Development of a Genomics-Based LAMP (Loop-Mediated Isothermal Amplification) Assay for Detection of *Pseudomonas fuscovaginae* from Rice

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Abstract

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The vast amount of data available through next-generation sequencing technology is facilitating the design of diagnostic marker systems. This study reports the use of draft genome sequences from the bacterial plant pathogen *Pseudomonas fuscovaginae*, the cause of sheath brown rot of rice, to describe the genetic diversity within a worldwide collection of strains representing the species. Based on a comparative analysis with the draft sequences, primers for a loop-mediated isothermal amplification (LAMP) assay were developed to identify *P. fuscovaginae*. The assay reported here reliably differentiated strains of *P. fuscovaginae* isolated from rice from a range of other bacteria that are commonly isolated from rice and other plants using a primer combination designated Pf8. The LAMP assay identified *P. fuscovaginae* purified DNA, live or heat-killed cells from pure cultures, and detected the bacterium in extracts or exudates from infected host plant material. The *P. fuscovaginae* LAMP assay is a suitable diagnostic tool for the glasshouse and laboratory and could be further developed for in-field surveys.

Pseudomonas is a large and diverse genus in the γ subclass of Proteobacteria (26). The genus contains plant pathogens, opportunistic human pathogens, plant commensals, biological control agents, and environmental microbes (3,17,37). The diversity of this group is reflected in the large pan-genome and relatively small core genome (26). *Pseudomonas fuscovaginae*, Miyajima, Tanii and Akita nom. rev., the cause of sheath brown rot disease of rice (27), belongs to the *P. fluorescens* group and the subgroup *P. asplenii*, based on partial sequences of four housekeeping genes (29). Sheath brown rot of rice was first reported in Hokkaido, Japan in 1982 (41) and has since been reported from most rice-growing regions of the world, including the Philippines (11), Mexico (13), Nepal (36), Madagascar (34), Latin America (47), and Australia (10). In addition to sheath browning, *P. fuscovaginae* can cause a systemic discoloration of sheath in seedlings, grain discoloration and panicle sterility in adult plants (47), and total yield loss with very severe infection (34). In addition to rice, *P. fuscovaginae* infects maize, sorghum, and wheat (13,14).

A large number of other bacterial species are associated with grain and glume discoloration in rice as well as sheath browning (2,9,19), and these symptoms are often associated with mixed microbial populations in the field. Symptoms of sheath brown rot caused by *P. fuscovaginae* can be confused not only with symptoms caused by other common bacterial pathogens of rice, including *Burkholderia glumae, B. cepacia, Pantoea ananatis*, and *Acidovorax avenae,* but also with some fungal pathogens of rice such as *Sarocladium* sp. and *Dreschlera* sp. (48). Accurate identification of causal organisms of diseases is fundamental to declaration of pest-free areas and for identification of resistant germplasm (1). Therefore, there is a need for a rapid, robust diagnostic assay for *Pseudomonas fuscovaginae* that could be deployed in the field.

Definitive diagnosis of the casual agent of sheath brown rot traditionally involves a polyphasic approach that includes pathogen isolation, morphological studies, and biochemical tests such as BACTID (5) and DNA sequencing (2,10). Recently, loop-mediated isothermal amplification (LAMP), a rapid and sensitive technique first developed by Notomi et al. (31), has been adopted widely as a diagnostic tool for human clinical samples (28). The amplification of DNA using this technique combines a *Bst* DNA polymerase with strand-displacement activity, four to six flanking primers, and amplification temperatures between 60 and 65°C (31). The technique is less sensitive to inhibitors than polymerase chain reaction (PCR) and, hence, is applicable for a wide variety of clinical and field samples (21). Recently, the technique has been used for the detection of human, animal, and plant pathogens (15,16,24,30,44–46). With no requirement for temperature cycling or secondary steps for visualizing results, the technique can be used in the field with minimal training, making LAMP more feasible for use in developing countries than conventional PCR (16). Diagnosis can be rapidly accomplished within 15 to 60 min. The availability of whole-genome sequences for many species of plant pathogens allows the rapid development of primers for assays such as LAMP, using coding sequences that are conserved within the target species. This comparative genomic approach, using draft genomes, to find selective targets for LAMP was recently used to develop a selective assay for *Erwinia amylovora* (7).

Here, we describe the use of draft genome sequences for *P. fuscovaginae* to assess genomic diversity of representative strains and guide the development of a selective and sensitive LAMP detection assay that is applicable for a global collection of *P. fuscovaginae*.

Materials and Methods

Whole-genome sequences of *P. fuscovaginae*. Draft whole-genomic sequences of *P. fuscovaginae* used included UPB0736 (accession number AIEU00000000) (32), CB98818 (accession number ALAQ01000000) (42), and DAR 77795 and DAR 77800 (Bioproject numbers PRJDB1417 and PRJDB 1418, respectively).

