Phylogenetic study and multiplex PCR-based detection of *Burkholderia plantarii*, *Burkholderia glumae* and *Burkholderia gladioli* using *gyrB* and *rpoD* sequences

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In order to develop a detection method for the rice pathogens *Burkholderia plantarii*, *Burkholderia glumae* and *Burkholderia gladioli*, the phylogeny of six plant-pathogenic *Burkholderia* species was analysed using the combined nucleotide sequences of *gyrB* and *rpoD*. *B. plantarii*, *B. glumae* and *B. gladioli* formed tight monophyletic branches supported by high bootstrap probabilities. The high sequence similarity revealed a close phylogenetic relationship between *B. glumae* and *B. plantarii*. *B. plantarii* strains were divided into three subclusters comprising rice strains, whereas the single *Vanda* strain occupied a unique position in the phylogenetic tree. The *gyrB* and *rpoD* sequences of all *B. glumae* strains examined were highly conserved. In contrast, *B. gladioli* strains demonstrated a far greater sequence diversity, but this diversity did not correlate with pathovar, host plant or geographical origin of the strains. A multiplex-PCR protocol using specific primers from the *gyrB* sequences was designed that allowed the specific detection and identification of *B. plantarii*, *B. glumae* and *B. gladioli* in rice seeds infected with these pathogenic species.

INTRODUCTION

Bacterial seedling rot (Uematsu *et al.*, 1976) and seedling blight (Azegami *et al.*, 1987) caused by *Burkholderia glumae* and *Burkholderia plantarii*, respectively, are devastating diseases of rice seedling cultivation in nursery boxes. During germination, these pathogens infect seeds and invade plumules through stomata and wounds and proliferate in the intercellular spaces of the parenchyma (Azegami *et al.*, 1988; Hikichi *et al.*, 1995). Proliferation of *B. glumae* and *B. plantarii* in the plumules leads to production of the toxic materials toxoflavin (Sato *et al.*, 1989; Yoneyama *et al.*, 1998)

and tropolone (Azegami *et al.*, 1985), resulting in rot and blight in rice seedlings, respectively.

Within *Burkholderia gladioli*, three pathovars, all of which are recognized as phytopathogenic bacteria, have been delineated: *B. gladioli* pv. *gladioli*, which causes gladiolus rot (Hildebrand *et al.*, 1973); *B. gladioli* pv. *alliiicola*, which causes onion bulb rot (Young *et al.*, 1978); and *B. gladioli* pv. *agaricicola*, which causes rapid soft rot of cultivated mushrooms (Lincoln *et al.*, 1991). *B. gladioli* has also been isolated from rice seedlings showing bleaching symptoms, suggesting that this bacterium also infects rice seedlings (Kato *et al.*, 1992). On the other hand, proliferation of *B. glumae* and *B. plantarii* is suppressed in rice seeds infected with *B. gladioli* (Miyagawa, 2000). Therefore, ecological studies on *B. gladioli* in rice plants are important to understand disease development caused not only by *B. gladioli* but also by

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The GenBank/EMBL/DDBJ accession numbers for the *gyrB* and *rpoD* gene sequences determined in this study are given in Table 1.

B. glumae and *B. plantarii*. Such ecological studies require efficient detection and identification of these three rice-pathogenic *Burkholderia* species. Although the diversity of *Burkholderia* species has been analysed using 16S rRNA gene sequences (Hu *et al.*, 2001; Salles *et al.*, 2002), the discriminatory power of this gene is too restricted to reveal the detailed phylogenetic relationships among *B. plantarii*, *B. glumae* and *B. gladioli*. The 16S rRNA gene has been widely used for designing taxonomically meaningful, highly specific PCR primers, providing enough sequence information to allow the analysis of both close and distant phylogenetic relationships among micro-organisms (Stackebrandt & Goebel, 1994). However, the degree of resolution obtained with 16S rRNA gene sequence analysis is not sufficiently discriminatory to permit resolution of intrageneric relationships among closely related micro-organisms, because of the extremely slow rate of evolution of the 16S rRNA gene (Yamamoto & Harayama, 1998). The genes encoding the β -subunit polypeptide of DNA gyrase (*gyrB*) and σ^{70} factor (*rpoD*) are estimated to evolve much faster than the 16S rRNA gene (Yamamoto & Harayama, 1998).

