Phenotypic and Molecular Analysis of a *Pasteuria* Strain Parasitic to the Sting Nematode[1]

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Abstract: Pasteuria strain S-1 was found to parasitize the sting nematode *Belonolaimus longicaudatus.* S-1 spores attached to several strains of *B. longicaudatus* from different geographical locations within the United States. However, they did not adhere to any of the following species: *Heterodera schachtii, Longidorus africanus, Meloidogyne hapla, M. incognita, M. javanica, Pratylenchus brachyurus, P. scribneri, P. neglectus, P. penetrans, P. thornei, P. vulnus,* and *Xiphinema spp.* The 16S rRNA genes from *Pasteuria* strain S-1 and *P. penetrans* strain Pp from Senegal were obtained by PCR amplification. A DNA sequence analysis showed that the S-1 16S rRNA had 96% or less similarity to the 16S rRNA genes from all previously reported *Pasteuria* species. Diverse phylogenetic methods all provided robust support for an association of Pasteuria strain S-1, *Pasteuria* strain NA parasitic to *H. glycines,* and *P. penetrans* strain Pp, to the exclusion of *P. ramosa.* In addition, our study showed intraspecific variation within *P. penetrans* as inferred by its 98% similarity to *P. penetrans* strain Pp.

Key words: Belonolaimus longicaudatus, microparasite, *Pasteuria* sp., *P. penetrans,* phylogeny, taxonomy, 16S rDNA sequence

Members of the genus *Pasteuria* are obligate, mycelial, endospore-forming bacterial microparasites of plant-parasitic nematodes and water fleas. Some strains have been described as promising candidates for biological control of plant-parasitic nematodes (Stirling, 1991; Chen and Dickson, 1998). Pasteuria spp. have been reported from many different climates and environments throughout the world (Sturhan, 1988; Ciancio et al., 1994). *Pasteuria penetrans* is the best-known species and has been shown to effectively suppress root-knot nematode populations in field and microplot experiments (Chen et al., 1996; Weibelzahl-Fulton et al., 1996). The extended survival in soil, host specificity, and tolerance to heat, desiccation, and chemicals (Chen and Dickson, 1998; Stirling, 1991; Bekal et al., 2000) make *P. penetrans* one of the more promising biological control agents for plant-parasitic nematodes.

Although more than 300 nematode species have been reported with *Pasteuria* spores attached to their cuticle, only four *Pasteuria* species have been described. *Pasteuria ramosa* is the only species that has not been observed to parasitize nematodes. It seems to parasitize only water fleas (*Daphnia* spp.) (Ebert et al., 1996). The three other species are all nematode parasites: *P. penetrans* on *Meloidogyne* spp., *P. thornei* on *Pratylenchus* spp., and *P. nishizawae* on the genera *Heterodera* and *Globodera* (Sayre and Starr, 1989). *Pasteuria* strain S-1 was found to be suppressive to the sting nematode *Belonolaimus longicaudatus* Rau in Florida (Giblin-Davis et al., 1995; 2000). The mature spore morphology and ultrastructure of strain S-1 differed considerably from the three other nematode-parasitizing *Pasteuria* species. Most notably, the sporangium and central body diameters were at least 0.7 µm and 0.5 µm wider than those of described *Pasteuria* species (Giblin-Davis et al., 2000).

Pasteuria are obligate microparasites and have not been cultured in artificial media (Williams et al., 1989). Therefore, most *Pasteuria* species have been described using traditional criteria such as host range, morphology, and ultrastructure (Mankau and Prasad, 1975; Sayre and Starr, 1989; Sayre and Wergin, 1977). Spore-filled nematodes that are considered suitable for studying *Pasteuria* are often difficult to obtain. Obtaining parasitized ectoparasitic nematodes is particularly difficult because they typically contain fewer spores than endoparasitic nematodes (Oostendorp et al., 1991). Although bacterial classification has traditionally relied on morphology and biochemical properties, it has expanded to include DNA-based analyses such as DNA base composition, DNA-DNA hybridization, and ribosomal gene sequence (Stackebrandt and Goepel, 1994; Goodfellow and O'Donnell, 1993). The 16S rDNA gene sequences have been used to identify bacterial species, and 16S rDNA has been sequenced for *Pasteuria* members. The first 16S rDNA sequence was obtained from *P. ramosa,* which allowed clarification of the taxonomy and phylogeny of the genus and showed its relatedness to *Alicocyclobacillus* (Ebert et al., 1996). Subsequently, the 16S rDNA gene of *P. penetrans* was sequenced and revealed 92% similarity with *P. ramosa.* This result supported the first classification of the genus and confirmed the specific status of *P. penetrans* (Anderson et al., 1999). More recently, a small subunit ribosomal gene was isolated from *Pasteuria* strain NA showing 97% and 91% similarity to *P. penetrans* and *P. ramosa,* respectively (Atibalentja et al., 2000).

