Validation of Species-Specific PCR Assays for the Detection of *Pantoea ananatis*, *P. agglomerans*, *P. allii*, and *P. stewartia*

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Abstract

Species of *Pantoea* represent a group of plant pathogenic bacteria that infect a variety of agro-economically important plant species. Among these, a complex of *P. ananatis*, *P. allii*, *P. agglomerans*, and *P. stewartii* subsp. *indologenes* cause center rot in onion, resulting in significant economic losses. As species of *Pantoea* are phenotypically closely related, identification of *Pantoea* species relies on the sequencing and phylogenetic analysis of housekeeping genes. To aid in rapid identification of *Pantoea* species, efforts have been made in developing species-specific primers to be used in PCR assays. In the current study, two *P. ananatis*, one *P. allii*, one *P. agglomerans*, and three *P. stewartii* published primers as well as newly developed *P. agglomerans* PagR primers were evaluated for their specificity against 79 *Pantoea* strains, belonging to 15 different species. To ensure that selected primers were evaluated against accurately identified species, sequencing and phylogenetic analysis of housekeeping gene *infB* were conducted. Thereafter, PCR assays using selected species-specific primers were performed. The results showed that previously described *P. ananatis*-specific PANA_1008; *P. allii*-specific allii-leuS; *P. stewartii*-specific PANST_rpoB, 3614galE, and DC283galE primers; and one newly designed *P. agglomerans*-specific PagR primer pair were highly specific for their target *Pantoea* species. They accurately identified these strains into their species and, in some cases, their subspecies level. The findings of the current study will facilitate rapid and reliable identification of *P. ananatis*, *P. agglomerans*, *P. allii*, and *P. stewartii.*

Keywords: conventional PCR assay, identification, Pantoea, speciesspecific primers

The genus *Pantoea* within the family *Erwiniaceae* (Adeolu et al. 2016) comprises a group of gram-negative, rod-shaped, yellow-pigmented bacteria that are distributed in varied ecological niches including soil (Selvakumar et al. 2008; Sulbaran et al. 2009), rivers (Morohoshi et al. 2007), insect gut (Dutta et al. 2014; Krawczyk et al. 2021; Maccollom et al. 2009), the phyllosphere (Lindow and Brandl 2003; Nadarasah and Stavrinides 2014), and the rhizosphere (Shariati et al. 2017). Some members of this genus have been reported as opportunistic human pathogens (Brady et al. 2010; Volksch et al. 2009), and many form close associations with crop plants and weeds as beneficial or commensalistic endo- or epiphytes (Gitaitis et al. 2002; Lu et al. 2021; Quecine et al. 2012; Rijavec et al. 2007). Nevertheless, *Pantoea* species are primarily known as plant pathogens that cause economically important diseases in many agriculturally important plant species including corn (Goszczynska et al. 2007; Paccola-Meirelles et al. 2001), onion (Gitaitis and Gay 1997), rice (Cother et al. 2004, Kini et al. 2017), sorghum (Cota et al. 2010), and wheat (Krawczyk et al. 2020).

Center rot of onion (*Allium cepa* L.) is a bacterial disease that is characterized by water-soaked lesions on leaves that develop into necrotic streaks, leading to stalk and internal scale rot of onion bulbs (Gitaitis and Gay 1997). The yield losses can occur both in the field and postharvest conditions with losses up to 100% (Walcott et al. 2002). *Pantoea ananatis* (Carr et al. 2010; Gitaitis and Gay 1997) and *P. agglomerans* (Edens et al. 2006; Hattingh and Walters 1981; Tho et al. 2015) were initially known as the causal agents of center rot of onion, but other *Pantoea* species such as *P. allii* (Brady et al. 2011; Edens et al. 2006) and *P. stewartii* subsp. *indologenes* pv. *cepacicola* (Koirala et al. 2021; Stumpf et al. 2018) have also been identified as pathogens of onion. Of these, *P. ananatis* and *P. agglomerans* are transmitted by *Thrips tabaci*, the onion thrips (Dutta et al. 2014), and as these pathogens are seed-borne (Brady et al. 2011; Goszczynska et al. 2006; Walcott et al. 2002), the management of the disease caused by *Pantoea* species is challenging, especially in the absence of resistant onion cultivars.

Rapid identification of *Pantoea* species using phenotypic characteristics is problematic due to the metabolic versatility and morphological similarity displayed by *Pantoea* species at an intraspecific and interspecies level (Coutinho and Venter 2009; Walterson and Stavrinides 2015), respectively. Furthermore, as different *Pantoea* species often coexist in the same onion tissue, (Vahling-Armstrong et al. 2016), species isolation and identification based solely on colony morphology or biochemical characteristics are unreliable. To circumvent this problem, a genotypic or PCR-based detection method has been developed to detect and identify a specific species of *Pantoea* (Asselin et al. 2016; Braun-Kiewnick et al. 2012; Gehring et al. 2014; Kini et al. 2021; Rahimi-Khameneh et al. 2019). These detection methods make use of a single PCR assay using species-specific primers to yield accurate taxonomic identification, thereby bypassing the need for sequencing. As conventional PCR is still a widely used tool that can rapidly process numerous samples at an economic cost, validation of these *Pantoea* species-specific primers against off-target *Pantoea* species would be useful and important for their application.

