this spore heterogeneity reveals a

concomitant cuticle heterogeneity between populations of *Meloidogyne* (Davies *et al.* 1994). *Meloidogyne incognita*, *M. javanica* and *M. arenaria* all reproduce by mitotic parthenogenesis (Triantaphyllou, 1985; Evans, 1998). Parthenogenesis is thought to have arisen with increased homozygosity because limited mobility in soil led to highly inbred populations where sexual reproduction no longer had any advantages (Dalmasso & Bergé, 1983; Trudgill *et al.* 1996; Trudgill, 1997). Concomitantly, the disadvantages of homozygosity may have been ameliorated by polyploidy. Such a reproductive strategy in biotrophic parasites is likely to increase host specialization and ultimately lead to an evolutionary 'dead end' (Trudgill *et al.* 1996; Evans, 1998). However, different species of root-knot nematodes have managed to maintain wide, and overlapping, host ranges and this has long been recognized to cause problems in their classification (Netscher & Taylor, 1979). More recently, molecular data have also confirmed the close relationship between different species of the mitotic parthenogenetic *Meloidogyne* (Blok *et al.* 1997; Stanton, Hugall & Moritz, 1997). It has been suggested that this reflects a relatively recent monophyletic origin, and that their wide distribution is a result of the activities of man (Trudgill *et al.* 1996). Although populations of root-knot nematodes appear to be comparatively homogeneous from studies using DNA, a considerable level of variation in the binding of *Pasteuria* spores is observed. This demonstrates that there is a considerable range of cuticular heterogeneity with regard to these characteristics exploited by the bacterium for attachment and suggests the ability of the nematodes to generate levels of cuticular heterogeneity at a faster rate than would be suggested by phylogenetic ontogeny. Observations of biological variation and polymorphism in host-parasite recognition systems usually underestimate the levels of the underlying biochemical variation (Frank, 1994) which have been presumably generated through an evolutionary 'arms race' between host and, in this instance, hyperparasite. For a parthenogenetically reproducing nematode, which can be considered as a clonal organism, this clearly presents a problem. The generation of cuticular variation, either through the switching of surface antigens (Grenache *et al.* 1996) or possibly the role of polyploidy, in these parthenogenetic nematodes is a crucial question that could shed light on the organization of the root-knot genome and how these biotrophic endophytes maintain a wide host range.

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