The objective of this research was to characterize the *Pasteuria* strain S-1 using both nematode attachment tests and 16S rDNA sequence analysis.

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This paper was edited by E. P. Caswell-Chen.

Materials and Methods

Nematode and bacterial strain origins: Soil containing spores of *Pasteuria* strain S-1 and *Pasteuria penetrans* strain Pp were originally obtained from nematode-suppressive soil sites at the Fort Lauderdale Research and Experiment station, Florida, and Dakar, Senegal, respectively. The sting nematode isolate used to propagate *Pasteuria* strain S-1 originated from infested turf-grass at the Tamarisk Golf Club, Rancho Mirage, California, and was cultured on excised corn roots (*Zea mays* L. cv. Golden Jubilee) (Huang and Becker, 1997). Sting nematode juveniles infected with S-1 were gnotobiotically cultured until adults were filled with mature spores (Bekal et al., 1999). *Pasteuria penetrans* strain Pp was reared on *Meloidogyne incognita* (Kofoid & White) Chitwood parasitizing tomatoes (*Lycopersicon esculentum* L. cv. Tropical) in a greenhouse pot culture.

Attachment test: Attachment tests of S-1 were conducted by examining the ability of spores to adhere to the cuticle of several different nematode species and several sting nematode isolates obtained from various locations in the United States. These potential hosts included one of each of the following nematode species: *H. schachtii* Schmidt, *Longidorus africanus* Merny, *M. hapla* Chitwood, *M. incognita, M. javanica* (Treub) Chitwood, *P. brachyurus* (Godfrey) Filipjev & Shuurmans Stekhoven, *P. scribneri* Steiner, *P. neglectus* Filipjev, *P. penetrans* (Cobb) Filipjev & Shuurmans Stekhoven, *P. thornei* Sher & Allen, *P. vulnus* Allen & Jensen, and *Xiphinema sp.* Cobb. All nematodes were obtained from the Department of Nematology greenhouse culture collection, University of California, Riverside. The attachment test included three *B. longicaudatus* isolates from California and Florida, and one each from Arkansas, Georgia, and North Carolina. Experiments included four replicates for each nematode species or isolate, and attachment was assessed by incubating nematodes in soil (Brown and Smart, 1985) infested with spores of *Pasteuria* strain S-1. For each nematode isolate, adult and (or) juvenile inoculum (100 to 1,000 nematodes) was incubated in 100 cm^3 soil infested with approximately 10,000 spores/cm^3. Soil endospore density was determined by using an incubation bioassay previously described for root-knot nematodes (Sayre and Wergin, 1977). Soil containing nematodes and *Pasteuria* spores was incubated at 25 ± 2 °C in darkness during which the soil was kept moist. After 2 weeks, nematodes were extracted from soil by sugar-flotation (Jenkins, 1964) and stained with 1% crystal violet. The presence or absence of attached endospores and the number of endospores per nematode were evaluated for 10 randomly selected nematodes from each replicate using an inverted microscope. Attachment data were analyzed with ANOVA. Means comparison was done with the *F*-test. Analyses were performed using StatView software (Abacus Concepts, Berkeley, CA).