In the present study, we analysed the phylogenetic diversity in the *gyrB* and *rpoD* gene sequences of these three rice-pathogenic *Burkholderia* species, in order to develop a specific and sensitive detection method.

METHODS

Bacteria and DNA preparation. Six *Burkholderia* species with validly published names, comprising a total of 108 strains, including 41 strains of *B. glumae*, 24 strains of *B. plantarii* and 37 strains of *B. gladioli*, were examined in this study (Table 1). Each bacterial isolate was grown aerobically in nutrient broth at 30 °C. Chromosomal DNA from the bacteria used as the PCR template was prepared using an AquaPure Genomic DNA Isolation kit (Bio-Rad), according to the supplier's instructions.

PCR amplification and sequencing of *gyrB* and *rpoD*. PCR of *gyrB* and *rpoD* was performed using primers UP-1E and AprU, and 70F2 and 70R2, respectively, as shown in Table 2. PCR was performed with a thermal cycler (TaKaRa) using PCR buffer (TaKaRa) containing 200 μ M of each of the dNTPs, 0.5 μ M of each primer, 0.2 μ g template DNA and 2.5 U *Ex-Taq* polymerase (TaKaRa), in a total volume of 40 μ l. A total of 35 amplification cycles were performed with template DNA denaturation at 94 °C for 1 min, primer annealing at 58 °C for 1 min and primer extension at 72 °C for 1 min. PCR products were electrophoresed on 1.0% agarose gels and purified using Quantum Prep Freeze 'N Squeeze DNA Gel Extraction spin columns (Bio-Rad), following the manufacturer's instructions. The nucleotide sequences of *gyrB* and *rpoD* were determined directly from the PCR fragments using primers M13R (5'-CAGGAAACAGCTATGACC-3') and M13(-21) (5'-TGTAACGACGCGCCAGT-3'), and 70Fs (5'-ACGACTGACCCGGTACGCATGTA-3') and 70Rs (5'-ATAGAAATAACCAGACGTAAGTT-3'), respectively. Sequencing was carried out using an ABI Automated DNA Sequencer model 373 (Applied Biosystems) and analysed using DNASIS-Mac software (Hitachi Software Engineering).

Data analysis. The nucleotide sequences of *gyrB* (768–801 bp) and *rpoD* (807–834 bp) genes were aligned and a phylogenetic tree was constructed using CLUSTAL W (DNA database of Japan; <http://www.ddbj.nig.ac.jp/search/clustalw-j.html>) with the neighbour-joining

method (Saitou & Nei, 1987), using genetic distances computed with Kimura's two-parameter model (Kimura, 1980). The neighbour-joining phylogenetic tree was drawn using TreeView. The nucleotide sequences of *gyrB* and *rpoD* from *Escherichia coli* K-12 were used as the outgroup for phylogenetic tree reconstructions.

Analysis of the phylogenetic diversity of *B. plantarii* strains.

Aliquots (40 μ l) of each PCR product from the genomic DNA of *B. plantarii* strains obtained using primers UP-1E and AprU were ethanol-precipitated and the pellets were dissolved in 10 μ l distilled water. The DNA in the solutions was digested with *SacI* and *HaeII* (both from TaKaRa) at 37 °C for 6 h, loaded onto horizontal 1.2% TAE agarose gels and stained with ethidium bromide after electrophoresis for detection of specific DNA fragments corresponding to the *gyrB* nucleotide sequences of the strains.

