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Variable-Number Tandem Repeats as Molecular Markers for Biotypes of *Pasteuria ramosa* in *Daphnia* spp.▽

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Variable-number tandem repeats (VNTRs) have been identified in populations of *Pasteuria ramosa*, a castrating endobacterium of *Daphnia* species. The allelic polymorphisms at 14 loci in laboratory and geographically diverse soil samples showed that VNTRs may serve as biomarkers for the genetic characterization of *P. ramosa* isolates.

Pasteuria spp. are endospore-forming bacteria that are obligate parasites of cladoceran crustaceans and nematodes that develop through a water- or a soilborne stage (18). Their coevolution with their respective hosts has provided an opportunity to explore the genetic basis of host-parasite relationships in aquatic and soil environments. The type species for the genus is *Pasteuria ramosa*, which is found in Europe and North America and is related to *Bacillus* spp. by 16S rRNA gene homology (7). It is an endoparasite of *Daphnia* species, planktonic crustaceans that play an important role in the food chains of ponds. A single waterborne endospore may infect, geminate, and proliferate in the body cavity of its host to generate up to 80 million endospores. Transmission occurs horizontally with the infection of new hosts by mature spores released from dead infected hosts. The cost of infection is high, since hosts are completely castrated (8). Infective spores can survive for extended periods in soils, where they form long-lasting spore banks (9).

The infectivity of a spore, i.e., the ability of a spore to infect and propagate within a particular specimen of a *Daphnia* species, is dependent on the lineage of the parasite and the host (4, 6, 14). Until now, studies of the population genetics, evolution, and epidemiology of *P. ramosa* have been limited by the lack of genetic markers to distinguish among isolates. Sequence information from *Pasteuria* species is limited primarily to *Pasteuria penetrans*, a bacterium infecting phytopathogenic nematodes (5, 16, 19, 20). Identification of individual strains of *P. ramosa* is difficult because molecular methods used for genotype analyses, such as PCR of randomly amplified polymorphic DNA or restriction fragment length polymorphism analysis, are adversely affected by contamination with the DNA of their hosts. Here we have identified genetic markers based on short tandem repeats that may be used to distinguish isolates and to address the evolution of genetic variants in different environments.

Variable-number tandem repeats (VNTRs) comprised of short sequence repeats (SSRs) constitute a rich source of polymorphism and have been used extensively as markers for discrimination between strains within prokaryotic DNAs (12, 21). VNTR loci have even been found in genetically highly homogenous pathogens, such as *Bacillus anthracis* (1, 10, 13).

In this study, we describe nine VNTRs in noncoding and putative coding regions of the *P. ramosa* genome. Two laboratory isolates and bacteria from 11 soil samples collected in the United Kingdom, Belgium, and Russia (Table 1) were typed using these markers to assess the extent of polymorphism at these loci.

A cosmid library containing 25- to 40-kb inserts was generated using high-molecular-weight DNAs isolated from vegetative cells of the laboratory isolate P1 of *Pasteuria ramosa*. Screening for marker genes for *P. ramosa* and *Daphnia* by PCR indicated that approximately 90% of the DNA was *P. ramosa* DNA. This library was subjected to pyrosequencing (15) and provided contigs representing 3.6 Mb (the predicted genome size is 4 to 4.5 Mb). We searched for repetitive DNA in these contigs by using Tandem Repeats Finder software (2; http://tandem.bu.edu/trf/trf.html). Short SSRs (repeat units of 3 to 6 nucleotides) were in a minority (6%) compared to repeats harboring 7 to 14 nucleotides (60%) or repeats of >15 nucleotides per unit (34%), which is rather uncommon for the relative abundance of prokaryotic SSRs (21). For DNA polymorphism analysis, we selected 14 SSRs harboring the largest number of repetitions in P1 (Table 2). Eight of these SSRs (indicated with asterisks in Table 2) were located within putative open reading frames (AMIGene Viewer [3; http://www.genoscope.cns.fr/agc/tools/amigene/Form/form.php]), but no significant similarities were found compared to the corresponding amino acid sequences in the protein sequence databases at the National Center of Biotechnology Information database (Bethesda, MD).