Therefore, in this study, we evaluated the specificity of the eight selected and newly designed *Pantoea* species-specific primers against the collection of 79 *Pantoea* strains, which included 15 different *Pantoea* species that were recovered from a wide range of plant hosts (25 plant hosts) and geographical regions (16 countries). To accurately assess the primers' specificity, species identity of the *Pantoea* collection used in this study was first verified by sequencing and phylogenetic analysis of the housekeeping gene *infB*. Afterward, the same genomic DNA extracted from the pure cultures of 79 *Pantoea* strains for the phylogenetic analysis was used

for the species-specific PCR assays. The findings of this study will provide valuable information that will aid in rapid and reliable species level identification of *P. ananatis*, *P. agglomerans*, *P. allii*, and *P. stewartii*.

The list of strains used in this study and their information is found in Table 1. The strains were streaked on Luria-Bertani agar plates (10 g/liter sodium chloride, 10 g/liter tryptone, 5 g/liter yeast extract, and 15 g/liter agar; pH 7.0) and incubated at 28°C for growth overnight. The resulting pure cultures were collected for genomic DNA extraction using the *Quick*-DNA Miniprep kit (Zymo Research). The purified DNA was quantified with the Eppendorf Biospectrometer basic and was adjusted to a final concentration of 40 to 50 ng/µl with 1× TE buffer (10 mM Tris-HCl and 1 mM EDTA; pH 8.0) and stored at 4°C until PCR.

Table 1. List of strains used in this study for the testing of *Pantoea* species-specific primers[a]

Lane in gel	Abbreviation used in Fig. 2	Species	Strain	Source	Location (country)	Year	Source/reference	*infB* GenBank accession
1	Pana	*P. ananatis*	BCC 0026	insect (Miriidae)	South Africa	NR	T. Coutinho Collection	OM272848
2	Pana	*P. ananatis*	LMG 20103	eucalyptus	South Africa	2000	De Maayer et al. 2010	OM272849
3	Pana	*P. ananatis*	LMG 2665^T	pineapple	Brazil	1928	LMG Bacteria Collection	OM272850
4	Pana	*P. ananatis*	CTB 1135	rice	Japan	1995	Kido et al. 2008	OM272851
5	Pana	*P. ananatis*	ATCC 35400	honey melon	U.S.A.	1981	Brady et al. 2008	OM272852
6	Pana	*P. ananatis*	LMG 5342	human	U.S.A.	1983	De Maayer et al. 2012	OM272854
7	Pana	*P. ananatis*	DAR 76143	rice	Australia	2004	NSW Plant Pathology & Mycology Herbarium	OM272855
8	Pana	*P. ananatis*	ICMP 10132	sugarcane	Brazil	1991	ICMP Culture Collection	OM272853
9	Pana	*P. ananatis*	BD 250	onion	South Africa	2004	T. Coutinho Collection	OM272856
10	Pana	*P. ananatis*	BD 442	maize	South Africa	2004	Weller-Stuart et al. 2017	OM272857
11	Pana	*P. ananatis*	PNA 97-1R	onion	U.S.A.	1997	Gitaitis and Gay 1997	OM272859
12	Pana	*P. ananatis*	CO119	NR	U.S.A.	1994	H. Shwartz Collection	OM272858
13	Pagg	*P. agglomerans*	LMG 1286^T	human	Zimbabwe	1967	Brady et al. 2008	OM272860
14	Pagg	*P. agglomerans*	LMG 2565	cereal	Canada	1979	Brady et al. 2008	OM272861
15	Pagg	*P. agglomerans*	BCC 0383	eucalyptus	Uruguay	2004	T. Coutinho Collection	OM272862
16	Pagg	*P. agglomerans*	BCC 0560	gypsophila	U.S.A.	1946	T. Coutinho Collection	OM272863
17	Pagg	*P. agglomerans*	LMG 2596	onion	South Africa	1977	Hattingh and Walters 1981	OM272864
18	Pagg	*P. agglomerans*	ATCC 13329	beet	U.S.A.	1984	Cooksey 1986	OM272865
19	Pagg	*P. agglomerans*	LMG 2554	runner bean	U.K.	1981	Brady et al. 2008	OM272866
20	Pagg	*P. agglomerans*	LMG 2572	wheat	Canada	1968	Brady et al. 2008	OM272867
21	Pagg	*P. agglomerans*	BD 1274	onion	South Africa	NR	Moloto et al. 2020	OM272868
22	Pagg	*P. agglomerans*	BD 823	daisy	South Africa	2008	T. Coutinho Collection	OM272869
23	Pagg	*P. agglomerans*	PNG 06-1	onion	U.S.A.	2006	UGA-CPES	OM272870
24	Pagg	*P. agglomerans*	Ptg016	onion	U.S.A.	2010	L. du Toit Collection	OM272871
25	Psi	*P. stewartii* subsp. *indologenes*	0696-21	sudangrass	U.S.A.	1996	Azad et al. 2000	OM272872
26	Psi	*P. stewartii* subsp. *indologenes*	MKB 0035	eucalyptus	South Africa	NR	T. Coutinho Collection	OM272873
27	Psi	*P. stewartii* subsp. *indologenes*	LMG 2632^T	foxtail millet	India	1960	Brady et al. 2008	OM272874
28	Pss	*P. stewartii* subsp. *stewartii*	LMG 2713	corn	U.S.A.	1963	Brady et al. 2008	OM272875
29	Pss	*P. stewartii* subsp. *stewartii*	LMG 2718	NR	U.S.A.	1982	Brady et al. 2008	OM272876
30	Psi	*P. stewartii* subsp. *indologenes*	LMG 2674	pineapple	Hawaii	1982	LMG Bacteria Collection	OM272877
31	Psi	*P. stewartii* subsp. *indologenes*	LMG 2630	guar gum powder	NR	1966	Brady et al. 2008	OM272878
32	Psi	*P. stewartii* subsp. *indologenes*	ICMP 12183	cassia	Brazil	1991	ICMP Culture Collection	OM272879
33	Psi	*P. stewartii* subsp. *indologenes*	LMG 2629	pearl millet	India	1963	LMG Bacteria Collection	OM272880
34	Psi	*P. stewartii* subsp. *indologenes*	BCC 1640/ BD 641	corn	South Africa	2005	T. Coutinho Collection	OM272881
35	Psi	*P. stewartii* subsp. *indologenes*	LMG 2635	maize	India	1981	LMG Bacteria Collection	OM272882
36	Psi	*P. stewartii* subsp. *indologenes*	BCC 1502/ BD 313	onion	South Africa	2002	T. Coutinho Collection	OM272883
37	Pall	*P. allii*	LMG 24202/ BD 309	onion	South Africa	2004	Brady et al. 2011	OM272884