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A full genomic sequence of a closely related Pseudomonad isolated from rice in Cambodia (DAR 77817) (9) was included as a comparison (Bioproject number PRDJB1419). Additionally, a draft genome sequence of a strain of *P. fuscovaginae* collected in the Philippines in 2000 (SE1; 12) was produced on an Illumina GAII sequencer at the University of California, Davis. Short-read trimming, filtering for quality, and shuffling were performed using Perl scripts from the FastX toolkit (version 0.0.13; http://hannonlab.cshl.edu/fastx_toolkit/). Reads with a quality score of less than 20 over 80% of the read were removed, and reads were trimmed to 55 bp. Reads were assembled de-novo using Velvet 1.1.06 (Zerbino and Birney 2008) with parameters optimized by VelvetOptimiser.pl, using a hash length of 45 and a k-mer length of 61.

Genomic diversity in *P. fuscovaginae*. *16s ribosomal RNA sequence comparison.* Sequences (1,394 bp) of the 16s ribosomal RNA (rRNA) gene from 22 *Pseudomonas* strains were generated and aligned using the ClustalW algorithm within MEGA 5.2 (40). The evolutionary history was inferred by using the maximum-likelihood method based on the Tamura-Nei model (39). Initial trees for the heuristic search were obtained by applying the neighbor-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood approach. All positions containing gaps and missing data were eliminated. In total, 1,313 positions were present in the final dataset. Evolutionary analyses were conducted in MEGA5 (5.2).

Multilocus sequence typing. The sequence of seven housekeeping genes (*acsA* [acetyl coenzyme A synthetase], *aroE* [shikimate dehydrogenase], *guaA* [GMP synthase], *mutL* [DNA mismatch repair protein], *nuoD* [NADH dehydrogenase I chain C, D], *ppsA* [phosphoenolpyruvate synthase], and *trpE* [anthralite synthetase component I]) from draft sequences of *P. fuscovaginae* strains and a *Pseudomonas* sp. from Cambodia were compared with the reference genome of *P. fluorescens* using multilocus sequence typing (MLST) 1.5 (http://cge.cbs.dtu.dk/services/MLST/) (23); additionally, the sequences of *rpoB* for each of the strains were used. The concatenated sequences were aligned using MEGA 5.2 (40) and the evolutionary history was inferred by using the maximum-likelihood method based on the JTT matrix-based model (20). Sequences for other *Pseudomonas* spp. (*P. syringae* pv. *tomato* DC3000 [ICMP 3443], *P. aeruginosa* DK2, *P. stutzeri* [ATCC 17588], *P. fluorescens* A506, *P. putida* BIRD1, *P. fragi* A22, and *P. chlororaphis* 06) were included for comparison.

Whole-genome comparison. Concatenated whole genomes of *P. fuscovaginae* and other *Pseudomonas* spp. (*Pseudomonas* sp. [DAR 77817], *P. syringae* pv. *tomato* DC3000 [ICMP 3443], *P. aeruginosa* DK2, *P. stutzeri* [ATCC 17588], *P. fluorescens* A506, and *P. putida* BIRD1) were compared and a phylogenetic tree was constructed using the programs EDGAR (6) and DARwin (33).

Plant growth and inoculation conditions. Seed of rice (*Oryza sativa* 'M202') were germinated overnight before being placed in a compost mix (pH 4 to 4.5) in 100-cm^2 pots in a controlled-environment glasshouse (65 to 70% relative humidity; 26 and 21°C day and night, respectively; and light at 400 to 600 W/m^2 for a maximum of 12 h). The pots were fertilized with a mixture of Algospeed (COMPO EXPERT; 176 g/liter) and Ferveg E13 (Angibaud-France; 12.5 g/liter). This stock solution was diluted (1%) and 1 liter of the diluted solution was applied once a week to 200 plants. The flag leaf sheath of 10 rice plants (growth stage 51, beginning of panicle emergence) was inoculated with *P. fuscovaginae* (DAR 77795) using a needle dipped in a cell suspension (approximately 1×10^6 cells/ml), as described by Adorada et al. (1). The plants were then misted for 24 h at 23°C in the dark before being returned to the glasshouse for symptom development. Five control plants were wounded with a needle dipped in sterile distilled water.

LAMP. *Primer design.* A MUMmer alignment strategy (22) was used to identify four conserved sequences (Table 1) shared between two disparate strains of *P. fuscovaginae* (SE1 from the Philippines and ICMP 9996 from Madagascar) but absent in the genome sequences of *P. syringae* B728A, *P. fulva* strain 12-X, *B. glumae* BGR1, *A. citrulli* AAC001, *Xanthomonas oryzae* pv. *oryzae* PXO99A, and *X. campestris* strain 8004 (accessions NC_007085, NC_015556, NC_012724 and NC_012721, NC_008752, NC_010717, and NC_007086, respectively). The corresponding regions were then extracted from the sequences of the above two strains of *P. fuscovaginae* plus an Australian strain (DAR 77795), the type strain from Japan (ICMP 5940), and a Chinese strain (CB 98818). These sequences were aligned with MEGA 5.2 (40) and the consensus sequence was used to design primers using LAMP Designer 1.02 (LAMP Designer; Premier Biosoft International; Table 2). These consisted of the forward outer (F3), reverse outer (B3), the forward and reverse inner primers (FIP and FIB, respectively) and the forward and reverse loop primers (LoopF and LoopB, respectively). Primers were synthesized by Integrated DNA Technologies. Initially, the F3 and B3 primers of each set were checked for amplification of DNA of strains DAR 77795, ICMP 9996, and SE1 by conventional PCR using *P. syringae* DNA as a negative control. Reactions (25 µl) consisted of 3 mM $MgCl_2$, 0.5 mM each dNTP, 4 µM F3 primer, 4 µM B3 primer, and 1 unit of *Taq* polymerase (New England BioLabs) in a 1× standard buffer (New England Biolabs). The temperature profile used was 3 min at 94°C; followed by 34 cycles of 30 s at 94°C, 30 s at 50°C, and 90 s at 72°C; and a final elongation cycle at 72°C for 3 min. PCR product (7 µl) was mixed with 3 µl of 5× loading dye and loaded on a 1% agarose gel containing 1× Gel Red (Biotium, Inc.). The gels were run at 85 V for 45 min and amplicons were visualized using a Syngene transilluminator (Synoptics Ltd.).