DNA Isolation: One hundred S-1-filled sting nematode adults recovered from the excised corn root cultures and 10 *P. penetrans* strain Pp-filled *M. incognita* females recovered from tomato pot cultures were hand-picked and placed in sterile water in a 1.5-ml microfuge tube. The nematode suspension was reduced to 50 µl after sedimentation, and the nematodes were homogenized in proteinase K extraction buffer (100 mM NaCl, 100 mM Tris pH 8.0, 50 mM EDTA, 1% β-mercaptoethanol, 1% sodium dodecyl sulfate, 100 µg/ml proteinase K) and brought to a final volume of 250 µl. The suspension was incubated in a water bath at 65 °C for 1 hour and agitated every 15 minutes. To release DNA from the bacterial spores, the suspension was added to a FastPrep tube with lysing matrix followed by agitation in a FastPrep FP120 (BIO 101, Vista, CA). The suspension was then centrifuged for 30 seconds at 10,000*g*, and the aqueous layer was transferred to a new tube. Total genomic DNA was then extracted and precipitated with the phenol-chloroform method (Sambrook et al., 1989).

16S rDNA amplification and electrophoresis: A partial nucleotide sequence (1341 nucleotides) of the S-1 16S rRNA gene region was obtained by PCR amplification of DNA extracted from the nematode using a bacteria-specific primer pair: 27 F (5′-AGAGTTTGATCSTGGC-TCAG-3′) and the 1392R (5′-ACGGGCGGTGTGTRC-3′) (Lane, 1991). Amplification reactions were performed in a 10-µl volume using the following final concentrations or total amounts: 5 ng DNA, 1 µM primers, 50 mM Tris (pH 8.3), 500 µg/ml bovine serum albumin, 0.25 mM dNTPs, 2.5 mM MgCl$_2$, and 0.5 units of *Taq* DNA Polymerase. Reactions were performed in glass capillary tubes using a 1002 Rapid Cycler (Idaho Technologies, Idaho Falls, ID). Amplifications were conducted under the following conditions: 95 °C for 2 minutes and then 95 °C for 10 seconds, 50 °C for 15 seconds, 72 °C for 30 seconds for 40 cycles, followed by 72 °C for 2 minutes. Amplification products were resolved on a 1.5% agarose gel and visualized by staining with ethidium bromide.

Cloning and sequencing: The 16S rDNA products were gel-purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and ligated into the pGEM-T vector (Promega). Plasmids were transformed into competent JM109 cells, and bacterial colonies containing plasmids with rDNA inserts were identified (Sambrook et al., 1989).

Sequence alignment and phylogenetic analysis: Sequences corresponding to 16S rRNA nucleotide positions 28 to 1,390 (according to the nomenclature of *Escherichia coli*) (Brosius et al., 1978) were determined for both *P. penetrans* strain Pp and *Pasteuria* strain S-1. Similarities among the 16S rDNA sequences from our clones and those deposited in GenBank were determined using BLAST (National Center for Biotechnology Information). All known *Pasteuria* sequences, including Pp and

S-1 data, were aligned with CLUSTAL W (Thompson et al., 1994) using default settings. We used a diverse array of phylogeny reconstruction methods to determine whether or not branching patterns were method- and (or) model-sensitive. Methods included maximum parsimony, minimum evolution, and maximum likelihood, including bootstrapping. All analyses were performed with PAUP 4.0b8 (Swofford, 1998). Maximum parsimony analyses included unweighted parsimony and transversion parsimony (i.e., zero weight for transitions). In both cases, gaps were coded as missing data. Minimum evolution analyses employed HKY85 distances (Hasegawa et al., 1985) and logdet distances (Lockhart et al., 1994). The latter were employed because a Chi-square test of base-compositional homogeneity (Swofford, 1998) revealed statistically significant nonstationarity among the sequences when constant sites were removed ($P = 0.012$). Maximum likelihood analyses employed the HKY85 model of sequence evolution and were performed with and without an allowance for a Γ distribution of rates. Maximum likelihood estimates of the transition to transversion (ts/tv) ratio and the shape parameter (α) of the Γ distribution were obtained for the original data and incorporated into subsequent bootstrap analyses (without Γ: ts/tv = 2.015418; with Γ: ts/tv = 2.978256, $\alpha = 0.13428$). All maximum parsimony and maximum likelihood searches, including bootstrapping, were branch-and-bound; starting trees were obtained by stepwise addition. Bootstrap searches with minimum evolution were heuristic, with tree bisection and reconnection branch swapping, because the branch-and-bound option is not available for minimum evolution. All bootstrap analyses included 500 replications. *Thermoactinomyces peptonophilus* was used as the outgroup in all analyses based on its relatedness to the *Pasteuria* genus (Yoon and Park, unpubl.). The sequence alignment, in NEXUS format, is available from the corresponding author.