38	Pall	*P. allii*	LMG 24203/ BD 377	onion	South Africa	2004	Brady et al. 2011	OM272885
39	Pall	*P. allii*	LMG 24248^T/ BD 390	onion	South Africa	2004	Brady et al. 2011	OM272886
40	Pall	*P. allii*	BCC 1493/ BD 304	onion	South Africa	2002	T. Coutinho Collection	OM272887
41	Pall	*P. allii*	20TX20	onion	U.S.A.	2020	S. Malla Collection	OM272888
42	Pall	*P. allii*	19UT001	onion	U.S.A.	2019	C. Nischwitz Collection	OM272889
43	Peuc	*P. eucalypti*	LMG 24197^T	eucalyptus	Uruguay	NR	Brady et al. 2008	OM272890
44	Peuc	*P. eucalypti*	BCC 0676	eucalyptus	South Africa	2006	T. Coutinho Collection	OM272891
45	Peuc	*P. eucalypti*	BCC 1489	onion	South Africa	2002	T. Coutinho Collection	OM272892
46	Peuc	*P. eucalypti*	BCC 0760	eucalyptus	Uruguay	2004	T. Coutinho Collection	OM272893
47	Peuc	*P. eucalypti*	20TX118	onion	U.S.A.	2020	S. Malla Collection	OM272895
48	Peuc	*P. eucalypti*	PNG09-2	onion	U.S.A.	2009	B. Dutta collection	OM272894
49	Pvag	*P. vagans*	LMG 24195	eucalyptus	Uruguay	2004	Brady et al. 2008	OM272896
50	Pvag	*P. vagans*	LMG 24196	eucalyptus	Argentina	2004	Brady et al. 2008	OM272897
51	Pvag	*P. vagans*	LMG 24199^T	eucalyptus	Uganda	2003	Brady et al. 2008	OM272898
52	Pvag	*P. vagans*	BCC 0662	sunflower	NR	NR	T. Coutinho Collection	OM272899
53	Pvag	*P. vagans*	BCC 1638	maize	South Africa	2005	T. Coutinho Collection	OM272900
54	Pvag	*P. vagans*	Ptg010 / C9-1	apple	U.S.A.	NR	L. du Toit Collection	OM272901
55	Pant	*P. anthophila*	LMG 2560	marigold	NR	1966	Brady et al. 2008	OM272902
56	Pant	*P. anthophila*	LMG 2941	crab apple	NR	1979	LMG Bacteria Collection	OM272903
57	Pant	*P. anthophila*	LMG 2558^T	balsam	India	1964	Brady et al. 2008	OM272904
58	Pant	*P. anthophila*	BCC 1643	maize	South Africa	2005	T. Coutinho Collection	OM272906
59	Pant	*P. anthophila*	BCC 1592	maize	South Africa	2005	T. Coutinho Collection	OM272905
60	Pant	*P. anthophila*	BCC 1651	maize	South Africa	2005	T. Coutinho Collection	OM272907
61	Pdis	*P. dispersa*	LMG 2749	human	NR	1979	LMG Bacteria Collection	OM272909
62	Pdis	*P. dispersa*	LMG 2603^T	soil	Japan	1979	Brady et al. 2010	OM272911
63	Pdis	*P. dispersa*	BCC 0210	eucalyptus	Thailand	2004	T. Coutinho Collection	OM272913
64	Pdis	*P. dispersa*	LMG 2602	sorghum	India	1970	LMG Bacteria Collection	OM272908
65	Pdis	*P. dispersa*	LMG 2605	cowpea	Tanzania	1965	LMG Bacteria Collection	OM272910
66	Pdis	*P. dispersa*	20TX134	onion	U.S.A.	2020	S. Malla Collection	OM282912
67	Pcon	*P. conspicua*	BCC 0541	milkpowder	U.K.	2008	T. Coutinho Collection	OM272915
68	Pcon	*P. conspicua*	LMG 24534^T	human	France	2006	Brady et al. 2010	OM242914
69	Psep	*P. septica*	LMG 5345^T	human	U.S.A.	1983	Brady et al. 2010	OM272916
70	Pbre	*P. brenneri*	LMG 5343^T	human	U.S.A.	1983	Brady et al. 2010	OM272917
71	Pbre	*P. brenneri*	LMG 24532	human	U.S.A.	2006	Brady et al. 2010	OM272918
72	Pcyp	*P. cypripedii*	BCC 0889	marula	South Africa	2007	T. Coutinho Collection	OM272920
73	Pcyp	*P. cypripedii*	LMG 2657^T	orchid	U.S.A.	1982	Brady et al. 2010	OM272919
74	Pwal	*P. wallisii*	LMG 26277^T	eucalyptus	South Africa	2006	Brady et al. 2012	OM272921
75	Pwal	*P. wallisii*	BD 946^T = LMG 26277^T	eucalyptus	South Africa	2006	Brady et al. 2012	OM272922
76	Pbej	*P. beijingensis*	BCC 1348	oyster mushroom	China	2012	Liu et al. 2013	OM272926
77	Pbej	*P. beijingensis*	BCC 1349	oyster mushroom	China	2012	Liu et al. 2013	OM272925
78	Pdel	*P. deleyi*	LMG 24200^T	eucalyptus	Uganda	NR	Brady et al. 2008	OM272923
79	Peucr	*P. eucrina*	LMG 2781^T	human	U.S.A.	1981	Brady et al. 2010	OM272924