After confirmation of DNA amplification with F3/B3, the full set of LAMP primers was evaluated. These included LAMP primer sets Pf3, Pf4, Pf7, Pf8, and Pf9, which were used for amplification of all samples shown in Table 3. Reactions (12 µl) were composed of 10 ng of template DNA, 7.2 µl of Isothermal Master Mix (Optigene Limited), 3.2 nM each F3/B3 primer, 0.32 µM each FIP/BIP primer, 1.6 µM each LoopF/LoopB primer, and 1.8 µl of water. All amplifications were performed in a Genie II (OptiGene Limited). The temperature profile was 65°C for 30 min followed by 85°C for 5 min to inactivate the enzyme. A reaction with no template DNA and one with DNA of *P. syringae* (10 ng/µl) were used as negative controls.

Sensitivity analysis. To determine the sensitivity of the LAMP assay, a 10-fold serial dilution (1×10^0 to 1×10^{-6}) of *P. fuscovaginae* SE1 DNA (10 ng/µl) was prepared. These dilutions (along with a no-DNA template control) were amplified in a real-time PCR machine (CFX Connect; Bio-Rad) under the following conditions: 1 ng of template DNA, 32 nM each F3/B3 primer, 0.32 µM each FIP/BIP primer, 1.6 µM each LoopF/LoopB primer, and 7.2 µl of Isothermal Master Mix (Optigene Limited) up to a total volume of 12 µl. The cycle consisted of 64 to 65°C for 1 h and a melt curve

Table 1. *Pseudomonas fuscovaginae* genes with conserved sequences in other *Pseudomonas* spp. identified through the genomic comparison of strains SE 1 and ICMP 9996 using DNA sequence analysis

Sequence name	Length (bp)	Function	Isolate	Accession number	Nucleic acid identity (%)
SEQ001	3246	Flagellar rod assembly protein/muramidase FlgJ	*P. protegens* Pf-5 chromosome, complete genome	NC_004129	77
SEQ371	396	Chromosomal replication initiator protein	*P. putida* UW4 chromosome, complete genome	NC_019670.1	83
SEQ483	531	Hypothetical protein	*P. brassicacearum* subsp. *brassicacearum* NFM421 chromosome, complete genome	NC_015379.1	88
SEQ992	420	Chromosomal replication initiator protein	*P. putida* UW4 chromosome, complete genome	NC_019670.1	85

cycle of 65 to 95°C (increments of 0.5°C per 5 s). All reactions were performed twice. A sensitivity assay was also performed on live cells. A 10-fold dilution of a suspension of *P. fuscovaginae* (DAR 77795) grown on King's B agar (KBA) was made in sterile distilled water. The serially diluted cells were used in a LAMP assay as described above, except the assay was performed in a StrataGene Real-Time PCR machine (MX3005) at 60°C. Each of the dilutions (100 µl) was spread on KBA and incubated for 48 h at 28°C to determine the concentration of cells in the suspension. The serial dilutions were replicated four times and the experiment was conducted twice.

Specificity analysis. To determine the specificity of the LAMP primers (Pf8), three pools of DNA from bacteria (10 per pool) isolated from rice seed were made (Table 4). LAMP reactions were run on these pools with and without DNA of *P. fuscovaginae* SE1 using the real-time LAMP parameters described above. All reactions were performed twice. Additionally, DNA from Pseudomonads isolated from rice in Cambodia were used individually in LAMP reactions (Table 4).

LAMP analysis of infected plants. A 5-mm^2 section of leaf sheath was excised from both inoculated and water control plants 5 days after inoculation (five replicate plants). The plant material was submerged in 200 µl of sterile distilled water in an Eppendorf tube and crushed using a sterile micropestle (Eppendorf International). A 10-fold serial dilution of the macerated tissue was made in sterile distilled water. Sample (1 µl) from each of the 10^{-1}, 10^{-2}, and 10^{-3} dilutions was used for the LAMP assay, as described above. A no-template control and an assay containing 10 ng of purified DNA of strain DAR 77795 were used as negative and positive controls, respectively. The assay was performed in a StrataGene Real-Time PCR machine (MX3005), as described above. All reactions were performed twice.

Results

Whole-genome sequence of *P. fuscovaginae* strain SE1. The genome statistics for *P. fuscovaginae* strain SE1 are shown in Table 5. This whole-genome shotgun project was deposited at DDBJ/EMBL/GenBank under the accession AQOH00000000.

