Detection and characterization of *Pasteuria* 16S rRNA gene sequences from nematodes and soils

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Various bacterial species in the genus *Pasteuria* have great potential as biocontrol agents against plant-parasitic nematodes, although study of this important genus is hampered by the current inability to cultivate *Pasteuria* species outside their host. To aid in the study of this genus, an extensive 16S rRNA gene sequence phylogeny was constructed and this information was used to develop cultivation-independent methods for detection of *Pasteuria* in soils and nematodes. Thirty new clones of *Pasteuria* 16S rRNA genes were obtained directly from nematodes and soil samples. These were sequenced and used to construct an extensive phylogeny of this genus. These sequences were divided into two deeply branching clades within the low-G + C, Gram-positive division; some sequences appear to represent novel species within the genus *Pasteuria*. In addition, a surprising degree of 16S rRNA gene sequence diversity was observed within what had previously been designated a single strain of *Pasteuria penetrans* (P-20). PCR primers specific to *Pasteuria* 16S rRNA for detection of *Pasteuria* in soils were also designed and evaluated. Detection limits for soil DNA were 100–10 000 *Pasteuria* endospores (g soil)⁻¹.

INTRODUCTION

Plant-parasitic nematodes cause significant yield losses of crops and other plants of economic value each year. Losses due to nematodes in 1994 were estimated to exceed \$1.5 billion for corn, soybean, wheat, cotton, peanut and vegetables combined (Koenning, 1999). There has been a steady decline in nematicides available for agronomic crops due to concern regarding environmental effects of these chemicals. As an example, methyl bromide is among the most effective means of controlling plant-parasitic nematodes. Methyl bromide is a potent degrader of ozone, however, and will be phased out of use in the United States by the year 2005 (Chitwood & Deshusses, 2001). In response to declining options for control of nematodes, environmentally safe alternatives to control nematodes are of great interest. Among the most promising of these alternatives are biocontrol agents that specifically target these pests.

Pasteuria represents a genus of Gram-positive, endospore-forming bacteria whose species have been observed

parasitizing the majority of economically important plant-parasitic nematodes (Sayer & Starr, 1988). Members of the genus *Pasteuria* have been reported from 323 nematode species belonging to 116 genera, including plant-parasitic, entomopathogenic, predatory and free-living nematodes (Chen & Dickson, 1998). The potential of one species, *Pasteuria penetrans*, to be an effective biocontrol agent for *Meloidogyne* species (root-knot nematodes) has been demonstrated (Sayer & Starr, 1988). In addition to *P. penetrans*, *Pasteuria thornei* and *Pasteuria nishizawae* have been reported to infect other plant-parasitic nematodes (Chen & Dickson, 1998; Oostendorp *et al.*, 1990). Variabilities in endospore size, endospore surface antigens and in the strength of endospore attachment to nematodes within the same population of *Pasteuria* species have been reported (Sharma & Davies, 1996). Very limited information on the molecular biology, phylogeny and ecology of *Pasteuria* species is available due to the current difficulty in cultivating these bacteria outside of the host nematode (Chen & Dickson, 1998). Development of a sensitive and specific tool for characterization of *Pasteuria*–nematode relationships, such as species-specific interactions between *Pasteuria* and nematodes, and for studying distribution of *Pasteuria* in soils would be of great benefit for evaluating the potential of this biocontrol agent as an alternative to nematicides (Stirling, 1991).

Analysis of 16S rRNA gene sequences is a well-established

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A table showing sequence similarities of all clones described in this paper is available as supplementary data in IJSEM Online (<http://ijs.sgmjournals.org>).

Table 1. Clones of the *Pasteuria* 16S rRNA gene from nematodes and soils

Clones (ca. 1400 bp) amplified with universal primers are marked with an asterisk; all other clones (ca. 600 bp) were amplified with specific primers.

