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Characterisation of *Pasteuria* Isolated from *Heterodera cajani* Using Morphology, Pathology and Serology of Endospores

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Summary

Pasteuria spp are potentially useful natural parasites of nematodes An isolate of *Pasteuria*, recovered from the pigeonpea cyst nematode, *Heterodera cajani*, was characterised using host range, spore morphometrics, and serology The spores of this isolate had a mean diameter of 2.36 µm, the smallest described so far on nematodes Based on spore encumberance on nematode cuticle, *Globodera rostochiensis*, *G pallida*, *Heterodera glycines*, *H trifolii*, *H schachtii*, and *Rotylenchulus reniformis* were hosts and *H carotae*, *H galeopsidis*, *Meloidogyne arenaria*, *M incognita*, *M javanica*, *M graminicola*, *M oryzae*, *M triticoryzae*, *M sasseri*, *M graminis*, and *M naasi* were non hosts Spore attachment on the nematode body was random except on *H trifolii*, where the spores selectively attached to the head region Marked heterogeneity in spore size and level of spore attachment within populations of nematode species was observed Variation in antigens present on the spore surface was confirmed by differential recognition of spores by monoclonal and polyclonal antibodies and Western blot analysis The parameters important in characterising species of *Pasteuria* are discussed in the light of the data reported The results suggest that host range and spore morphometrics are not adequate for the characterisation of species

Key words Bacteria – Biological control – Cyst nematodes – Host range – Immunology – Morphology – Pigeonpea – Reniform nematode – Root-knot nematodes

Introduction

The *Pasteuria* group of gram-positive, endospore-forming bacteria are hyperparasites of plant-parasitic nematodes and water fleas (*Daphnia* spp). All the economically important genera of plant-parasitic nematodes have an association with these bacteria (*Sayre* and *Starr*, 1988) and some isolates have potential for use in the biomanagement of nematode pests (*Stirling*, 1991). The taxonomy of the hyperparasite remains unclear but it is probably made up of a number of species and isolates which differ in their host ranges and virulence. The life-cycles, host ranges and spore morphologies are considered important characters in classifying these bacteria (*Sayre* et al., 1991; *Ciancio* et al., 1994) and using these criteria, four species of *Pasteuria* have been described so far: 1. *Pasteuria penetrans* (Thorne) *Sayre* and *Starr*, 1985, parasitic on *Meloidogyne incognita* and other root-knot nematodes, 2. *P. thornei* *Starr* and *Sayre*, 1988, on *Pratylenchus brachyurus*, 3. *P. nishizawae* *Sayre*, *Wergin*, *Schmidt* and *Starr*, 1991, which parasitises the cyst nematodes *Heterodera glycines*, *H. elachista*, *H. trifolii*, and *Globodera rostochiensis* (*Sayre* et al., 1991), and 4. *P. ramosa* *Metchnicoff*, 1888, a parasite of water fleas *Daphnia* spp. (*Sayre* et al., 1983).

Two populations of *Pasteuria* described from India are distinct in their relatively wide host range across populations of root-knot, cyst, and reniform nematode species (*Bhattacharya* and *Swarup*, 1988; *Sharma*, 1985; *Sharma* and *Swarup*, 1988; *Sharma* and *Sharma*, 1989). The bacterial population originally isolated from *Heterodera mothi* in northern India also parasitised populations of *Heterodera cajani*, *H. graminis*, *H. sorghi*, *H. zeae*, *H. avenae* and *Meloidogyne incognita* (*Sharma*, 1985; *Bhattacharya* and *Swarup*, 1988; *Sharma* and *Swarup*, 1988). Another field population of *P. penetrans* parasitic on *H. cajani*, *M. javanica*, and *Rotylenchulus reniformis*

was found in southern India (*Sharma* and *Sharma*, 1989). The objective of this study was to describe the *Pasteuria* population from southern India based on spore morphometrics, pathology and serology.

Materials and Methods

Nematodes and bacterial endospores

The *Pasteuria* population was collected from females of *Heterodera cajani* and designated PPHc. The nematode females were recovered from soil samples collected from a pigeonpea field at the research farm of the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) Asia Center, Andhra Pradesh, India (*Sharma* and *Sharma*, 1989). The bacterial population was cultured for over a year on *H. cajani* growing on pigeonpea cultivar ICPL 87 in 15-cm diameter pots in a glasshouse. The spores of *Pasteuria* were obtained from the nematode females for use in different experiments. *Heterodera cajani* females infected with a population of *Pasteuria* were crushed in 0.5 ml water in 1.5 ml Eppendorf tubes and the number of spores was determined with a haemocytometer. The spore density per female was estimated in three separate batches of 13, 7, and 17 females. Range and mean spore density per female were calculated. Spores were also collected from a population of *Pasteuria penetrans* (PP1) routinely cultured on *Meloidogyne incognita* race 2 (originating from North Carolina State University, USA) at Rothamsted Experimental Station (*Davies* et al., 1994).