Results

Attachment tests: S-1 did not attach to any of the plant-parasitic nematode species tested except *B. longicaudatus* (Table 1). In contrast, S-1 attached to all sting nematode isolates (Table 1). The number of spores attached to the different sting nematode isolates varied. They were lower for the NC, F3, and AR strains than for F1, F2, Tm, Di, Ae, and GA ($P < 0.05$). More than 50% of the nematodes had S-1 attached to their cuticle. Florida (except F3) and California strains showed the highest endospore attachment.

16S rDNA sequences: Approximately 1.3kb of rRNA gene sequence was amplified by PCR from both *Pasteuria* strain Pp and strain S-1 genomic DNA. Restriction digestions with *Apa* I revealed differences between Pp and S-1 DNA clones, whereas no polymorphism occurred among clones of the same *Pasteuria* strain (data

TABLE 1. Attachment of *Pasteuria sp.* S–1 to different isolates of *Belonolaimus longicaudatus* and several plant-parasitic nematode species.

B. longicaudatus isolates	Nematode origin (city, state)	Nematode inoculum[a]	Endospore number[b]	Attachment rate (%)[c]
AR	Hope, AT	320 ± 57	6.5 ± 3.6 a	75
Ae	Rancho Mirage, CA	511 ± 52	16.7 ± 9.1 c	95
Di	Rancho Mirage, CA	207 ± 48	11.5 ± 7.2 c	92
Tm	Rancho Mirage, CA	723 ± 81	19 ± 8.5 c	96
F1	Fort Lauderdale, FL	571 ± 62	15.5 ± 8.5 c	82
F2	Gainesville, FL	102 ± 33	12.3 ± 6.2 c	85
F3	Lake Alfred, FL	612 ± 58	5.1 ± 3.2 a	65
GA	Athens, GA	433 ± 71	7.7 ± 4.3 b	72
NC	Raleigh, NC	96 ± 29	4.4 ± 3.1 a	51

[a] Values are means followed by the standard deviation.
[b] Mean comparison with the *F*-test; numbers followed by the same letter are not significantly different at $P \le 0.05$.
[c] Attachment rate (%) measured from 40 nematode specimens.

not shown). The nucleotide sequences corresponding to the 16S rRNA nucleotide position 28 to 1,390 were determined for both the *P. penetrans* strain Pp and *Pasteuria* strain S-1 (GenBank accession numbers AF375881 and AF254387, respectively). Restriction maps performed on the Pp and S-1 sequences for *Apa* I sites matched the restriction digest patterns from the 16S PCR product of both strains.

Using BLAST searches (NCBI), S-1 and Pp showed high similarity to other *Pasteuria* rDNA sequences. S-1 showed 96% similarity with previously published *P. penetrans* (GenBank accession number AF077672) (Anderson et al., 1999), 93% with *P. ramosa* (GenBank accession number U34688) (Ebert et al., 1996), and 96% with *Pasteuria* strain NA (GenBank accession number AF134868) (Atibalentja et al., 2000). The sequence of Pp showed 98% similarity with *P. penetrans,* 93% with *P. ramosa,* and 96% with *Pasteuria* sp. NA.

CLUSTAL generated an alignment of 1,389 bp, including 98 positions with a gap in one or more species. Of these, 55 positions are gaps between *Thermoactinomyces* and *Pasteuria* and 43 positions exhibit gaps among species/strains of *Pasteuria*. Additionally, the 16S rDNA sequence alignment detected several variable and conserved regions (data not shown). Uncorrected genetic distances ranged from 0.006 to 0.083 among the ingroup taxa and 0.130 to 0.143 between ingroup taxa and the outgroup *Thermoactinomyces* (Table 2). HKY85 distances ranged from 0.006 to 0.089 among the ingroup taxa and 0.143 to 0.161 between ingroup taxa and the outgroup.

Figure 1 shows the maximum likelihood tree (-ln likelihood = 3351.2877) that was obtained with the HKY85 model (without Γ) of sequence evolution. *Pasteuria ramosa* is excluded from a clade (A in Fig. 1) that includes *P. penetrans* and the two undescribed *Pasteuria* species strains S-1 and NA. Within clade A, *P. penetrans* strain P100 and *P. penetrans* strain Pp are sister-taxa (B in Fig. 1), as are *Pasteuria* strain S-1 and *Pasteuria* strain

TABLE 2.   Genetic distances determined from 1,389-bp sequence alignment of *Pasteuria* spp. and *T. peptonophilus*.