Clone	Source
Np3, Np4, Np6, Np13, Np16, Np17	DNA extracted from endospores taken from female nematodes of <i>Meloidogyne arenaria</i> Race 1 (P-20) collected from peanut plants in North Central Florida and raised in tomato plants in a greenhouse
Nf3*, Nf7*, Nf9*, Nf21*	DNA extracted from endospores taken from female nematodes of <i>Meloidogyne arenaria</i> Race 1 (P-20) collected from peanut plants in North Central Florida and raised in tomato plants in a greenhouse
NCp2A5, NCp2B5, NCf4*, NCf8*, NCp9	<i>P. penetrans</i> <i>in vitro</i> cultures (Gerber & White, 2001) originally from <i>Meloidogyne arenaria</i> Race 1 (P-20) collected from peanut plants in North Central Florida
SI1, SI6, SI10	Soil 1. Golf course at the Gainesville Golf and Country Club, Gainesville, FL, USA, infested with sting nematodes (<i>Belonolaimus longicaudatus</i>)
SII4, SII8, SII12	Soil 2. Nursery at the Gainesville Golf and Country Club, Gainesville, FL, USA, infested with root-knot nematodes (<i>Meloidogyne</i> species)
SIH6	Soil 3. Gainesville Country Club Golf Course, Gainesville, FL, USA, infested with <i>Tylenchus</i> species
SIV7, SIV8	Soil 4. Golf course at Fort Lauderdale Research and Education Center, Fort Lauderdale, FL, USA, infested with sting nematodes (<i>Belonolaimus longicaudatus</i>)
SV24, SV25, SV26, SV27, SV35, SV36	Soil 5. Golf course at the Gainesville Golf and Country Club, Gainesville, FL, USA infested with lance nematode (<i>Hoplolaimus</i> species)

and sensitive tool for detection and phylogenetic analysis of bacteria (Stahl, 1997). Use of 16S rRNA gene sequences to identify *Pasteuria* species and to assess the diversity of these bacteria within a nematode population or soil sample would be of great benefit in understanding nematode–parasite interactions and *Pasteuria* ecology. Development of 16S rRNA-based phylogeny and detection systems requires compilation of 16S rRNA gene sequences from a variety of representatives of the genus, yet only five 16S rRNA gene sequences for *Pasteuria* species could be retrieved from GenBank at the time of this study (Anderson *et al.*, 1999; Atibalentja *et al.*, 2000; Bekal *et al.*, 2001; Ebert *et al.*, 1996).

In this study, 16S rRNA gene sequences were amplified from *Pasteuria* endospores extracted from *Meloidogyne* nematodes or soil samples and their phylogenetic relationships were established using maximum-parsimony analysis. Two sets of primers specific for the *Pasteuria* 16S rRNA gene were designed for detection and characterization of *Pasteuria* species from nematodes and nematode-infested soils, and a phylogeny of these sequences was developed. This work greatly extends previous 16S rRNA phylogenies of *Pasteuria*, the most extensive of which include only two *Pasteuria* sequences (Anderson *et al.*, 1999; Atibalentja *et al.*, 1999).

METHODS

Bacteria sources and soils. *P. penetrans* P-20 endospores were collected from root-knot nematode *Meloidogyne arenaria* Race 1 as described by Anderson *et al.* (1999). *In vitro* cultures of *Pasteuria* originally collected from *Pasteuria*-infected *M. arenaria* Race 1

nematodes from peanut plants in North Central Florida were prepared as described by Gerber & White (2001). Soils 1–5 (sources of clones) are described in Table 1. Soil 6 was taken from a residential garden in Gainesville, FL, USA. Endospore-filled *Meloidogyne* females were collected from greenhouse-cultivated tomato roots and ruptured with a plant tissue grinder to form an endospore suspension (Oostendorp *et al.*, 1990).

DNA isolation. *Pasteuria* endospore DNA was extracted as described by Atibalentja *et al.* (2000). Soil and nematode DNA was isolated using an UltraClean soil DNA kit (MoBio) according to the manufacturer's instructions. For each sample, 0.25 g soil was used for extraction. For quantitative detection of endospores, *P. penetrans* endospores were spiked into soil 6 at 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10^1 endospores g^{-1} and DNA was purified as described above.