Table 2. Loop-mediated isothermal amplification primers developed for the detection of *Pseudomonas fuscovaginae*[a]

Primer set	Single primer	Sequence (5′–3′)	Relative position
Pf3	F3	GAATTCCGTTCCTATGATTCCT	1,080
	B3	TTGAGCAGACTCATGATTCAG	1,346
	FIP	TCACGCACAAACTGTTCTGGTTGCAGAGCAATAATCGCTAT	…
	BIP	AAAGGCCGGGTACGCAACTCTGGTAGCTCTTCATCTGT	…
	LoopF	GCCGACTTCAGCACATCT	1,174
	LoopB	TACGCCAGCAAGATCACG	1,236
Pf4	F3	CAGGACGCCATCAAGTAC	2,778
	B3	CCGGTCATCGAAATGGTATAG	3,076
	FIP	AGGTTCAGGGTATTGTTCTGGCGAGCTACACACTGACCGA	…
	BIP	TGGTCGACTCGTCGGGTAATGGTCTGTACCGTAAAGGT	…
	LoopF	GCCCATGAGATTGCCCTT	2,894
	LoopB	GTGATGGACAACACCGTCA	2,970
Pf7	F3	CATGGGTTGCTCCTTTGAT	69
	B3	CACATTGCTGTGAGCCTT	302
	FIP	ATTGTGCGCGTGTAGGCATAGCGTATCCTGTGCATTGT	…
	BIP	CAATCCCGGAACGCTGGTTAAAGATGGCATCGAACAGC	…
	LoopF	AACGGTGTTTGCTGGTAGT	175
	LoopB	CGTCAGGCAACGTCTGAT	239
Pf8	F3	TGTGTTCAGTTCCTTCATCG	197
	B3	TAACAGCGTCACGTTCTTC	473
	FIP	GATCATCAGCGGGACTGTCAGGGTGAGGTCTACGAGAAATTC	…
	BIP	CGACGGCGATTTCGTTGCGTTATACAACAACGCTTGCC	…
	LoopF	TGAGAAACGCCTTCTGGTC	306
	LoopB	TGTGCTGTGCCTGATTCTT	365
Pf9	F3	TCGTCGAGGTCGATATGG	138
	B3	GCGAAGAGGAAGAACTCAG	372
	FIP	CCTCGGAACGCTGAAGAGCTTACGCATGTATTGCTGACT	…
	BIP	CGAGCACCTGTTGGTGGATTGTTCAACGACTTCCTGG	…
	LoopF	CACCAGCCGATGGTAGAC	196
	LoopB	TCGCCGGGATATTCCATTTC	280

[a] Primer sets Pf3 and Pf4 were based on sequence SEQ001, Pf7 was based on SEQ371, Pf8 was based on SEQ483, and Pf9 was based on SEQ992.

Table 3. Isolates of *Pseudomonas fuscovaginae*, *Pseudomonas* sp., and *P. syringae* used in the initial specificity testing of loop-mediated isothermal amplification (LAMP) primers[a]

					LAMP primer set				
Accession number	Species	Origin	Host	Notes	Pf3	Pf4	Pf7	Pf8	Pf9
DAR 77795	*P. fuscovaginae*	Australia	*Oryza sativa*	DNA	+	+	–	+	+
DAR 77797	*P. fuscovaginae*	Australia	*O. sativa*	DNA	+	+	nt	+	–
DAR 77817	*Pseudomonas* sp.	Cambodia	*O. sativa*	DNA	–	–	nt	–	–
DAR 77794	*Pseudomonas* sp.	Cambodia	*O. sativa*	DNA	–	–	nt	–	–
UPB264b	*P. fuscovaginae*	Burundi	*O. sativa*	Heat-killed cells	+	+	nt	+	–
ICMP 5940	*P. fuscovaginae*	Japan	*O. sativa*	Heat-killed cells	+	+	nt	+	–
UPB407	*P. fuscovaginae*	Burundi	*Leersia hexandra*	Heat-killed cells	+	–	nt	–	–
UPB0735	*P. fuscovaginae*	Burundi	*O. sativa*	Heat-killed cells	+	+	nt	+	–
ICMP 9996	*P. fuscovaginae*	Madagascar	*O. sativa*	Heat-killed cells	+	±	nt	+	–
UPB898	*P. fuscovaginae*	Colombia	*O. sativa*	Heat-killed cells	–	–	nt	+	–
SE1	*P. fuscovaginae*	Philippines	*O. sativa*	Heat-killed cells	+	–	–	+	+
NCPPB 1242	*P. syringae* pv. *syringae*	Australia	*Panicum milaceum*	DNA	–	–	–	–	–

[a] All assays were conducted at least twice; nt = not tested.

The version described in this article is the first version, AQOH01000000.