Multiplex PCR. To detect and discriminate *B. plantarii*, *B. glumae* and *B. gladioli* using single-step PCR, primers (Table 2) developed for the *gyrB* gene of the bacteria were used for development of a multiplex-PCR protocol. PCR was performed with one cycle of 94 °C for 2 min and 35 cycles of 94 °C for 1 min, 63 °C for 1 min and 72 °C for 1 min. Aliquots (9 μ l) of each PCR product were loaded onto horizontal 1.5% TAE agarose gels and stained with ethidium bromide after electrophoresis for detection of 597, 529 and 479 bp DNA fragments corresponding to the *gyrB* nucleotide sequences of *B. plantarii*, *B. glumae* and *B. gladioli*, respectively.

Analysis of rice seeds infected with *B. plantarii*, *B. glumae* and *B. gladioli*.

To test whether infection of rice seeds with *B. plantarii*, *B. glumae* and *B. gladioli* could be detected using multiplex PCR, rice seeds naturally infected with *B. plantarii* and *B. glumae* were obtained from Dr H. Miyagawa, National Agricultural Research Center for Western Region, Japan. Flowering spikelets of rice plants cultivated in pots were inoculated with a suspension of *B. plantarii* MAFF 302391, MAFF 302907, MAFF 302924, MAFF 311030 or MAFF 301723^T, *B. glumae* MAFF 301169^T or *B. gladioli* MAFF 302386, MAFF 302543, MAFF 302918, MAFF 302919 or T-1 at 1.0×10^8 c.f.u. ml⁻¹ by spray application at 10 ml per plant, and the resultant rice seeds infected with each strain were used in this study. Non-infected rice seeds were used as a control. One gram of rice seeds was ground in a mortar with 1.0 ml distilled water and the suspension was then placed in a microtube and centrifuged at 1000 g for 1 min. The supernatant was held at 100 °C for 8 min and then centrifuged at 12 000 g for 3 min. Twenty-five microlitres of the supernatant was used as a template for the multiplex PCR.

RESULTS AND DISCUSSION

Phylogenetic diversity of *B. plantarii*, *B. glumae* and *B. gladioli*

The phylogeny of six plant-pathogenic *Burkholderia* species (*Burkholderia andropogonis*, *Burkholderia caryophylli*, *Burkholderia cepacia*, *B. plantarii*, *B. glumae* and *B. gladioli*) was analysed using the combined nucleotide sequences of the *gyrB* and *rpoD* genes. These species all represented different clusters in the resulting phylogenetic tree (Fig. 1). The combined *gyrB* and *rpoD* nucleotide sequences showed high similarity and the sequence similarity between *B. glumae* MAFF 301169^T and *B. plantarii* MAFF 301723^T was 96.2%, indicating a close phylogenetic relationship between the two species. Furthermore, the *B. plantarii*, *B. glumae* and *B. gladioli* clusters were supported by high bootstrap probabilities, indicating that they each form a tight monophyletic branch (Fig. 1).

Table 1. *Burkholderia* strains analysed and classifications established according to nucleotide sequences of *gyrB* and *rpoD*

ATCC, American Type Culture Collection; GTC, Gifu Type Culture Collection, Japan; KU, Kyushu University, Japan; KUCS, Kochi University, Japan; LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Gent, Belgium; MAFF, Micro-organisms Section of the MAFF Gene Bank.