PCR amplification and RFLP. 16S rRNA gene sequences were amplified with primers 27F or 39F, and 1388R (Anderson *et al.*, 1999; Atibalentja *et al.*, 2000; Lane, 1991). Primers 617F, 654R and

Table 2. Primers for amplification of *Pasteuria* 16S rRNA gene sequences

Name	5'→3' sequence	Reference
27F	AGAGTTTGATCMTGGCTCAG	Lane (1991)
39F	GCGGCGTGCCTAATACA	Atibalentja <i>et al.</i> (2000)
617F	CGTGTRAGCGTTCGAAACGGT	This study
654R	TACCGCACTCAAGATGCACCG	This study
1166R	CGCCGGCTGTCTCTCCAA	This study
1388R	ACGGGCGGTGTGTACAAG	Atibalentja <i>et al.</i> (2000)

Fig. 1. Location of primers and restriction sites for characterization of *Pasteuria* 16S rRNA genes. Positions are indicated relative to the 16S rRNA gene sequence of the type strain of *P. penetrans* (GenBank accession no. AF077672).

1166R (Table 2) were designed for specific amplification of *Pasteuria* 16S rRNA gene sequences on the basis of unique and consensus sequences among *Pasteuria* and related sequences. DNA was amplified using HotStarTaq Master Mix (Qiagen) in a GeneAmp PCR system 2400 (Perkin-Elmer Applied Biosystems) under the following conditions: initial enzyme activation and DNA denaturation of 15 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, 45 s at 61 °C and 45 s extension at 72 °C, and a final extension at 72 °C for 7 min. PCR products amplified with primers 27F (or 39F) and 1388R were verified by digestion with *Bam*HI and *Eco*RV, products amplified with 39F and 654R were verified by digestion with *Bam*HI, and products amplified with primers 617F and 1166R were verified by digestion with *Stu*I (Fig. 1). Amplification products were purified with the PCR Purification kit (QIAquick; Qiagen) and were cloned into the pCRII-TOPO cloning vector (Invitrogen). For RFLP assays, individual colonies were screened by direct PCR amplification of the target DNA fragment from *Escherichia coli* clones and the desired PCR products were subjected to RFLP analysis using the restriction enzyme *Hha*I and resolved by 2.0 % agarose gel electrophoresis.

Sequencing and phylogenetic analysis. 16S rRNA gene clones exhibiting different RFLP patterns were selected for sequencing. Selected amplification products were subjected to direct sequencing after cleaning with the QIAquick PCR Purification kit (Qiagen). Sequencing was carried out in the DNA Sequencing Core Laboratory at the University of Florida, Gainesville, Florida, USA. Six of the resulting sequences generated with universal primers were assembled to produce contigs of ca. 1400 bases and sequences of ca. 600 bases amplified with specific primers were initially aligned using the PILEUP function of the GCG (Genetics Computer Group, 1999). Final alignments were constructed by visual inspection using PAUP 4.0b8 (Swofford, 2001). Representative sequences of *Pasteuria* were aligned with other low-G+C, Gram-positive bacterial species and maximum-parsimony analysis was conducted with *Streptomyces griseus* as the outgroup. The regions involving characters 1–30, 72–114 and 1463 to the end of the sequences were unalignable and therefore were not included in the maximum-parsimony analysis. Phylogenetic trees were built by a maximum-parsimony method using PAUP 4.0b8. Trees were constructed using heuristic searches with 10 random stepwise additions of taxa and by tree-bisection reconnection branch-swapping. The characters were weighted to give more weight to characters with lower levels of homoplasy. Bootstrap supports were obtained by heuristic searches of the reweighted trees with 100 or 1000 replicates.