Genomic diversity of *P. fuscovaginae*. The *P. fuscovaginae* strains clustered with isolate strain of *P. asplenii* (with a bootstrap value of 96%) and are clearly different from the representative sequences from the other species of *Pseudomonas*, based on the phylogeny generated from the 16s rRNA gene sequences (Fig. 1). The *Pseudomonas* sp. (ICMP 17674 = DAR 77817) isolated from rice in Cambodia (9) was more closely related to *P. putida. P. fuscovaginae* strains ICMP 9996, CB98818, DAR 77795, ICMP 5940, and DAR 77800 clustered closely within two subclades of

Table 5. Draft genome assembly statistics for *Pseudomonas fuscovaginae* strain SE 1

Total PF reads	24,345,762
Total Mbp reads used	1,232
Sequence coverage	188×
N50	92,359
Number of contigs	692
Maximum contig length (bp)	305,205
Total contig length (bp)	6,558,325

Table 4. Bacterial strains used in this study to establish loop-mediated isothermal amplification (LAMP) assay sensitivity[a]

Strain	Country of origin	Host	Species	Notes, source
NCPPB3112	Brazil	*Canna indica*	*Acidovorax avenae* pv. *avenae*	NCPPB[b]
94-21	United States	*Citrullus lanatus*	*A. avenae* pv. *citrulli*	R. Walcott
3549	United States	*Saccharum officinarum*	*Burkolderia andropogonis*	L. E. Claflin
O187	United States	*Allium cepa*	*B. gladioli*	H. F. Schwartz
B473	United States	*Phaseolous* sp.	*Curtobacterium flaccumfaciens*	H. F. Schwartz
O121	United States	*A. cepa*	*Enterobacter* sp.	H. F. Schwartz
O174	United States	*A. cepa*	*Enterobacter* sp.	H. F. Schwartz
112	United Kingdom	*Pyrus communis*	*Erwinia herbicola*	L. E. Claflin
O158	United States	*A. cepa*	*Pantoea ananatis*	H. F. Schwartz
ATCC10844	United States	*Lactuca sativa*	*Pseudomonas marginalis*	H. F. Schwartz
NCPPB 1242	United States	*Panicum milaceum*	*Pseudomonas syringae* pv. *syringae*	L. E. Claflin
M108	United States	*Solanum lycopersicum*	*Pseudomonas syringae* pv. *syringae*	H. F. Schwartz
M72	United States	*Capsicum annuum*	*P. syringae* pv. *syringae*	H. F. Schwartz
ATCC13223	United States	*Phaseolus coccineus*	*Pseudomonas viridiflava*	H. F. Schwartz
K60	United States	*S. lycopersicum*	*Ralstonia solanacearum*	J. E. Leach
NCPPB 2417 (4429)	New Zealand	*Sorghum bicolor*	*Xanthomonas axonopodis* pv. *holcicola*	L.E. Claflin
2919	Réunion Island	*Mangifera* sp.	*X. axonopodis* pv. *mangiferaeindicae*	L. Gagnevin
X-1	United States	*Pelargonium* sp.	*X. campestris* pv. *pelargonii*	J. E. Leach
85-10	United States	*Capsicum* sp.	*X. campestris* pv. *vesicatoria*	A. J. Bogdanove
X11-5A	United States	*Oryza sativa* cv. Lemont	*X. oryzae*	C. Gonzales
KACC10331	Korea	*O. sativa*	*X. oryzae* pv. *oryzae*	S. H. Choi
BLS413	Philippines	*O. sativa*	*X. oryzae* pv. *oryzicola*	C. M. Vera Cruz
136M	Philippines	*O. sativa*	Unknown, isolated from seed	C. M. Vera Cruz
SHU 36	Philippines	*O. sativa*	Unknown, isolated from seed	C. M. Vera Cruz
SHU 50	Philippines	*O. sativa*	Unknown, isolated from seed	C. M. Vera Cruz
SHU 100	Philippines	*O. sativa*	Unknown, isolated from seed	C. M. Vera Cruz
SHU 147	Philippines	*O. sativa*	Unknown, isolated from seed	C. M. Vera Cruz
SHU 178	Philippines	*O. sativa*	Unknown, isolated from seed	C. M. Vera Cruz
SHU 202	Philippines	*O. sativa*	Unknown, isolated from seed	C. M. Vera Cruz
SHU 222	Philippines	*O. sativa*	Unknown, isolated from seed	C. M. Vera Cruz
SHU 268	Philippines	*O. sativa*	Unknown, isolated from seed	C. M. Vera Cruz
SHU 303	Philippines	*O. sativa*	Unknown, isolated from seed	C. M. Vera Cruz
M136	Mali	*O. sativa*	*Xanthomonas* sp., isolated from rice	V. Verdier
SHU 36	Philippines	*O. sativa*	*Xanthomonas* sp., isolated from seed	C. M. Vera Cruz
SHU 50	Philippines	*O. sativa*	*Xanthomonas* sp., isolated from seed	C. M. Vera Cruz
SHU 100	Philippines	*O. sativa*	*Xanthomonas* sp., isolated from seed	C. M. Vera Cruz
SHU 147	Philippines	*O. sativa*	*Xanthomonas* sp., isolated from seed	C. M. Vera Cruz
SHU 178	Philippines	*O. sativa*	*Xanthomonas* sp., isolated from seed	C. M. Vera Cruz
SHU 202	Philippines	*O. sativa*	*Xanthomonas* sp., isolated from seed	C. M. Vera Cruz
SHU 222	Philippines	*O. sativa*	*Xanthomonas* sp., isolated from seed	C. M. Vera Cruz
SHU 268	Philippines	*O. sativa*	*Xanthomonas* sp., isolated from seed	C. M. Vera Cruz
SHU 303	Philippines	*O. sativa*	*Xanthomonas* sp., isolated from seed	C. M. Vera Cruz
MB232	Cambodia	*O. sativa*	*Pseudomonas* sp., isolated from plant	E. Cother
MB234	Cambodia	*O. sativa*	*Pseudomonas* sp., isolated from plant	E. Cother
MB235	Cambodia	*O. sativa*	*Pseudomonas* sp., isolated from plant	E. Cother
MB236	Cambodia	*O. sativa*	*Pseudomonas* sp., isolated from plant	E. Cother
MB239	Cambodia	*O. sativa*	*P. fulva,* isolated from plant	E. Cother
MB245	Cambodia	*O. sativa*	*Pseudomonas* sp., isolated from plant	E. Cother
MB246	Cambodia	*O. sativa*	*Pseudomonas* sp., isolated from plant	E. Cother
MB247	Cambodia	*O. sativa*	*Pseudomonas* sp., isolated from plant	E. Cother
MB248	Cambodia	*O. sativa*	*Pseudomonas* sp., isolated from plant	E. Cother
MB249	Cambodia	*O. sativa*	*Pseudomonas* sp., isolated from plant	E. Cother
MB250	Cambodia	*O. sativa*	*Pseudomonas* sp., isolated from plant	E. Cother
MB251	Cambodia	*O. sativa*	*Pseudomonas* sp., isolated from plant	E. Cother
MB290	Cambodia	*O. sativa*	*P. plecoglossicida,* isolated from plant	E. Cother
MB292	Cambodia	*O. sativa*	*P. tolaasii*, isolated from plant	E. Cother
DAR 49869	United States	*Quercus* sp.	*P. putida*	Herb DAR
DAR 73902	Australia	Soil	*P. putida*	Herb DAR
DAR 77215	Australia	Soil	*P. putida*	Herb DAR