Endospore attachment assay and pathology

To obtain a standardised attachment assay, the rate of *Pasteuria* spore encumbrance on second-stage juveniles of *H. cajani* was determined by treating 0.1 ml of 10^3 juveniles per ml suspension with 0.1 ml of 10^3, 10^4, 10^5, and 10^6 endospore suspension in previously salinized Eppendorf tubes (*Sambrook* et al., 1989). The Eppendorfs were spun in a centrifuge (Hermle Z 230 M) at 10,000 g for a minium of three minutes (*Hewlett* and *Dickson*, 1993). The juveniles were removed from the tubes with a pipette and samples of 20 juveniles in a drop of water were placed on glass slides and observed at high magnification with a BH 2 Olympus microscope. The number of spores adhering to each nematode was counted and the experiment was repeated. The range, mean, and standard error were calculated and a spore concentration for the standard attachment assay determined. Populations of second-stage juveniles of *Globodera rostochiensis*, *G. pallida*, *Heterodera glycines*, *H. carotae*, *H. galeopsidis*, *H. trifolii*, *H. schachtii*, *R. reniformis*, *Meloidogyne javanica*, *M. graminicola*, *M. oryzae*, *M. triticoryzae*, *M. sasseri* and *M. naasi* were tested form their ability to attach to the *Pasteuria* population. Cysts of *G. rostochiensis* and *G. pallida* were incubated at 18 °C in potato root diffusates. Cysts of other nematodes were incubated at optimum temperatures. In some cases (*H. carotae*, *H. schachtii*, and *H. galeopsidis*), juveniles were obtained by breaking the cysts. To determine the host range, 0.1 ml of 10^3 second-stage juveniles per ml suspension of each of these nematodes and vermiform stages of *R. reniformis* were separately exposed to 0.1 ml of endospore suspension in water in salinized Eppendorf tubes. The Eppendorf tubes were centrifuged and nematodes were observed with a microscope as described previously. Number of juveniles with spores attached, range and mean spore number per juvenile, and the standard errors of means were calculated. Frequency distributions of spores on each of the different nematode populations were constructed according to the following groupings: 1 = no spore, 2 = 1–6, 3 = 7–12, 4 = 13–18, 5 = 19–24, and 6 = 25 and more spores per nematode.

Spore morphology and morphometrics

Light microscopy. Second-stage juveniles of *H. cajani* encumbered with spores of the *Pasteuria* population were mounted in water on a glass slide and spores were observed at × 1000 magnification. Measurements of endospore diameter and central body diameter were taken using an eyepiece micrometer on an Olympus BH 2 microscope.

Scanning Electron Microscopy (SEM) (*Shepherd* and *Clark*, 1986). The encumbered juveniles of *H. cajani* were fixed in 3.0% glutaraldehyde in 0.05 M phosphate buffer (pH 7.4), dehydrated in ethanol series, critical point dried using carbon dioxide, mounted on aluminium stubs, sputter coated with gold and examined with a Hitachi S 450 scanning electron microscope at 10 kV. Morphometric data were obtained by measuring images on the photomicrographs. Mean endospore diameter, mean central body diameter, endospore height, endospore flatness (ratio of height and diameter), proportion of spore occupied by the central body (ratio of central body diameter and spore diameter), and standard errors of means were calculated.

Coulter Counter Studies. To assess the spore size and distribution in a large sample, a Coulter Counter™ (model ZM) was used. It operates on the principle of electrical zone sensing. The Coulter Counter consisted of a main electronic unit, a sampling stand, and a vacuum control unit. Orifice tubes which could measure particle size between the range of 1 and 42 µm were used. Electrolyte (0.9% sodium chloride) solution was filtered before use and a background count was performed to establish that the electrolyte was free from any contaminating particles. Endospores of *Pasteuria* were collected from infected *H. cajani* females. The endospore sample was dispersed in the electrolyte on the sample platform and the stirrer was set to maximum speed to avoid any air bubbles. The reset/count control on the panel was set to count total number of spores as well as to determine their size.