Maximum-likelihood analyses were performed using the program TREE-PUZZLE version 5.0 obtained from <http://www.tree-puzzle.de/> (Strimmer & von Haeseler, 1996). Maximum-likelihood was performed using the Hasegawa-Kishino and Yano (Hasegawa *et al.*, 1985) model of substitution with the transition/transversion ratio and nucleotide frequencies estimated from the dataset. The quartet-puzzling tree search algorithm with 10 000 puzzling steps was used and the neighbour-joining method (Saitou & Nei, 1987) was used for estimation of the tree parameters. A uniform-rate model of rate heterogeneity was assumed. Frequencies of puzzling steps in the quartet-puzzling tree search were used as a measure of confidence, which are highly correlated to bootstrap values (Strimmer &

von Haeseler, 1996). A sequence similarity matrix of all sequences described in this study was constructed using the distance matrix function in the RDP PHYLIP interface (Maidak *et al.*, 2001).

RESULTS AND DISCUSSION

PCR amplification and RFLP analysis of *Pasteuria* 16S rRNA genes

Approximately 1400 bp of 16S rRNA genes was amplified from DNA preparations of *in vivo* and *in vitro* (Gerber & White, 2001) *P. penetrans* endospores resulting in clones Nf3, Nf7, Nf9, Nf21, NCf4, NCf8 (Table 1). These sequences shared 97.5–100.0 % homology with previously reported *P. penetrans* 16S rRNA gene sequences and were used to design PCR primers that would specifically amplify *Pasteuria* 16S rRNA genes. Based on consensus regions of these sequences, primer sets 617F/1166R and 39F/654R (Fig. 1) were thought to be *Pasteuria*-specific and were used in the subsequent amplification of *Pasteuria* sequences from soil and nematodes. A supplementary table showing sequence similarities of all clones described in this paper is available as supplementary material in IJSEM Online at <http://ijs.sgmjournals.org/>.

All *Pasteuria* sequences amplified with universal bacterial primers 27F (or 39F) and 1388R shared a few highly conserved restriction sites, including *Bam*HI (nucleotide position 246 based on *E. coli* numbering), *Eco*RV (697) and *Stu*I (1001) (Fig. 1). No related non-*Pasteuria* rRNA gene sequences included in the original alignment (Fig. 2) contained these digestion sites at these locations (as determined *in silico*; data not shown), suggesting that these restriction sites may be useful in preliminary identification of *Pasteuria* clones. Digestion of clones produced from amplification products using these primers provides a presumptive test for *Pasteuria* sequences before the DNA sequence is determined.

Amplification with primer set 39F/654R or 617F/1166R resulted in amplification of 16S rRNA gene sequences from all soils and nematodes tested that were known to harbour *Pasteuria* (Fig. 3). All amplification products were initially verified by restriction digestion with either *Bam*HI or *Stu*I; 24 were sequenced (Table 1) and confirmed to be closely related to previously reported *Pasteuria* sequences (Anderson *et al.*, 1999; Atibalentja *et al.*, 2000).

The region amplified by primers 39F and 654R is more variable between different clones than the region amplified by 617F and 1166R, such that PCR products amplified with

Fig. 2. Phylogenetic placement of *Pasteuria* species within the low-G+C, Gram-positive division. Analysis based on 1349 nt sequences from positions 39–1388 (based on *E. coli* numbering) with *Streptomyces griseus* as the outgroup taxon. Numbers above nodes represent bootstrap values (100 replicates). GenBank accession numbers for sequences from '*Sulfobacillus yellowstonensis*', '*Sulfobacillus montserratensis*', '*Sulfobacillus ambivalens*' and '*Bacillus caldolyticus*' are AY007665, AY007663, AY007664 and Z26924, respectively.

these primers exhibited a greater diversity of RFLP patterns (Fig. 4). Although a variety of RFLP patterns was observed for clones from individual nematode and soil samples, the majority of clones from each sample exhibited a single dominant RFLP pattern. For example, only 5 out of 40 clones showed different RFLP patterns in the Np series (data not shown).