[a] All strains were negative with LAMP primer Pf8.
[b] National Collection of Plant Pathogenic Bacteria.

the phylogenetic tree based on multilocus *s*equence typing of the eight concatenated gene sequences from *P. fuscovaginae* and other *Pseudomonas* spp. (Fig. 2). *P. fuscovaginae* strain SE1 clustered with *P. stutzeri* (with strong bootstrap support) while *Pseudomonas* sp. (DAR 77817) was distinct, clustering with the *P. syringae* pv. *tomato* strain. All *P. fuscovaginae* strains clustered with strain SE1 from the Philippines based on the concatenated sequences of the entire genomes (Fig. 3). Additionally, strain SE1 was least related to other *P. fuscovaginae* strains. The *Pseudomonas* sp. strain from Cambodia clustered with *P. putida* (Fig. 3).

LAMP bioassay. Four highly conserved sequences among *P. fuscovaginae* strains but divergent from other bacterial species were identified after MUMmer alignment of available *P. fuscovaginae* and other bacterial genomes. LAMP primer design software (LAMP Designer; Premier Biosoft) generated five best-match primers for each of the five sequences. Five of the initial F3/B3 primers consistently amplified (primer sets Pf3, Pf4, Pf6, Pf7, and Pf8) a single band of approximately 200 bp. Therefore, full sets of LAMP primers were generated based on these sequences. The primer sequences and their relative position in the target are shown in Table 2. No amplification was observed in negative controls that lacked template DNA or contained *P. syringae* DNA (Table 3). Primer set Pf7 failed to amplify DNA from strains DAR 77795 and SE1 and, therefore, was excluded from further analysis. Primer set Pf9 amplified DNA from DAR 77795 and SE1 but failed to amplify DNA from any of the other *P. fuscovaginae* strains. Primer set Pf4 consistently amplified DNA from the Australian *P. fuscovaginae* strains DAR 77795 and DAR 77797 and the Japanese strain ICMP 5940 but gave inconsistent results for strains from the Philippines, Colombia, Madagascar, and Burundi. The primer sets Pf3 and Pf8 amplified most of the tested *P. fuscovaginae* strains consistently. However, primer set Pf3 failed to amplify Colombian strain UPB 898 from rice while the primer set Pf8 failed to amplify DNA from the Burundi (UPB 407) strain from the aquatic weed *Leersia hexandra.*

Primer set Pf8 consistently amplified DNA from strain SE1 when mixed with DNA from a range of *Pseudomonas* spp. Furthermore, amplification was not detected with this primer set when *P. fuscovaginae* DNA was not included in the pool (negative control). Additionally, amplification was not detected with genomic DNA from other Pseudomonads from rice. When the DNA of *P. fuscovaginae* strain SE1 was serially diluted, the assay detected a concentration of DNA at 1 pg/µl in all samples tested. Using live cells of strain DAR 77795, the assay detected approximately 16 bacterial