[a] NR = Not recorded.

To verify the taxonomic identification of the strains belonging to the collection, a housekeeping gene *infB* (translation initiation factor β-subunit, Brady et al. 2008) was sequenced and phylogenetically examined for all 79 strains (Table 1). A total volume of 25 µl of PCR reaction was used for the amplification of the *infB* gene. Per reaction consisted of 12.5 µl of 2× GoTaq Green Master Mix (Promega Corporation), 2.5 µl of 10 µM forward primer, 2.5 µl of 10 µM reverse primer, 6.5 µl of nuclease-free water, and 1 µl of template DNA, and the reaction was run on the FlexCycler^2 (Analytik Jena AG, Jena, Germany) according to the cycling condition suggested by Brady et al. (2008).

The *infB* PCR product was column-purified using the Monarch PCR and DNA clean-up kit (NEB, Ipswich, MA) and was sequenced at Eurofins Genomics LLC (Louisville, KY). The *infB* gene sequences of the type species of *Pantoea* were downloaded from GenBank database and aligned with the sequences resulting from the collection using MAFFT v 7.48 (Katoh et al. 2002). The overhangs were trimmed in BioEdit v 7.2 (Hall 1999). The best-fit evolutionary model was searched by jModelTest v 2.1.10 (Darriba et al. 2012; Guindon and Gascuel 2003), and the maximum-likelihood phylogenetic tree was constructed using online PhyML v 3.0 (Guindon and Gascuel 2003). The 1,000 replicate bootstrap analysis was conducted to test the reliability of the branches. Lastly, the tree was visualized and edited in MEGA X (Kumar et al. 2018).

A total of eight primer pairs consisting of two *P. Ananatis*-, one *P. allii*-, two *P. agglomerans*-, and three *P. stewartii*-specific primers were evaluated against 79 *Pantoea* strains. The sequences, annealing temperature, and expected PCR amplicon size of the species-specific primers are listed in Table 2. In addition to previously published primers, a pair of *P. agglomerans*-specific primers was newly designed for this study. The *pagR* gene region had been previously used to design *P. agglomerans*-specific qPCR assays (Braun-Kiewnick et al. 2012). The *pagR* gene from the publicly available, complete genome of *P. agglomerans* strain C410P1 (GenBank genome accession number: NZ_CP016889, *pagR* gene locus tag in C410P1: BEE12_RS09685) was used to conduct NCBI nucleotide BLAST search against the genomes, and sequences were deposited in GenBank. A total of 95 nucleotide sequences of *P. agglomerans pagR* and its homologs were downloaded and aligned using Clustal W (Thompson et al. 1994) function in BioEdit v 7.2 (Hall 1999). The conserved regions in the *pagR* gene that were distinctive to *P. agglomerans* were manually searched on which the *P. agglomerans*-specific PCR primers were designed using Geneious Prime 2022.0.1 (https://www.geneious.com).