Ten primer sets were designed to amplify these 14 SSRs (Table 2) by using Primer3 software (17; http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Bacterial DNA extraction from infected *Daphnia* cells was carried out with an EZNA tissue DNA kit (Peqlab) according to the manufacturer's instructions. For pond sediment samples, successful detection of microbial DNA requires adequate purification from the coextracted contaminants that inhibit PCR, such as humic and fulvic acids (22); therefore, we used a SoilMaster DNA extrac-

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TABLE 1. *P. ramosa* isolates used in this study

Isolate	Origin	Geographic region, site of isolation[a]	Year of isolation
P1	Lab strain	Gaarzerfeld, Germany	1997
P3	Lab strain	Tvärminne, Finland	2002
Moscow Zoo 97	Sediment	Moscow, Russia, zoological garden	1997
Moscow NJK 97	Sediment	Moscow, Russia, Novodevichiy Monastery	1997
Moscow V 97	Sediment	Moscow, Russia, Vorantzovsky Park	1997
Oxford Pond 8 96	Sediment	Oxford, United Kingdom, pond 8	1996
Oxford Pond 8 97	Sediment	Oxford, United Kingdom, pond 8	1997
Oxford Pond 11 97	Sediment	Oxford, United Kingdom, pond 11	1997
Oxford Pond 12 97	Sediment	Oxford, United Kingdom, pond 12	1997
Oxford Pond 17 97	Sediment	Oxford, United Kingdom, pond 17	1997
Belgium OM1	Sediment	Heverlee, Belgium, pond OM1	2006
Belgium OM3	Sediment	Heverlee, Belgium, pond OM3	2006
Belgium Neeryse	Sediment	Neerijse, Belgium	2006

[a] In Russia, samples were collected from three places in Moscow, namely, the zoological garden, the Vorantzovsky Park of the German Embassy (7 km south of the zoo), and a pond close to the Novodevichiy Monastery (4 km south of the zoo). UK samples were collected from four ponds located within 1 to 2 km of each other, 25 km south of Oxford. Belgium samples came from two adjacent ponds in Heverlee and one pond in Neerijse, a village close to Heverlee.

tion kit (Epicentre). Endospores of *P. ramosa* in pond sediments were subjected to mechanical disruption before extracting the DNA. Bead mill homogenization was carried out with a high-speed (5,000 rpm) bead beater (BioSpec Products, Inc.) after suspending 200 mg of soil samples in 250 μl of soil DNA extraction buffer and 2 μl of proteinase K (50 μg/μl) in tubes containing glass beads (0.5-, 0.1-, and 1-mm diameter). Tubes were subjected to bead beating at 5,000 rpm for one cycle of 10 s, one cycle of 20 s, and three cycles of 30 s successively and then centrifuged at 4,500 rpm for 15 min at 10°C. DNAs were extracted from the supernatant following the kit procedure. PCR amplifications were performed in 25-μl volumes containing 1× PCR buffer [Tris-HCl, pH 8.7, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$], a 200 μM concentration of each deoxynucleoside triphosphate, a 200 nM concentration of each primer, 0.5 U of HotStarTaq DNA polymerase (QIAGEN GmbH), and 2 μl of template DNA. The PCR cycling conditions were as follows: 15 min at 94°C; 42 cycles of 30 s at 94°C, 30 s at 50°C (primer-specific annealing temperature), and 1 min at 72°C; and a final elongation step for 10 min at 72°C.

Polymorphism was checked for each of the 14 SSRs in the two laboratory isolates by sequencing the PCR products (Fasteris SA, Inc.). Five SSRs, all situated within putative coding regions, did not show variation in the number of repeats, an observation which has been confirmed with four other laboratory isolates (originating from the United States, United Kingdom, Russia, and Belgium). The nine other SSRs (shown in bold in Table 2) showed polymorphisms and were chosen to study diversity in field samples. For genotyping, forward primers were fluorescently labeled. Allele sizes were determined by separation of the PCR products in an ABI PRISM 310 DNA sequencer (Applied Biosystems). Fragment lengths were assigned by Genemapper, using a GeneScan-500 (6-carboxytetramethylrhodamine) size standard. The results are presented in Table 3. In some samples, more than one allele was found for a given primer set. The allele numbers ranged from three for Pr SSR3 to eight for Pr SSR4. We did not find any correlation between the repeat copy number and the allelic variability (Spearman rank test; rho = −0.42; $P = 0.26$). For some loci, e.g., Pr SSR3, distinct alleles were found for each of the three geographical locations studied. Others showed polymorphism within a studied location (Pr SSR4) or between two samples collected from the same pond during successive years (Pr SSR6, Oxford, pond 8).

For the sequence amplified by the primer set Pr2, which was located within an open reading frame, length variation did not change the reading frame for the putative encoded protein. However, it is known that VNTRs have the potential to affect metabolic regulation, antigenic variation, or environmental adaptation (11). Moreover, extragenic VNTRs can have pro-