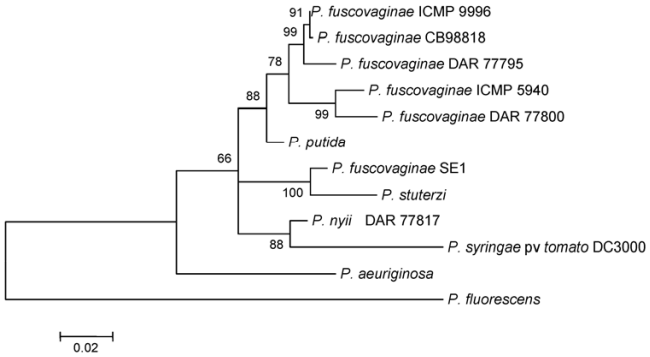

Fig. 2. Molecular phylogenetic analysis of *Pseudomonas* species by maximum-likelihood (ML) method using the genes *acsA* (Acetyl coenzyme A synthetase), *aroE* (Shikimate dehydrogenase), *guaA* (GMP synthase), *mutL* (DNA mismatch repair protein), *nuoD* (NADH dehydrogenase I chain C, D), *ppsA* (Phosphoenolpyruvate synthase), *trpE* (Anthralite synthetase component I), and *rpoB* (β subunit of RNA polymerase). The evolutionary history was inferred by using the ML method. The tree with the highest log likelihood (–10,674.52) is shown.

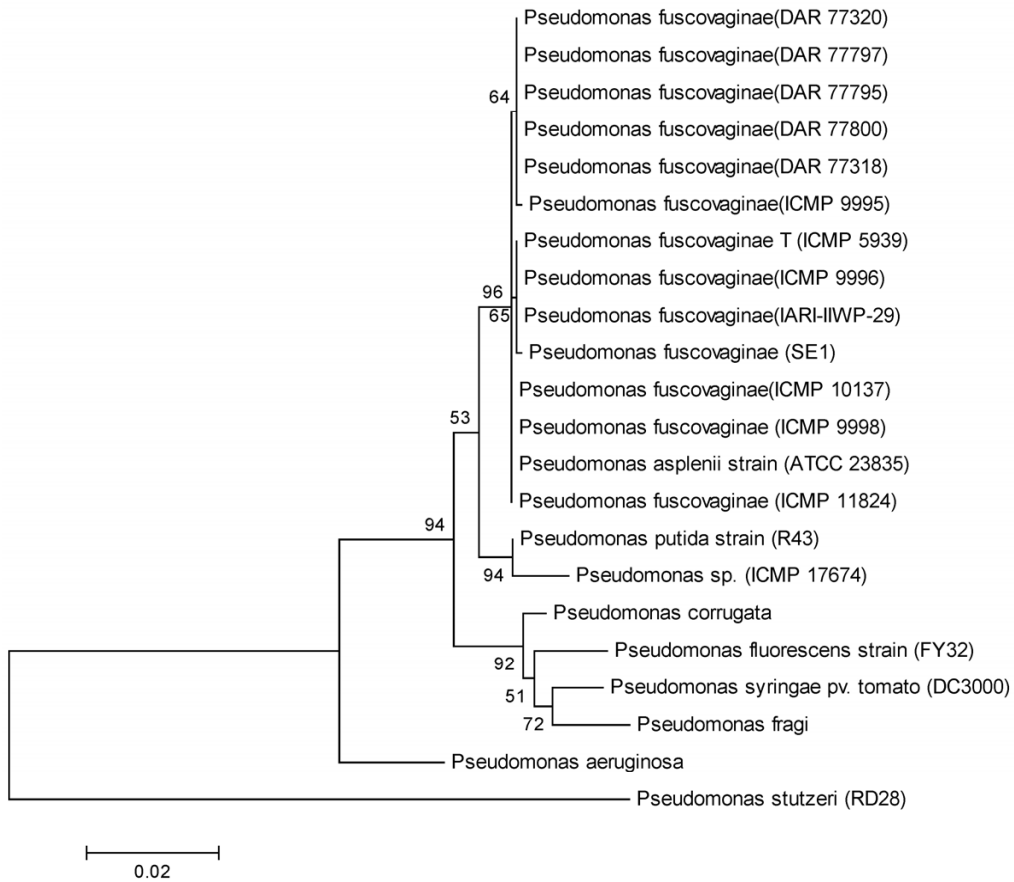

Fig. 1. Molecular phylogenetic analysis of the 16s ribosomal RNA gene inferred using the maximum-likelihood method. The tree is drawn to scale, with branch lengths measured in number of substitutions per site. Only bootstrap values (1,000 replicates) greater than 50% are shown next to branches.

cells in a 1-µl aliquot in all samples tested. The bacterial cell concentration and the number PCR cycles required to reach the minimum threshold were correlated ($R^2 = 0.9722$; Fig. 4).

Rice plants inoculated with *P. fuscovaginae* strain DAR 77795 showed typical symptoms of brown sheath rot 5 days after inoculation. Serial dilutions of all the inoculated plant extract showed amplification at all dilutions, whereas there was no amplification from the water-inoculated controls.

Discussion

The number and diversity of whole-genome sequences of plant-pathogenic bacteria continues to increase rapidly, generating huge quantities of data. Many of these genomes are in a draft form and require extensive annotation and comparative analysis. However, draft genomes can be used to find sequences that are unique to species or pathovars of bacterial plant pathogens (7,24,25). Comparative programs such as EDGAR (6) have been used to rapidly identify genomic regions that are unique to a particular species or pathovar which, in turn, can then be used to design LAMP primers (7). The inclusivity of the assay designed using this genomic approach is dependent on the selection of strains that represent the genetic diversity within the group to be analyzed. This is particularly important for Pseudomonads, which are a biologically and genetically diverse group (38).