Table 2. List of *Pantoea* species-specific primers used in this study

Target species	Name	Sequence	Target gene	Amplicon size (bp)	Annealing temperature (°C)	Extension time (s)	Source
Pantoea species	infB-01_F infB-02_R	ATYATGGGHCAYGTHGAYCA ACKGAGTARTAACGCAGATCCA	Translation initiation factor IF2 encoding gene (*infB*)	1124	55	60	Brady et al. 2008
P. ananatis	PANAN_gyrB_fwd PANAN_gyrB_rev	GATGACGARGCCATGCTGC GATCTTGCGGTATTCGCCAC	DNA gyrase β-subunit encoding gene (*gyrB*)	423	58	30	Kini et al. 2021
	PANA_1080_61F PANA_1080_1009R	ACCCTGTCCCCGTTGGCACTGT AATGATGCCCACTGTTGAAGGAAT	Hypothetical protein encoding gene	949	55	60	Asselin et al. 2016
P. allii	allii-leuS181F allii-leuS235R	GTCCGGACACCTTCTATGGCGCAA GGTTGCTTGCGGAAGCCTGCAGT	Leucine t-RNA ligase encoding gene (*leuS*)	54	60	30	Rahimi-Khamenech et al. 2019
P. agglomerans	PANAG_infB_fwd PANAG_infB_rev	GATGACGARGCCATGCTGC TGTCCGGCGTGCCGGCTG	Translation initiation factor IF2 encoding gene (*infB*)	730	58	60	Kini et al. 2021
	PagR_210F PagR_626R	TCAGAGTTTACGTTGTGTTGAAG GTGTGGCGTTATTCACTCCGAGC	LuxR family transcriptional regulator protein gene	416	55	30	This study
P. stewartii	PANST_rpoB_fwd PANST_rpoB_rev	CACCGGTGAACTGATTATCG GTCCTGAGGCATCAATGTGT	RNA polymerase β-subunit encoding gene (*rpoB*)	559	58	30	Kini et al. 2021
P. stewartii subsp. *indologenes*	3614galE 3614galEc	CGACCTGTTTGCCTCTCACC CATCAGCTTGGAGGTGCCG	UDP-glucose 4-epimerase encoding gene (*galE*)	250	68 → 63	30	Gehring et al. 2014
P. stewartii subsp. *stewartii*	DC283galE DC283galEc	CGACCTGTTTGCCTCTCACT CATCAGCTTGGAGGTGCCA		250	68 → 63	30	Gehring et al. 2014

For species-specific PCR assays, 10 ul of reaction was used, which was composed of 5 µl of 2× GoTaq Green Master Mix, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 2 µl of nuclease-free water, and 1 µl of template DNA. The PCR reactions were run with the following conditions: 2 min initial denaturation at 95°C, 30 cycles of 1 min denaturation at 95°C, 1 min annealing at a primer-specific temperature (Table 2), 30 s extension at 72°C (1 min/kb), and a final extension at 72°C for 5 min until it holds at 4°C.

For the amplification of *P. stewartii* subsp. *indologenes* and subsp. *stewartii*-specific *galE* gene, a two-step PCR cycling condition, previously described by Gehring et al. (2014), was used with a slight modification. The first step consisted of 10 cycles of denaturation at 95°C for 45 s, annealing at 68°C for 30 s, and extension at 72°C for 30 s. The second step used 20 cycles of the same conditions but with a lower annealing temperature of 63°C.

A volume of 5 µl of PCR product was visualized in 1% TBE (130 mM Tris-HCl, 45 mM boric acid, and 2.5 mM EDTA; pH 8.0) agarose gel stained with SYBR Safe dye (Thermo Scientific). The PCR reactions were loaded in a uniform manner for all assays, as indicated by the lane number in Table 1. The amplicon sizes of the PCR assays using previously described species-specific primers were checked against the PCR product sizes that were described in the original literatures (Asselin et al. 2016; Gehring et al. 2014; Kini et al. 2021; Rahimi-Khameneh et al. 2019).

Species identity verification of the *Pantoea* collection.

The strains used in this study were largely represented by the four target species (*P. ananatis*, *P. agglomerans*, *P. allii*, and *P. stewartii* subsp. *indologenes*) that were isolated from symptomatic plant hosts, including corn, millet, onion, pineapple, and rice (Table 1). However, other plant associated *Pantoea* species like *P. anthophila*, *P. cypripedii*, *P. deleyi*, *P. dispersa*, *P. eucalypti*, *P. vagans*, and *P. wallisii* and clinical strains of *P. brenneri*, *P. consipcua*, *P. eucrina*, and *P. septica* have also been included.

Prior to the assessment of specificity of selected primers, species identity of our *Pantoea* collection which was previously established (data not shown) was re-evaluated to ensure that the primers are tested against correctly identified species. For this step, genomic DNA extracted from pure cultures of 79 *Pantoea* strains (Table 1) were utilized for the sequencing of the *infB* gene, a housekeeping gene that is frequently used for the identification of *Pantoea* species (Brady et al. 2008, 2010, 2011). The GenBank accession numbers of the *infB* gene sequences generated from this study are also included in Table 1. Subsequently, the maximum-likelihood phylogenetic analysis of the *infB* sequences of 79 strains against that of known, type *Pantoea* species showed that our collection of *Pantoea* strains were in fact accurately identified (Fig. 1) with one exception. Two strains, BCC 1348 and BCC 1349, grouped with the *Erwinia* branch instead of *Pantoea* branches, indicating closer association of these two strains as well as the type strain of *P. beijingensis* LMG27579 to the species of *Erwinia* rather than that of *Pantoea*. However, grouping of *P. beijingensis* with *Erwinia* species agrees with the findings of Rezzonico et al. (2016) and Xu et al. (2021), and *P. beijingensis* is likely to be reclassified as *E. beijingensis*.