Serological studies

Indirect immunofluorescence. Monoclonal antibodies produced against a population of *P. penetrans* (PP1) isolated from *M. incognita* (*Davies* et al., 1994) were tested for their ability to recognize *Pasteuria* spores obtained from *H. cajani* females. A clean spore suspension was collected from infected nematode females and a 15 µl spore suspension was added to multiwell slides (ICN Flow) coated with poly-1-lysine (*Harlow* and *Lane*, 1988). Spores were allowed to adhere to the slides and the slides were incubated separately in either tissue culture supernatants containing five monoclonal antibodies (*Davies* et al., 1994) or a rabbit polyclonal antibody (*Persidis* et al., 1992) for two hours at 37 °C in a humid chamber. Recognition of spores by the antibodies was done by probing the spores with secondary antibodies, anti-mouse or anti-rabbit as appropriate, conjugated to fluorescein isothiocynate (FITC) (*Davies* et al., 1994). Spores not exposed to the first antibody but incubated in the second FITC antibody conjugate served as negative controls. Immunofluorescence intensity on 25–30 spores taken at random was recorded (*Davies* et al., 1994).

Polyacrylamide gel electrophoresis and *immunoblotting*. Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate (SDS-PAGE)-was performed using a 12% (w/v) separating gel (pH 8.8) and 4% (w/v) stacking gel (pH 6.8). Spores (10^6 in 100 µl water) of two populations of *Pasteuria* (PPHc and PP1) were diluted 1:1 (v/v) with sample buffer (50 mM Tris/HCl pH 6.8, 2% SDS w/v, 2% 2-ME, 10% glycerol and 0.002% bromophenol blue w/v) and heated for 2 min at 100 °C before 20 µl of the solution was loaded onto the stacking gel. Prestained SDS-PAGE molecular weight markers (Sigma; Cat No SDS-7B) were

run on each gel. After electrophoresis the gel slabs were electro-blotted onto nitrocellulose membranes (Bio-Rad) in continuous buffer (*Davies* and *Lander*, 1992) using a Semi-phor™ blotting system (Hoeffer Scientific Instruments). Membranes were blocked with 2% BSA in PBST (2% bovine serum albumen w/v and 0.05% v/v Tween in PBS).

Results

Endospore attachment assay and pathology

Nematode females infected with *Pasteuria* were dull yellow, as compared to healthy shining white or brown cysts of *H. cajani*, and infected females contained between 0.5×10^5 and 0.8×10^5 spores per female (mean 0.6×10^5 per female). Spores readily attached to second-stage juveniles (Table 1), and the attachment of spores to each juvenile increased 10-fold when the spore density was increased from 10^4 to 10^5; a further increase in spore density to 10^6 resulted in 5-times more adhesion of spores to juveniles. All subsequent attachment tests were done in a final spore concentration of 5×10^5 spores per ml. The endospores attached to juveniles of *G. rostochiensis*, *G. pallida*, *H. glycines*, *H. trifolii*, *H. schachtii*, and vermiform stages of *R. reniformis* in addition to juveniles of the original *H. cajani* host. Mean number of spores per nematode was greatest on *G. pallida* and smallest on *H. glycines* (Table 2 and 3). All the juveniles of *G. pallida* and *G. rostochiensis* were encumbered with spores; however the spore number per juvenile varied markedly (Table 2 and 3). The relative frequency distribution of spore encumbered juveniles indicated a high level of interspecific and intraspecific variation in spore attachment; for example, 65% of juveniles of *G. pallida* had more than 12 spores attached while 90% of juveniles of *G. rostochiensis* had 12 or less spores attached per juvenile. In contrast, *R. reniformis* had 5% of the population with more than 24 spores per individual, whereas 55% of the population were not encumbered with any spores. No spore attachment was found on juveniles of 13 populations of *Meloidogyne* species or *H. carotae* and *H. galeopsidis* despite increasing centrifugation time (Table 2).