Bacterial species and strains	Corrected and uncorrected genetic distances[a]					
P. penetrans P100	—	0.00600	0.02993	0.02693	0.07646	0.13976
P. penetrans Pp	0.00604	—	0.03001	0.03155	0.03032	0.14342
Pasteuria sp. S-1	0.03074	0.03082	—	0.02920	0.08256	0.14239
Pasteuria sp. NA	0.02759	0.03248	0.03001	—	0.07722	0.14333
P. ramosa	0.08167	0.08616	0.08859	0.08250	—	0.12951
T. peptonophilus	0.15630	0.16101	0.15963	0.16079	0.14324	—

[a] Uncorrected distances above the diagonal; HKY85 distances below the diagonal.
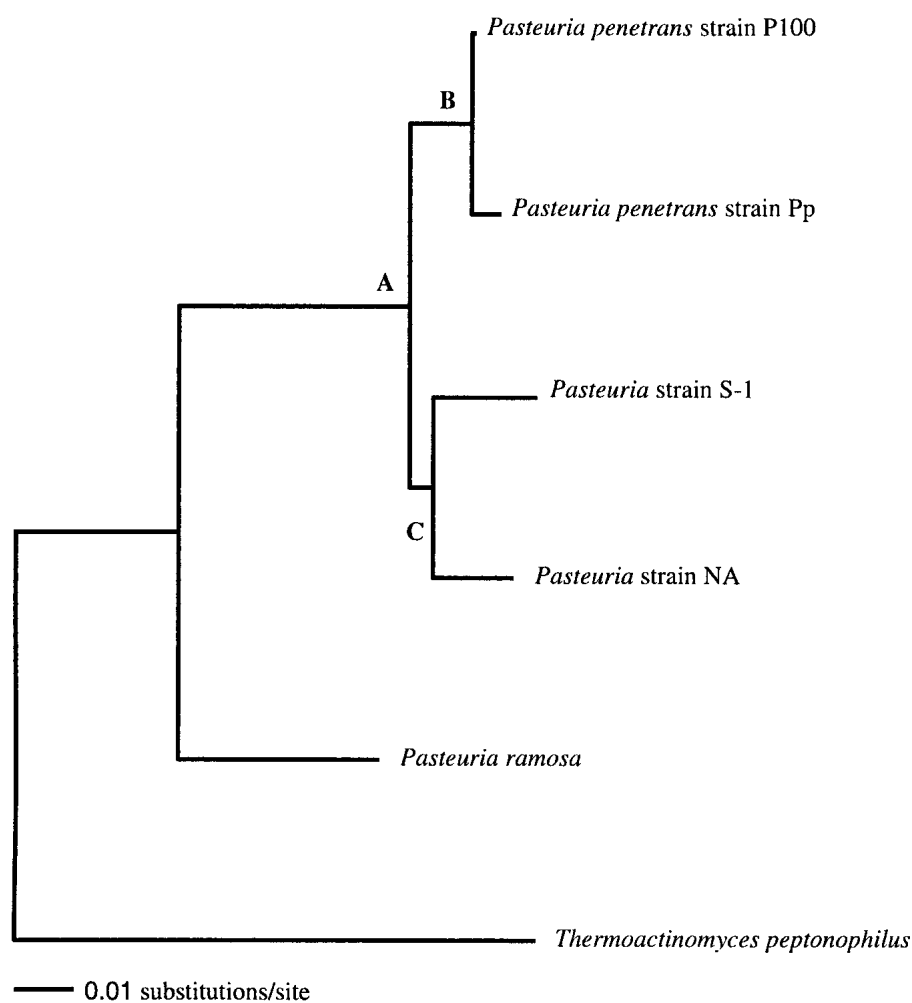

FIG. 1.   Maximum likelihood phylogram for the 1,389-bp data set derived from 16S rRNA genes of *Pasteuria* strain S-1, *P. ramosa* (Ebert et al., 1996), *P. penetrans* strain P100 (Anderson et al., 1999), *P. penetrans* strain Pp, and *Pasteuria* strain NA (Atibalentja et al., 2000). *Thermoactinomyces peptonophilus* was used as the outgroup. Bootstrap support percentages for clades A, B, and C are given in Table 3 for different phylogenetic methods.