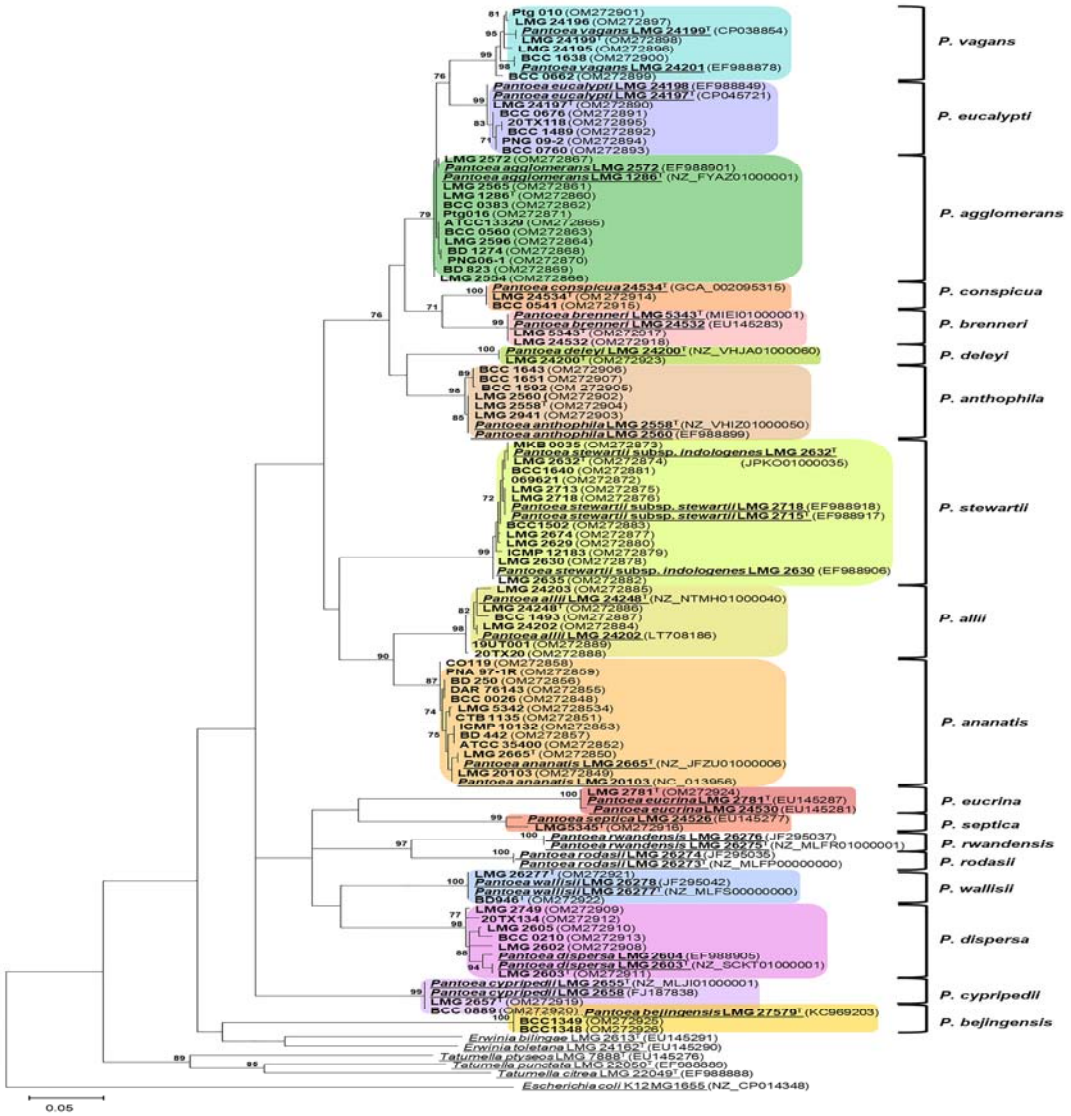

Fig. 1. A maximum-likelihood phylogenetic tree based on the partial *infB* gene sequences (615 bp) of *Pantoea* strains used in this study (highlighted by colored boxes). The NCBI GenBank accession numbers are specified in the brackets, and underlined names indicate the reference sequences that are downloaded from GenBank. The bootstrap values below 70% are not shown, and *Escherichia coli* K12 was used as an outgroup.

P. ananatis-specific PCR assay.

The *P. ananatis*-specific primers, PANA_1080_61F and PANA_1080_1009R, designed by Asselin et al. (2016) were highly specific for *P. ananatis* (Fig. 2A, lane 1 to 12). These primers did not amplify any amplicon from *P. allii* DNA samples, a phylogenetically closely related taxon of *P. ananatis*, nor from the DNA belonging to any other species. Furthermore, Asselin et al. (2016) demonstrated that PANA_1080 primers could specifically detect *P. ananatis* from environmental (onion) DNA samples that were mixed with other onion pathogenic bacteria and showed that the specificity of PANA_1080 primers were superior to other published *P. ananatis* primers that were not able to differentiate between *P. ananatis* and *P. allii* (Carr et al. 2010; Figueiredo and Paccola-Meirelles 2012; Gitaitis et al. 2002). The second pair of *P. ananatis*-specific primers tested in this study, PANAN_gyrB_fwd and

PANAN_gyrB_rev (Kini et al. 2021), have accurately identified DNA samples of *P. ananatis* (Fig. 2B, lane 1-12). However, the PCR also resulted in unspecific amplification of the template DNA belonging to multiple *Pantoea* species (Fig. 2B, lane 13 to 79). The similarity in the amplicon size (below 500 bp marker) between *P. ananatis* and that of other species (for example, lane 28 and 29) can make visual calling of the species identity difficult.