Strain	Geographical origin	Species name of host plant	Code no.	DDBJ accession no.	
				<i>gyrB</i>	<i>rpoD</i>
<i>Burkholderia andropogonis</i>					
ATCC 23061 ^T	USA	<i>Sorghum vulgare</i>	—	AB190570	AB190667
ATCC 19311	USA	<i>Dianthus caryophyllus</i>	—	AB190571	AB190668
MAFF 311215	Tsukuba, Japan	<i>Zea mays</i>	—	AB190572	AB190669
<i>Burkholderia caryophylli</i>					
ATCC 25418 ^T	USA	<i>Dianthus</i> sp.	—	AB190573	AB190670
MAFF 311278	Shiga, Japan	<i>Dianthus</i> sp.	—	AB190574	AB190671
<i>Burkholderia cepacia</i>					
ATCC 25416 ^T	USA	<i>Allium cepa</i>	—	AB190575	AB190672
MAFF 302528	Toyama, Japan	Soil	—	AB190576	AB190673
<i>Burkholderia glumae</i>					
112 ^a	Iwate, Japan	<i>Oryza sativa</i>	G1	AB190577	AB190674
MAFF 311194 ^a	Toyama, Japan	<i>Oryza sativa</i>	G2	AB190617	AB190714
Pg-10 ^a	Toyama, Japan	<i>Oryza sativa</i>	G3	AB190616	AB190713
MAFF 311200	Toyama, Japan	<i>Oryza sativa</i>	G4	AB190615	AB190712
MAFF 311199	Yamaguchi, Japan	<i>Oryza sativa</i>	G5	AB190614	AB190711
MAFF 311198	Shiga, Japan	<i>Oryza sativa</i>	G6	AB190613	AB190710
MAFF 311197	Kyoto, Japan	<i>Oryza sativa</i>	G7	AB190612	AB190709
MAFF 311196	Nagano, Japan	<i>Oryza sativa</i>	G8	AB190611	AB190708
MAFF 311192	Toyama, Japan	<i>Oryza sativa</i>	G9	AB190610	AB190707
MAFF 302931	Miyagi, Japan	<i>Oryza sativa</i>	G10	AB190609	AB190706
MAFF 302928	Iwate, Japan	<i>Oryza sativa</i>	G11	AB190608	AB190705
MAFF 302880	Aomori, Japan	<i>Oryza sativa</i>	G12	AB190607	AB190704
MAFF 302878	Miyagi, Japan	<i>Oryza sativa</i>	G13	AB190606	AB190703
MAFF 302876	Toyama, Japan	<i>Oryza sativa</i>	G14	AB190605	AB190702
MAFF 302874	Ibaraki, Japan	<i>Oryza sativa</i>	G15	AB190604	AB190701
MAFF 302829	Shimane, Japan	<i>Oryza sativa</i>	G16	AB190603	AB190700
MAFF 302828	Kumamoto, Japan	<i>Oryza sativa</i>	G17	AB190602	AB190699
MAFF 302826	Ehime, Japan	<i>Oryza sativa</i>	G18	AB190601	AB190698
MAFF 302825	Nagasaki, Japan	<i>Oryza sativa</i>	G19	AB190600	AB190697
MAFF 302746	Ibaraki, Japan	<i>Oryza sativa</i>	G20	AB190599	AB190696
MAFF 302744	Fukuoka, Japan	<i>Oryza sativa</i>	G21	AB190598	AB190695
MAFF 302615	Indonesia	<i>Oryza sativa</i>	G22	AB190596	AB190693
MAFF 302616	Indonesia	<i>Oryza sativa</i>	G23	AB190597	AB190694
MAFF 302552	Kumamoto, Japan	<i>Oryza sativa</i>	G24	AB190595	AB190692
MAFF 302462	Tsukuba, Japan	<i>Vigna radiata</i>	G25	AB190594	AB190691
MAFF 302437	Ohita, Japan	<i>Oryza sativa</i>	G26	AB190593	AB190690
MAFF 302417	Tsukuba, Japan	<i>Vigna radiata</i>	G27	AB190592	AB190689
MAFF 302394	Miyagi, Japan	<i>Oryza sativa</i>	G28	AB190591	AB190688
MAFF 301682	Ibaraki, Japan	<i>Oryza sativa</i>	G29	AB190590	AB190687
MAFF 301441	Hiroshima, Japan	<i>Oryza sativa</i>	G30	AB190589	AB190686
MAFF 301389	Akita, Japan	<i>Oryza sativa</i>	G31	AB190588	AB190685
MAFF 301388	Yamagata, Japan	<i>Oryza sativa</i>	G32	AB190587	AB190684
MAFF 301387	Kumamoto, Japan	<i>Oryza sativa</i>	G33	AB190586	AB190683
MAFF 301386	Fukuoka, Japan	<i>Oryza sativa</i>	G34	AB190585	AB190682
MAFF 301172	Shizuoka, Japan	<i>Oryza sativa</i>	G35	AB190584	AB190681
MAFF 301171	Kagawa, Japan	<i>Oryza sativa</i>	G36	AB190583	AB190680
MAFF 301169 ^T	Ehime, Japan	<i>Oryza sativa</i>	G37	AB190582	AB190679
MAFF 301099	Fukushima, Japan	<i>Oryza sativa</i>	G38	AB190581	AB190678

Table 1. cont.