TABLE 2. Primer sequences and repeat motif attributes of 14 *P. ramosa* SSRs

Primer name	Primer sequence	SSR name[c]	SSR motif	Repeat size (nt)	No. of repetitions in isolate P1	Smallest-largest no. of repetitions	Size range of replicons (nt)	No. of alleles
Pr1 fwd	ACCTAAAGAACAGGAATATCTGGA	**Pr SSR1**	AAACTAACA	9	7	3–9	195–249	5
Pr1 rev	GCATGGAATGATTTTTGCTG							
Pr2 fwd[a]	CTGCTGGATGGATGGACTACGTGA	**Pr SSR2.1***	CCTGGTAAA	9	4	3–4	259–304	3
Pr2 rev	ACCGGTCCCGTAGGTATAGG	**Pr SSR2.2***	CATCCTGGTGGTCCTTGG	18	3	2–4		
Pr3 fwd	GGACCAATCGAACCAGGTAT	**Pr SSR3***	GG(A/G)CCGATG[b]	9	7	5–8	356–383	3
Pr3 rev	AACGGTTTCTTCGCTTGTTG							
Pr4 fwd	GGTAACCCTGGATGTCCTGA	**Pr SSR4**	TT(A/G)CTTTA[b]	8	16	10–19	329–393	8
Pr4 rev	ATCCCGTTACAAATGGGACA							
Pr5 fwd[a]	CCCTAAAGGAGACCCAGGAG	Pr SSR5.1*	TGGAGCACC	9	3			1
Pr5 rev	TGAATCGCACTATTACTTGGAAA	Pr SSR5.2*	AAAGGCGAT	9	17			
Pr6 fwd	AACATAAGGGATTAAGGAATGTC	**Pr SSR6**	TTTTTCTTTTCT	12	6	1–6	235–295	4
Pr6 rev	TGGAAAAGAAAAGGCATTAGC							
Pr7 fwd	AACGTACTGACAAACCAAACCA	**Pr SSR7**	AACAACC(T/C)C[b]	9	11	4–11	109–172	7
Pr7 rev	AATTTTTCTTAGATTGCTAGGTTGA							
Pr8 fwd	GCATCAAATACAAAAACAAATGAAG	**Pr SSR8**	AGAATATGAAGAAGATGC	18	4	4–8	386–458	5
Pr8 rev	TGTTTCTCTCGCGTTTCCTT							
Pr9 fwd	ATACGACGAACGGAAcAAGA	**Pr SSR9**	AGCAACAAC	9	5	2–8	151–205	6
Pr9 rev	AACCAAAGAATTAACGCCATT							
Pr10 fwd[a]	CATTACTGATTAAGCCGGAATCTA	Pr SSR10.1*	GTTGCTCCG	9	4			1
Pr10 rev	TCGCAAGCTAATATACCAGGAA	Pr SSR10.2*	ACAGGACCATTTATACCC	18	2			
		Pr SSR10.3*	GTAGGACCTGTACGTCCA	18	2			

[a] Primers that amplify more than one closely located repeat motif.
[b] Imperfect repeats. The nucleotide at the left in parentheses can be replaced by the one at the right.
[c] *, located within putative coding region. SSRs in bold were found to be polymorphic.

TABLE 3. Amplicon sizes of fragments containing SSRs found in *P. ramosa* isolates

Strain	Amplicon size (nt) with primer							
	Pr1	Pr2	Pr3	Pr4	Pr6	Pr7	Pr8	Pr9
Lab P1	213 231	286	374	369	295	172	386	178
Lab P3	222 249	304	383	393	283	145	422	169
Moscow Zoo 97	195 249	259 286	374 383	329 369 377	271	109 118 163 172	404	169
Moscow NJK 97	195	259	374	361 369	271	109 145 163 172	404 422	169
Moscow V 97	249	286 304	374	369	271	109 145 154 163 172	458	169
Oxford Pond 8 96	195	259 286	374	369	271 283	109 145 154 163	440	169
Oxford Pond 8 97	195	286	374	369	235 271	109 145 154 163	440	169
Oxford Pond 11 97	195	286	383	369	235 271	109 145 154 163	440 458	169
Oxford Pond 12 97	195	286 304	374 383	369	271	109 145 163 172	440	151 169
Oxford Pond 17 97	195	259	374 383	369 377	271	109 145 154 163	440	169
Belgium OM1	231	286	356	321	271	109 136 145 154 163	404	187 196 205
Belgium OM3	231	259 286	356	321 353	271	109 145 154 163	404	187 196
Belgium Neeryse	231	304	356	321 345	271	109 145 154 163	404	178

nounced effects on adjacent gene expression (21). The biological significance of *P. ramosa* VNTRs is unknown, but the identification of VNTRs can be a starting point for such research.

These VNTRs are the first molecular markers reported that have allowed the differentiation of populations of *Pasteuria* spp. as a function of environmental distribution. Moreover, the use of VNTRs for analyzing *P. ramosa* spore diversity in sediment samples raises the possibility of in situ analysis without isolating bacteria. This approach will facilitate epidemiological, genetic, and ecological studies of this nonculturable bacterium and will be valuable in determining the basis for host preference and virulence of *Pasteuria* spp. as parasites of phytopathogenic nematodes.

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