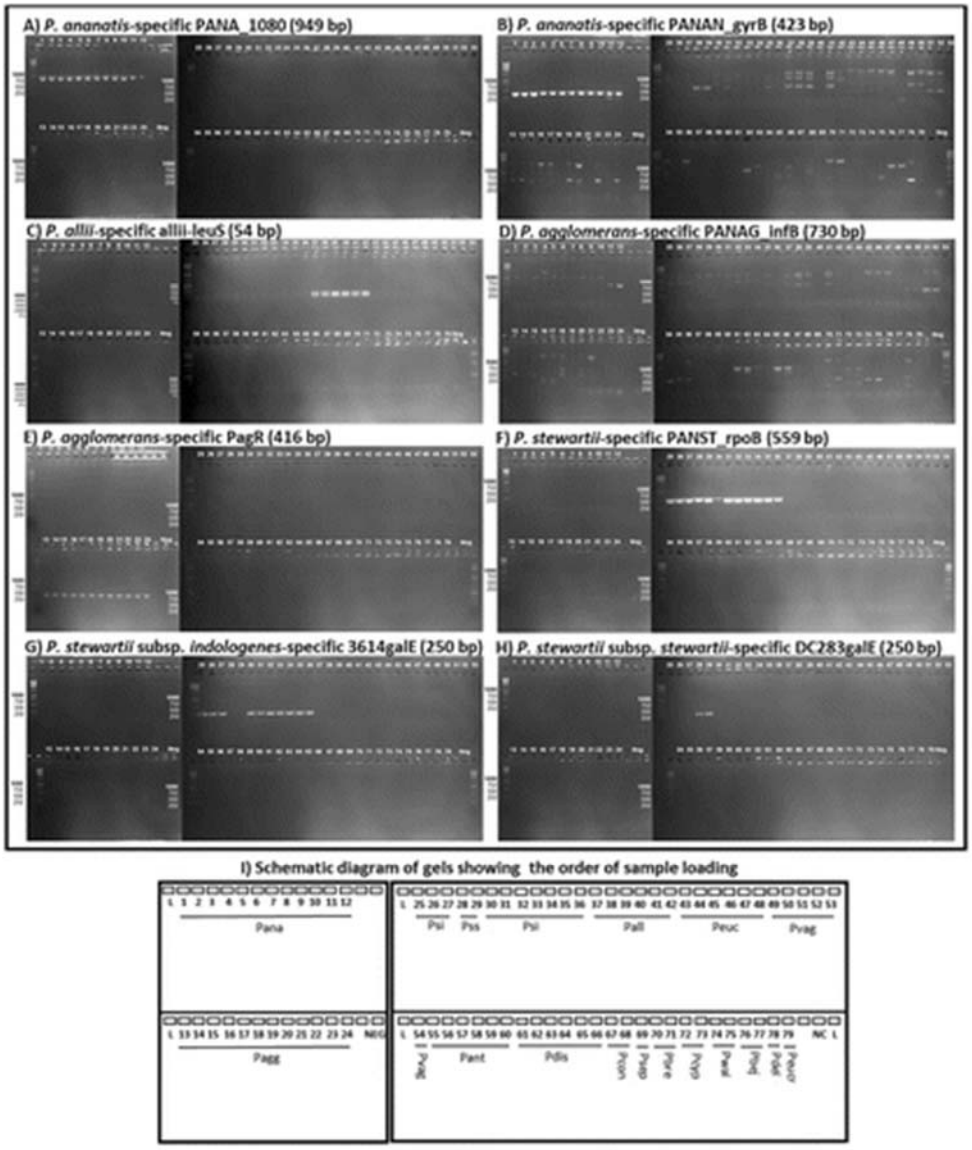

Fig. 2. Gel images of PCR assay using **A,** *Pantoea ananatis* specific primers PANAN_gyrB; **B,** *P. ananatis* specific primers PANA_1080; **C,** *P. allii* specific primers allii-leuS; **D,** *P. agglomerans* specific primers PANAG_infB; **E,** *P. agglomerans* specific primers PagR; **F,** *P. stewartii* specific primers PANST_rpoB; **G,** *P. stewartii* subsp. *indologenes* specific primers 3614galE; **H,** *P. stewartii* subsp. *stewartii* specific primers DC283galE; and **I,** a schematic diagram of gels illustrating the order of samples loaded into the wells. Abbreviations are as follows: L = DNA 1 kb ladder (Thermo Scientific) which was loaded on the left end of each gel except for **C,** for which DNA 1 kb plus ladder (Thermo Scientific) was used; Pana = *P. ananatis*; Pagg = *P. agglomerans*; Psi = *P. stewartii* subsp. *indologenes*; Pss = *P. stewartii* subsp. *stewartii*; Pall = *P. allii*; Peuc = *P. eucalypti*; Pvag = *P. vagans*; Pant = *P. anthophila*; Pdis = *P. dispersa*; Pcon = *P. conspicua*; Psep = *P. septica*; Pbre = *P. brenneri*; Pcyp = *P. cypripedii*; Pwal = *P. wallisii*; Pbej = *P. beijingensis*; Pdel = *P. deleyi*; Peucr = *P. eucrina*; and NC = Negative Control.