TABLE 3.   Bootstrap support percentages for clades in Figure 1 with different phylogenetic methods.

Clade[a]	Maximum Parsimony		Minimum Evolution			Maximum Likelihood	
	Unweighted	Transversion	HKY85 distances	Logdet distances w/ constant sites	Logdet distances w/o constant sites	W/o Γ	W/Γ
A	100[b]	100	100	100	100	100	100
B	100	88	100	100	100	100	100
C	60	41	54	64	57	54	47

[a] Clades on phylogenetic tree in Figure 1.
[b] Bootstrap support percentages are based on 500 sampling replicates conducted with PAUP 4.0b8.

NA (C in Fig. 1). The maximum likelihood tree (-ln likelihood = 3308-3183) with HKY85 + Γ is not shown but differs from the tree in Figure 1 in the phylogenetic placement of *Pasteuria* strain S-1, which is the sister-taxon to clade B. Bootstrap analyses with maximum likelihood, maximum parsimony, and minimum evolution all provide 100% bootstrap support for clade A (Table 3). Clade B was also robust, and bootstrap support ranged from 88 to 100% (mean = 98%). Bootstrap support for clade C was weak and ranged from 41 to 64% (mean = 54%).

Discussion

On the basis of the ribosomal RNA gene analysis and previously published scanning and transmission electron microscopy studies (Giblin-Davis, 2000), strain S-1 is different from previously described *Pasteuria* species. The divergence of the S-1 strain is supported through its *B. longicaudatus* specificity. The inability of S-1 spores to adhere to other plant-parasitic nematodes expands the range of nematode species tested (Giblin-Davis et al., 1995). Moreover, ultrastructure and sporogenesis studies of S-1 support a species distinction (Giblin-Davis, 2000). In addition, the variable host specificity observed among members of *P. penetrans* within *Meloidogyne* species (Stirling, 1985; Davies et al., 1994) suggests that alternative classification methods based on 16S rDNA sequence analysis provide a more rigorous approach to distinguish among *Pasteuria* species.

Although the overall genetic variation of the small unit of the ribosomal gene was relatively low in the *Pasteuria* clade compared with other actinomycetes (Kreuze et al., 1999), sequence alignment illustrated variable and conserved regions that could be used in defining specific *Pasteuria* primers. To date, more than 300 plant-parasitic nematode species are known to be associated with *Pasteuria* suppression or attachment (Chen and Dickson, 1998). Genetic variation of the genus is poorly explored and has not been used extensively in species identification. In the past, identification of *Pasteuria* was challenging and questionable because of their obligate parasitic life cycle, and that has restricted taxonomic analysis to morphometric and ultrastructural criteria. The two new sequences presented in this manuscript add to the limited number of identified *Pasteuria* 16S rDNA sequences. Collectively, these sequences could lead to the development of *Pasteuria* specific primers, which would provide a much-needed, culture-independent approach to identify these obligately associated bacteria. Ribosomal sequences of phenotypically described species such *P. thornei* on *Pratylenchus* spp., *P. nishizawae* on *Heterodera* and *Globodera* (Sayre and Starr, 1989), and different strains of *Pasteuria* members such as the L-1 strain of *Hoplolaimus galeatus* (Giblin-Davis, 1990) are obvious candidates for amplification using *Pasteuria* specific primers.

Previous rDNA sequence analysis of *P. penetrans* did not show any intraspecific variation between the strains P100 and P20 (Anderson et al., 1999), but our research revealed significant dissimilarity among strains of *P. penetrans* from Florida and from Senegal. Exploring such variation could help clarifying cross-reactions observed for other strains of *P. penetrans* (Chen and Dickson, 1998). However, studies to analyze *Pasteuria* genetic diversity using less conserved genetic regions might be more appropriate. Intergenic spacers of the ribosomal genes that have been used to study variation in actinomycetes as well as many other organisms (Borneman and Triplett, 1997; White et al., 1998) could be useful in exploring intraspecific variation of the *Pasteuria* genus.

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