Table 1. Effect of the spore density of *Pasteuria*, isolated from females of *Heterodera cajani*, in suspension on the numbers attached to juveniles of *Heterodera cajani*

Spore density per ml	Number of spores attached	
	Range	Mean (SE)
1×10^6	8–44	22.3 (2.15)
5×10^5	2–25	9.9 (1.26)
1×10^5	0–14	4.4 (0.82)
5×10^4	0–6	1.3 (0.36)
1×10^4	0–2	0.4 (0.15)

Table 3. Range and mean density of *Pasteuria* spores attached to vermiform stages of six nematode species in host range tests

Nematode	Number of spores attached	
	Range	Mean (SE)
Globodera		
pallida	2–37	17.3 (2.08)
rostochiensis	2–16	3.5 (0.86)
Heterodera		
glycines	0–2	0.5 (0.17)
schachtii	0–9	2.0 (0.62)
trifolii	1–8	2.9 (0.45)
Roylenchulus		
reniformis	0–51	5.7 (2.62)

Nematode	Origin	% nematodes with					
		0	1–6	7–12	13–18	19–24	>24
Globodera							
pallida	UK	0	15	20	30	20	15
rostochiensis	UK	0	35	55	10	0	0
Heterodera							
cajani	India	0	0	15	30	25	30
glycines	USA	85	15	0	0	0	0
schachtii	UK	65	30	5	0	0	0
trifolii	UK	30	65	5	0	0	0
Rotylenchulus							
reniformis	India	65	25	10	0	0	5

Table 2. Relative frequency distribution of number of *Pasteuria* spores attached to different populations of nematodes in standard attachment assays containing 5×10^5 endospores

The following nematodes were all tested but no spores were observed to adhere: *Heterodera carotae* (UK), *H. cruciferae* (UK), *H. galeopsidis* (UK), *H. goettingiana* (UK); *Meloidogyne arenaria* R1, *M. graminicola* (Bangladesh), *M. graminicola* (India); *M. graminicola* (Ivory Coast), *M. graminicola* (Surinam), *M. graminicola* (USA), *M. incognita* R2 (USA), *M. javanica* R1 (India), *M. javanica* R3 (India), *M. naasi* (origin unknown), *M. oryzae* (origin unknown), *M. sasseri* (USA), *M. triticoryzae* (India)

Spore morphology and morphometrics

Several different methods were used to measure spore size; light microscopy and SEM were labour intensive techniques but could be used for obtaining estimates on small numbers of spores. There was a large degree of variation in the morphology of the endospores; measurements obtained by SEM were smaller than those obtained by light microscopy (Table 4). The endospore diameter, as measured by SEM, was between 1.85 μm and 2.54 μm and height ranged between 0.73 μm and 1.77 μm (Table 4). Endospore flatness between 0.29 and 0.40 gave spores a saucer shaped appearance (Fig. 1c), while many with flatness ratio greater than 0.5 were typically cup-shaped (Fig. 1a and 1b). Mean diameter of the central body wa 48% of the spore diameter and mean spore height wa about 56% of mean spore diameter; ratio of spore heigh and diameter ranged between 0.29 and 0.90 (Table 4). The majority of spores had lost their exosporium exposin the top of the central body and episporial fibers (Fig. 1t,

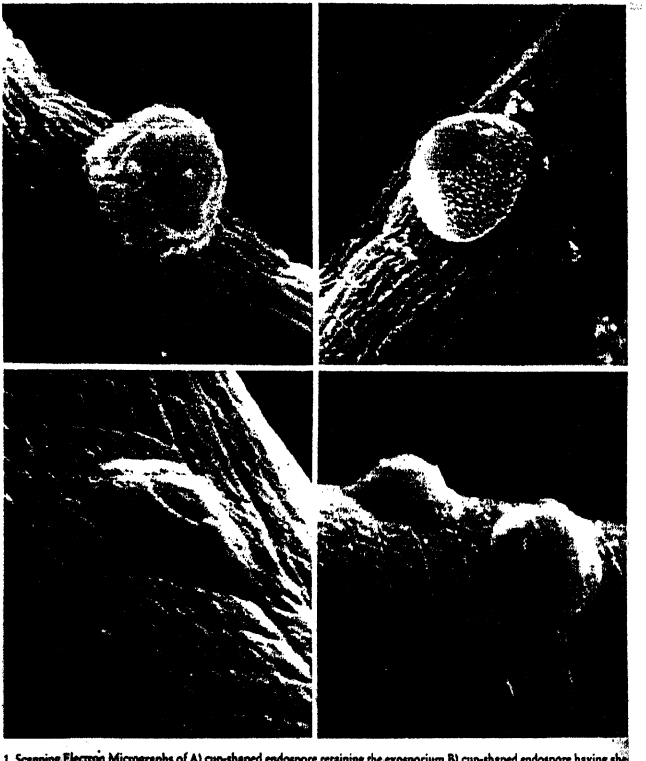

Fig. 1. Scanning Electron Micrographs of A) cup-shaped endospore retaining the exosporium B) cup-shaped endospore having she exosporium C) flattened saucer-shaped endospore embedded into the nematode cuticle D) upper endospore cup-shaped, lospore possible intermediate stage of attachment between the cup- and the saucer-shaped endospore.