Phylogenetic relationships between *P. penetrans* and other bacterial species

To confirm the placement of *Pasteuria* within the low-G+C, Gram-positive division and its association with the family *Alicyclobacillaceae*, a phylogenetic tree was constructed with maximum-parsimony from sequences available through GenBank and those from this study amplified from endospores and culture (Fig. 2). A total of 208

characters was excluded from the maximum-parsimony analysis yielding a matrix of 1389 characters. The matrix used in the phylogenetic analysis included 714 constant characters, 535 parsimony informative characters and 140 parsimony uninformative characters. The most parsimonious tree obtained had a tree length of 842 changes, a consistency index of 0.65, a retention index of 0.81, a rescaled consistency index of 0.53 and a homoplasy index of 0.35. According to maximum-parsimony, *Pasteuria* sequences were placed in a clade that contained previously described *Pasteuria*, *Alicyclobacillus*, *Sulfobacillus* and *Bacillus* species. This result is in general agreement with previously reported phylogenetic analyses (Anderson *et al.*, 1999; Atibalentja *et al.*, 2000; Ebert *et al.*, 1996).

Pasteuria species have been characterized as belonging to the *Alicyclobacillus* group by GenBank and the

Fig. 3. PCR products amplified from nematode and soil samples using primers 39F and 654R (top) and primers 617F and 1166R (bottom). Lanes: M, 100 bp molecular size marker (Promega); 1, DNA extracted from *Meloidogyne arenaria* Race 1 known to harbour *P. penetrans*; 2, soil 1; 3, soil 2; 4, soil 3; 5, soil 4; 6, soil 5; and 7, no template control.

Alicyclobacillaceae family by the taxonomic outline of the *Archaea* and *Bacteria* of *Bergey's Manual of Systematic Bacteriology* (<http://www.cme.msu.edu/bergeys/>). However, inclusion of additional *Pasteuria*, *Alicyclobacillus* and *Sulfobacillus* sequences resulted in a clear delimitation of a distinctive clade for *Pasteuria* from the rest of the *Alicyclobacillus* group (bootstrap value of 100). This distinction suggests that a re-evaluation of the placement of *Pasteuria* within the family *Alicyclobacillaceae* may be in order.

In our analysis, *Pasteuria ramosa* branched deeply within the *Pasteuria* clade and two additional major clades of *Pasteuria* could be distinguished (Fig. 2). One clade (*P. penetrans* clade 1; bootstrap support of 99 %) includes *Pasteuria* that parasitize root-knot nematodes, including the type species for *P. penetrans* (GenBank no. AF077672; Anderson *et al.*, 1999) and our isolates that parasitize root-knot nematodes.

Fig. 4. *HhaI* digestion RFLPs of 39F/654R amplification products from individual clones of the *Pasteuria* rRNA gene from nematodes or soils. Lanes: M, 100 bp molecular size marker; 1, SV25; 2, SV24; 3, SV35; 4, Np3; 5, Np36; 6, Np4; and 7, Np16. SV series clones are from soil sample 5 (infested with lance nematodes); N series clones are from root-knot nematodes infected with *P. penetrans*.

The second clade (clade 2; bootstrap support of 75 %) includes *Pasteuria* that infect the cyst nematode *Heterodera glycines* (AF134868; Atibalentja *et al.*, 2000; Sayre *et al.*, 1991) and a strain that infects the sting nematode *Belonolaimus longicaudatus* (AF254387; Bekal *et al.*, 2001).

Using primers 39F and 654R designed for specific amplification of *Pasteuria* 16S rRNA gene sequences, 25 partial sequences of approximately 600 bp each of *Pasteuria* from different sources were included in a more comprehensive phylogenetic analysis of this bacterial group (Fig. 5). *Thermoactinomyces dichotomicus* was used as the outgroup. In this analysis, the alignment was edited to exclude the incomplete beginnings and ends of the sequences and yielded a matrix of 611 characters. The matrix used in phylogenetic analysis included 181 constant characters, 85 parsimony informative characters and 345 parsimony uninformative characters. The most parsimonious tree had a tree length of 476 changes, a consistency index of 0.94, a retention index of 0.84, a rescaled consistency index of 0.80 and a homoplasy index of 0.05.