***P. allii*-specific PCR assay.**

P. allii-specific primers, allii-leuS181F and allii-leuS235R, were designed for Taqman-based quantitative real-time PCR (qRT-PCR) by Rahimi-Khameneh et al. (2019). However, in this study, these primers were used in conventional PCR reactions. In addition to four housekeeping genes (*atpD*, *gyrB*, *infB*, and *rpoB*) that are frequently employed in the multilocus sequence analysis for description of novel *Pantoea* species (Brady et al. 2008, 2010, 2011), the gene *leuS* (leucine t-RNA ligase encoding gene) has also been identified as a reliable marker for analyzing phylogenies of the genus *Pantoea* (Tambong et al. 2014). The allii-leuS181F and allii-leuS235R primers utilized the polymorphic nucleotides in *leuS* gene to allow differentiation of *P. allii* strains from rest of the *Pantoea* species. Despite small amplicon size, a distinct band (~50 bp) was produced for the reactions containing the DNA templates of *P. allii* strains (Fig. 2C, lane 37 to 42). A faint band, approximately 1,000 bp in size, was seen in lane 24 (Fig. 2C), but due to the size difference between the two amplicons, the use of allii-leuS181F and allii-leuS235R as diagnostic qRT-PCR should not be affected.

***P. agglomerans*-specific PCR.**

For the detection of *P. agglomerans*, two pairs of primers were evaluated. The PCR assay of the first pair, PANAG_infB_fwd and PANAG_infB_rev, did not yield a *P. agglomerans*-specific 730-bp product as reported by Kini et al. (2021). The presence of multiple bands across the *Pantoea* species suggests that these primers may not be specific for their target gene *infB* and thus failed to detect *P. agglomerans* (Fig. 2D, lane 13 to 24). The second pair, PagR_210F and PagR_626R, was designed for this study based on previous finding that *pagR* (LuxR family transcriptional regulator encoding gene) is a useful gene region for identifying *P. agglomerans*, specifically; it is chromosomally encoded in both the plant pathogenic and nonpathogenic strains of *P. agglomerans* by Rezzonico et al. (2009). Braun-Kiewnick et al. (2012) designed a qPCR assay based on 28 *pagR* gene sequences extracted from different *P. agglomerans* strains. However, the forward and reverse primers of this qPCR assay were designed on highly conserved regions of the *pagR* gene belonging to both *P. agglomerans* and the *Enterobacter* species. This would allow the amplification of the *pagR* gene in species other than *P. agglomerans*. In the 10 years since the original publication, many additional *Pantoea*, as well as other bacterial species genomes, have become available in public databases. Thus, we designed a new pair of conventional PCR primers on variable regions of the *pagR* gene to differentiate *P. agglomerans* from its phylogenetically close relative *P. vagans*, as well as from the *Enterobacter* species. For this purpose, a total of 85 *pagR* gene sequences were identified and downloaded from GenBank which is composed of 40 *P. agglomerans*, 10 *P. vagans*, three *Citrobacter* sp., and 42 *Enterobacter* spp. The nucleotide identity within *P. agglomerans* ranged from 96 to 100%, whereas similarity shared between *P. agglomerans* and non *P. agglomerans pagR* sequences was below 82%. Therefore, despite the presence of *pagR* homolog in other species, PagR_210F and PagR_626R primers were designed on the regions that were conserved within *P. agglomerans* but were polymorphic in other species. The PCR guided by PagR_210F and PagR_626R primers was highly discriminatory, where amplicons (416 bp) were only seen in the samples containing *P. agglomerans* DNA (Fig. 2E, lane 13 to 24).

***P. stewartii*-specific PCR.**

All three primer sets tested were highly specific for *P. stewartii* at varying levels. PANST_rpoB_fwd and PANST_rpoB_rev primers were able to unambiguously detect both

subspecies of *P. stewartii* (Fig. 2F, lane 25 to 36), whereas 3614galE primers and DC283galE primer pairs each distinguished its target subspecies, *P. stewartii* subsp. *indologenes* (Fig. 2G, lane 25 to 27 and 30 to 36) and *P. stewartii* subsp. *stewartii*, respectively (Fig. 2H, lane 28 to 29). According to Gehring et al. (2014), a subspecies-level identification of *P. stewartii* strains is crucial for distinguishing *P. stewartii* subsp. *indologenes* contaminants from the quarantine pathogen and causal agent of Stewart's wilt of maize, *P. stewartii* subsp. *stewartii* (Stewart 1897), in maize seeds. Similarly, some strains of *P. stewartii* subsp. *indologenes* are pathogenic on onion (Koirala et al. 2021; Stumpf et al. 2018), and a PCR test using 3614galE primers will aid in identifying *P. stewartii* subsp. *indologenes* from other *Pantoea* species that may also be present in the onion tissue.

In this study, we showed that the use of *Pantoea*-specific primers in conventional PCR assays accurately identified four *Pantoea* species: *P. ananatis*, *P. allii*, *P. agglomerans*, and *P. stewartii* subsp. *indologenes*. Out of the eight primer pairs, *P. ananatis*-specific PANA_1008; *P. allii*-specific allii-leuS; *P. agglomerans*-specific PagR; and *P. stewartii*-specific PANST_rpoB, 3614galE, and DC283galE primers were highly specific for their target *Pantoea* species when tested against 14 different *Pantoea* species. Furthermore, the species calls made by these PCR assays correlated with the species identity resolved by the sequencing and phylogenetical analysis of the *infB* gene. As demonstrated in this study, PCR assays using these primers will serve as an effective and sufficient tool in screening of *P. ananatis*, *P. allii*, *P. agglomerans*, and *P. stewartii* strains.

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