Table 4 Measurements of *Pasteuria* endospores with scanning electron microscope and light microscope

Parameter	Range	Mean	(SE)
Spore[1]			
Diameter[3]	1 85–2 54	2 2›	(0 08)
Diameter[4]	2 00–2 80	2 36	(0 06)
Height[3]	0 73–1 77	1 23	(0 01)
Height[3]/diameter[3]	0 29–0 90	0 56	(0 07)
Central body[2]			
Diameter[3]	0 95–1 30	1 07	(0 04)
Diameter[4]	1 00–1 60	1 33	(0 04)
Central body/spore			
Diameter[3]	0 40–0 62	0 48	(0 02)
Diameter[4]	0 42–0 70	0 57	(0 02)

[1] Spore diameter measurements are inclusive of parasporal fibers
[2] Spore diameter measurements exclude parasporal fibers
[3] Measurements (μm) with scanning electron microscope
[4] Measurements with light microscope

1c and 1d), but a few spores adhering to nematodes had not lost their exosporium (Fig 1a) The Coulter counter was useful for obtaining a size distribution on a large number of spores, the diameter of over 107 K particles was measured and over 99% of these had a diameter of 3 4 um or less Unfortunately, the orifice of tubes to meas ure particle size smaller than 3 4 μm could not be used as they became blocked and required repeated cleaning

Serology

Indirect immunofluorescence of spores showed that the polyclonal antibody (Pab PP1) recognised all the spores whereas the monoclonal antibodies recognised only up to 15% of the spores (Table 5) Western blot analysis using Pab PP1 showed several large differences between spore extracts of *Pasteuria* from root knot nematodes (*M incog nita*) and those from *H cajani* although shared antigens between the two populations of spores were also present (Fig 2)

Table 5 Indirect immunofluorescence of *Pasteuria* spores (per centage) from *Heterodera cajani* using monoclonal (Mab) and polyclonal (Pab) antibodies raised against whole spores of *Pas teuria* from *M incognita* (*Persidis* et al , 1992)

Antibody	% positive	% negative
Mab 12	12	88
Mab 53	10	90
Mab 84	12	88
Mab 117	15	85
Mab 134	0	100
Pab PP1	100	0
Control	0	100

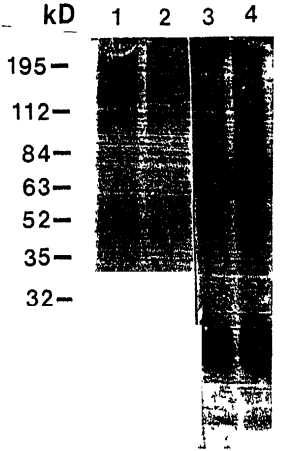

Fig 2 Western blot of *Pasteuria* spores isoalted from *Heterodera cajani* (lanes 1 and 2) and *Meloidogyne incognita* (lanes 3 and 4) probed with a polyclonal antibody raised to whole *Pasteuria* spores isolated from *M incognita* (Persidis et al 1992)

Discussion

The *Pasteuria* spores recovered from females of *H ca jani* were different from the descriptions of those from the three *Pasteuria* species hitherto reported on plant parasitic nematodes (*Sayre* et al , 1991) the endospores were much smaller than those of other *Pasteuria* so far described (Table 4 and 6), and, were also able to adhere to several different nematode genera (Table 3) Spore sizes measured by SEM were slightly smaller than those obtained by light microscopy, due to slight shrinkage of the specimens dur

Table 6 Endospore diameter of *Pasteuria* populations reported on *Heterodera* species

Heterodera	Spore diameter (range)	Reference
avenae	NA (4–5)	*Davies* et al (1990)
cajani	NA (4–5)	*Sharma* and *Sharma* (1989)
fici	4 9 (4 5–5 0)	*Abrantes* and *Vovlas* (1988)
goettingiana	5 0 (4 4–5 3)	*Sturhan* et al (1994)
glycines	5 3 NA	*Sayre* et al (1991)
glycines	5 0 NA	*Noel* and *Stanger* (1994)
mothi	5 0 NA	*Sharma* and *Swarup* (1988)