In this study, we report the draft genome of a strain of *P. fuscovaginae* (SE1) from the Philippines, which was used in conjunction with previously published genomes of other strains of the bacterium (32,42) to develop a robust LAMP assay. This assay was specific to *P. fuscovaginae*-associated sheath brown rot of rice. The 16s rRNA gene is traditionally used to ascribe a bacterial strain to a genus (35) but it is incapable of resolving strains below the genus level (4,43). The initial phylogenetic analysis of the 16s rRNA gene of *Pseudomonas* spp. (Fig. 1) showed that the strains chosen for this study clustered with the *P. fuscovaginae* type strain. This supports the work of Cother et al. (10), who reported the presence of *P. fuscovaginae* in Australia. Strain SE1, first isolated from the Philippines (12) and shown to be highly pathogenic to rice (1), is also a member of this group. However, based on MLST, the SE1 strain is more closely related to *P. stutzeri*. MLST is often used to increase the resolution of phylogenetic analysis using several housekeeping genes (4,29,43). Finally, concatenated genes from the available draft genomes of *P. fuscovaginae* supported the inclusion of strain SE1 within the species, and helped to define the diversity among the species. This knowledge was used to develop the LAMP assay. Relative to conventional PCR, LAMP is highly specific (due, in part, to the use of six primers), it is an isothermal reaction, and the DNA yields allow visualization using in-tube staining (25). However, the high yield of DNA also presents a problem, because DNA contained in aerosols may yield false-positive results. This can be avoided by the use of systems that do not require the tube to be opened after amplification (such as the Genie II system), or the use of a system where the dye is brought into contact with the DNA after amplification by centrifugation or shaking (18). These approaches will be particularly important when attempting to deploy these assays in the field.

The use of strain SE1 (the most divergent strain for which a draft whole-genome sequence is available) in the initial identification of target genomic DNA contributed to the specificity of the LAMP assay. No false positives were observed when the assay was tested with DNA from over 30 strains of other bacteria often associated with rice and rice seed. Furthermore, the assay differentiated closely related strains that were isolated from hosts other than rice and from the closely related *Pseudomonas* sp. recently reported from Cambodia (9). The assay detected a concentration of 16 cells of *P. fuscovaginae* per LAMP reaction, which is a similar level of sensitivity reported for the detection of *E. amylovora* (8). The high sensitivity of LAMP assays is useful when determining the presence of a potential human pathogen in water or clinical samples; however, this may be a drawback for application in the field for routine identification or epidemiological studies of plant pathogens, because there are many opportunities for cross-contamination.

In summary, using comparative genomics of a worldwide collection of strains of *P. fuscovaginae*, we developed LAMP primers that are specific for strains of *P. fuscovaginae* isolated from rice in South America, Asia, Australia, and Africa. The availability of draft whole-genome sequences increases the number of available targets for the development of LAMP diagnostics, which provides opportunity for greater specificity or inclusivity and wider applications for the assays in the future. These primers could be used to confirm isolate identity of bacteria from culture collections and for epidemiological studies and disease surveys, or to assist in the restriction of movement of this important pathogen into new areas of rice production.

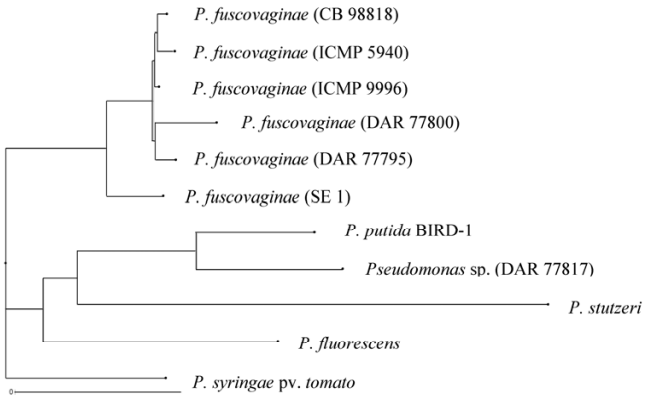

Fig. 3. Phylogenetic tree of *Pseudomonas fuscovaginae* based on the concatenated sequences of the genomes using the programs EDGAR and DARWin (5,32).

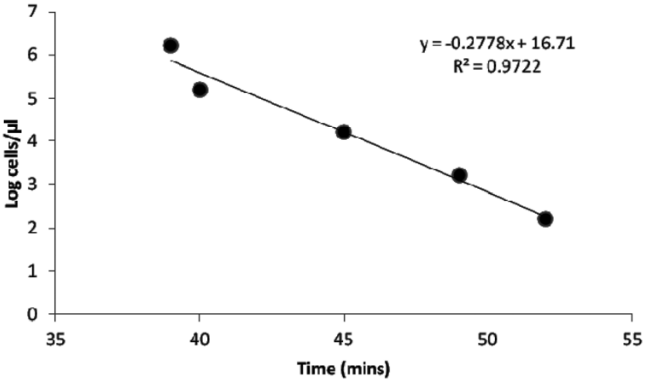

Fig. 4. Correlation between time required to detect *Pseudomonas fuscovaginae* by loop-mediated isothermal amplification assay and the number of bacterial cells per microliter.

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