This analysis revealed diversity among the different *Pasteuria* sequences (Fig. 5). As in Fig. 2, *P. ramosa* was positioned on a deep branch of the phylogenetic tree and the two additional *Pasteuria* clades were separated. Bootstrap support for the *P. penetrans* clade (clade 1) was 99 %; however, support for clade 2 was quite low (67 %).

Clade 1 consists of sequences from strains (including the type strain of *P. penetrans*) known to infect root-knot nematodes and clones predominantly from soils infested with root-knot nematodes. It is not known at this time whether all clade 1 strains belong to the species *P. penetrans*. Barring 16S rRNA gene sequence similarity of less than 97 % (Stackebrandt & Goebel, 1994), physiological and genetic studies are required to confirm that two strains are from different species. Due to the current lack of appropriate laboratory cultivation systems for *Pasteuria*, these tests are difficult at best. Possible differences in infectivity in nematodes are also difficult to assess at this time. These uncertainties will be greatly reduced when efficient systems for *Pasteuria* cultivation are developed (Gerber & White, 2001).

Five subclades can be distinguished within clade 1. Subclades 1a, 1b and 1d have good bootstrap support values of 98, 94 and 93 % respectively, suggesting that each is a distinctive clade within clade 1. Subclades 1c and 1e are supported by lower bootstrap values, 78 and 80 %, respectively, which may compromise their phylogenetic position as individual clades within clade 1. Ten sequences (Np and Nf series; Table 1) generated from endospores from a single race of root-knot nematode (*Meloidogyne* species) were originally thought to belong to a single strain previously designated *P. penetrans* P-20 (Oostendorp *et al.*, 1990; Anderson *et al.*, 1999). In our analysis, ten P-20 sequences fell into three different subclades (1a, 1b and 1e).

Fig. 5. Phylogenetic relationships among *Pasteuria* species. Phylogenetic reconstruction based on maximum-parsimony using heuristic search of 16S rRNA gene sequences (PAUP, version 4.0b8). Analysis based on ca. 600 nt sequences from positions 39–654 with *Thermoactinomyces dichotomicus* as the outgroup taxon. Numbers above nodes represent bootstrap values (1000 replicates).

Most sequences from soils 2 and 5 separated into a distinct subclade (1c).

Clade 2 includes sequences from clones taken from soils infested with a variety of nematodes, including root-knot, sting and cyst nematodes. This clade also includes previously described strains that infect cyst, lance and sting nematodes. It is not known at this time if clade 2 *Pasteuria* strains exclusively attack nematodes other than root-knot nematodes. A more complete understanding of relationships between phylogenetic groups of *Pasteuria* and the nematodes they infect would be very important in the accurate design and implementation of *Pasteuria*-based systems to control nematodes.

Clade 2 includes SIV7 and SIV8, clones obtained from soil 4 (which harboured sting nematodes), and SV36, obtained from soil 5 (known to harbour lance nematodes). Also included in clade 2 are sequences from a previously described cyst nematode-infecting *Pasteuria* (GenBank no. AF134868) (Atibalentja *et al.*, 2000) and a sting nematode-infecting *Pasteuria* (AF254387) (Bekal *et al.*,

2001). Bootstrap support for this clade is low (67%), such that confidence that clade 2 is a single clade is not high. It is possible that clade 2 should be divided into two individual clades and additional full sequences from this clade would help clarify the topology of the tree and define the clade. Clade 2 may be separated into at least two subclades (Fig. 5). Bootstrap support for separation of clade 2 into these two subclades is very strong and the sequence similarities between representatives of the two subclades are low (less than 93%). As currently defined, 16S rRNA gene sequence similarity may not be used to define species, but may be used to determine whether two strains do not belong to the same species if they share less than 97% similarity (Stackebrandt & Goebel, 1994). On this basis, it is likely that subclades 2a and 2b represent different species.