NA, not available

ing dehydration and fixation. *Ciancio* et al. (1994) measured spore diameters of *Pasteuria* populations on 52 different species of nematodes; the smallest endospore was found on *Acrobeloides* species and had a mean diameter 2.7 µm, which is larger than the spores observed from *H. cajani* in this study. There was a marked variation in the ratio of spore height to diameter on spores adhering to nematodes. Some spores were raised, "cup-shaped" while others were flatter, "saucer-shaped", with a smaller height to diameter ratio. There are two possible explanations for these observations, either the spore populations are made up of two distinct morphological types, or a maturation process occurs with the "cup-shaped" spores settling and spreading onto the surface of the nematode cuticle during the attachment process. Similar variation in the shape of spores adhering to nematodes has been recorded previously (*Noel* and *Stanger*, 1994; *Sayre* et al., 1991). Spore density per female of *H. cajani* was much lower than that reported on root-knot, and other cyst nematodes.

The spores showed marked differences in their ability to attach to different species of nematodes (Table 2). In the majority of cases spores adhered randomly to the cuticle of the nematodes with the exception of *H. trifolii* where spores had a preference to attach to the anterior region, in particular to the lip region. Marked heterogeneity in the numbers of spores attached per nematode was observed on all nematode populations and was greatest on *R. reniformis*. Spores of *Pasteuria* recovered from cyst nematodes have not previously been found to adhere to *R. reniformis* (*Singh* and *Dhawan*, 1990; *Sayre* et al., 1991). The attachment of spores to *G. pallida*, *G. rostochiensis*, *H. cajani* and *R. reniformis* can be broadly divided into four distinct groups; no recognition, low recognition (between 1 to 12 spores per nematode), medium recognition (between 13 and 24 spores per nematode) and high recognition (greater than 24 spores per nematode). These differences could be due to either spore heterogeneity or cuticle heterogeneity, both of which have previously been observed in spore baiting experiments using monoclonal antibodies (*Davies* et al., 1994). No spore attachment on *M. javanica* was observed despite the fact that the original field population of this isolate was found to attack *M. javanica* (*Sharma* and *Sharma*, 1989). Continuous culturing on *H. cajani* presumably resulted either in the selection of a subpopulation of *Pasteuria* with small spores and an inability to recognize the cuticle of *M. javanica* or the susceptibility of the nematode had changed. The original field population had larger spores with a diameter of 4–5 µm (*Sharma* and *Sharma*, 1989). *Ratnasoma* and *Gowen* (1991) found that highest spore attachment was associated with a particular spore size and it varied between nematode species; *M. javanica* was encumbered by spores with a diameter between 3–5 µm. *Davies* et al. (1994) suggested that culturing the bacterium on a particular host reduces the variability present in the original population.

It was not possible to observe differences between spores using immunofluorescence with the polyclonal antibody, however, the monoclonal antibodies subdivided the spores into two groups; whether or not each monoclonal antibody was recognising the same or a different sub-population was difficult to tell. Western blot analysis showed large differences between *Pasteuria* isolated from root-knot nematodes and those isolated from *H. cajani*; previous studies of three *Pasteuria* populations cultured on root-knot nematodes showed a high degree of conservation between each of the populations, although some small qualitative and quantitative differences could be seen (*Davies* et al., 1992).

The results presented here suggest that the characterisation of *Pasteuria* into different species on the basis of host range and spore morphometrics is unsatisfactory because these parameters are prone to variability. The *Pasteuria* population from *H. cajani* described here has clearly changed from the original field population due to continual culturing on one particular nematode. Currently there is no method available to isolate the *Pasteuria* population from fields other than with a bait nematode, the use of which inadvertently selects a sample of the original population and therefore misrepresents the true diversity present in the field. The results suggest that host specificity of the *Pasteuria* populations could be an artefact of the method of isolation. *Ciancio* et al. (1994) concluded that no definite criteria are available to separate *Pasteuria* isolates into species or pathotypes. *Pasteuria* populations can be divided into two groups, those that complete their life-cycle in the juvenile (*P. thornei* group; PT group) and those that do not produce spores until the nematode has matured (*P. penetrans* group; PP group). *Pasteuria nishizawae* is a member of the PP group. *Pasteuria* populations reported by *Davies* et al. (1990) on *H. avenae*, and by *Sturhan* et al. (1994) on *H. goettingiana*, belong to the PT group. The population on *H. cajani* also belongs to the PP group. Serology is an excellent technique for studying diversity of *Pasteuria* and may ultimately lead to a method of identifying *Pasteuria* which is predictive of host range (*Davies*, 1994). However, further classification of *Pasteuria* populations requires more information, particularly analysis of their DNA.

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