The number of characters used in this analysis was not high (85 parsimony informative characters) due to the relatively small amplification products and conserved regions within the amplicon. The robustness of branches formed in this analysis is therefore tenuous, although the topology of the tree did not change significantly when the near-full-length

sequences presented in Fig. 2 were subjected to a similar analysis using the same region of sequence as those in Fig. 5 (analysis not shown). Robustness of the subclades discussed above will be tested as more sequences become available.

Maximum-likelihood analysis obtained by the quartet-puzzling method revealed a similar topology for the phylogenetic tree presented in Fig. 5, although some changes in the major clades were observed and supports for the main clades were lower (data not shown). Using this method, clade 1 has a support of 59 %, greater than the 50 % recommended by the developers of the quartet-puzzling method. Clade 2 was not supported and subclades 2a and 2b became individual clades. Inside clade 1, each of the subclades exhibited frequencies of puzzling steps in the range 85–93, confirming the subclades achieved by maximum-parsimony. Moreover, subclades 1c and 1e received supports of 89 and 93 %, respectively, possibly reaffirming their compromised identities as subclades in the maximum-parsimony analysis.

PCR detection of *Pasteuria* in soils and nematodes with *Pasteuria*-specific primers and their use in detection of *Pasteuria* in soils

The *Pasteuria*-specific PCR primers developed as part of this study were also evaluated for specific detection of *Pasteuria* in soils and nematodes. Detection and enumeration of *Pasteuria* will facilitate studies to determine the efficacy of their commercial use as biocontrol agents as new systems for laboratory cultivation of *Pasteuria* are developed (Gerber & White, 2001). Detection of sequences in serial dilutions of endospores added to soil 6 was consistently several orders of magnitude lower with primer set 617F/1166R than with 39F/654R (data not shown). The lowest detection limit for *P. penetrans* 16S rRNA genes in soil 6 using primer set 617F and 1166R was 100 endospores (g soil)⁻¹ (Fig. 6). The primer sets proved to be specific for *Pasteuria* strains, as determined by sequence analysis (data not shown).

For initial screening (presumptive confirmation) of 16S rRNA gene clones prior to sequencing, PCR products can be verified by digestion with the restriction enzymes *Bam*HI or *Stu*I, depending on the primer set used. All *Pasteuria* sequences reported to date, including those found in this study, have a single *Bam*HI site in the 39F/654R

amplification product and a single *Stu*I site in the 617F/1166R amplification product. For differentiation between different *Pasteuria* clones, digestion of individual 39F/654R products with *Hha*I is more informative than digestion of 617F/1166R due to greater sequence variability in this region between different clones. Evaluation of PCR primers for specific amplification and detection of these individual clades and subclades is currently being carried out.

In addition to providing information on the phylogeny of this potentially important biocontrol agent, the work presented here should form the basis for further studies that will elucidate the distribution and concentration of *Pasteuria* in soils and nematodes, and assist in defining host ranges of various strains and species of *Pasteuria*. Previous studies have shown that broader groups of *Pasteuria* exhibit preferences with regard to host nematodes (Davies *et al.*, 1994). Additional studies are now needed to define host ranges of the various clades and subclades and to define potentially different species within these clades. Ultimately, however, a complete understanding of the relationship between *Pasteuria* and their nematode hosts will require an efficient system for laboratory cultivation.

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Fig. 6. PCR detection of *P. penetrans* spiked into soil 6. Primer set 617F/1166R was used. Serial dilutions of endospores spiked into 1 g soil: 1, 10⁷; 2, 10⁶; 3, 10⁵; 4, 10⁴; 5, 10³; 6, 10²; and 7, 0; M, 100 bp molecular size marker;

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