Strain	Geographical origin	Species name of host plant	Code no.	DDBJ accession no.	
				<i>gyrB</i>	<i>rpoD</i>
MAFF 301094	Okayama, Japan	<i>Oryza sativa</i>	G39	AB190580	AB190677
MAFF 301093	Nagano, Japan	<i>Oryza sativa</i>	G40	AB190579	AB190676
MAFF 106541	Kanagawa, Japan	<i>Oryza sativa</i>	G41	AB190578	AB190675
<i>Burkholderia gladioli</i>					
MAFF 301064	Chiba, Japan	<i>Freesia</i> sp.	L1	AB190618	AB190715
MAFF 301580	Shizuoka, Japan	<i>Dendrobium</i> sp.	L2	AB190619	AB190716
MAFF 301585	Tochigi, Japan	<i>Miltonia</i> sp.	L3	AB190620	AB190717
MAFF 301588	Tochigi, Japan	<i>Cymbidium</i> sp.	L4	AB190621	AB190718
MAFF 302385	Shizuoka, Japan	<i>Gladiolus</i> sp.	L5	AB190622	AB190719
MAFF 302386	Ibaraki, Japan	<i>Oryza sativa</i>	L6	AB190623	AB190720
MAFF 302409	Hokkaido, Japan	<i>Phaseolus angularis</i>	L7	AB190624	AB190721
MAFF 302418	Tsukuba, Japan	<i>Vigna radiata</i>	L8	AB190625	AB190722
MAFF 302424	Fukuoka, Japan	<i>Cymbidium</i> sp.	L9	AB190626	AB190723
MAFF 302515	Toyama, Japan	<i>Tulipa</i> sp.	L10	AB190627	AB190724
MAFF 302533	Toyama, Japan	Soil	L11	AB190628	AB190725
MAFF 302534	Toyama, Japan	Soil	L12	AB190629	AB190726
MAFF 302537	Tsukuba, Japan	Onion	L13	AB190630	AB190727
MAFF 302543	Ibaraki, Japan	<i>Oryza sativa</i>	L14	AB190631	AB190728
MAFF 302544	Chiba, Japan	<i>Oryza sativa</i>	L15	AB190632	AB190729
MAFF 302545	Chiba, Japan	<i>Oryza sativa</i>	L16	AB190633	AB190730
MAFF 302911	Yamagata, Japan	<i>Oryza sativa</i>	L17	AB190634	AB190731
MAFF 302914	Yamagata, Japan	<i>Oryza sativa</i>	L18	AB190635	AB190732
MAFF 302918	Yamagata, Japan	<i>Oryza sativa</i>	L19	AB190636	AB190733
MAFF 302919	Yamagata, Japan	<i>Oryza sativa</i>	L20	AB190637	AB190734
MAFF 311209	Okinawa, Japan	<i>Gladiolus</i> sp.	L21	AB190638	AB190735
ATCC 10248 ^T	USA	<i>Gladiolus</i> sp.	L22	AB190639	AB190736
T-1 ^b	Fukuoka, Japan	<i>Oryza sativa</i>	L23	AB190641	AB190738
H-1 ^b	Fukuoka, Japan	<i>Oryza sativa</i>	L24	AB190642	AB190739
E-14 ^b		<i>Oryza sativa</i>	L25	AB190643	AB190740
LMG 18157	USA	Cystic fibrosis patient	L26	AB220893	AB220904
R-2602 ^c	Canada	Cystic fibrosis patient	L27	AB239176	AB239182
R-11809 ^c	Italy	Cystic fibrosis patient	L28	AB239177	AB239183
R-8654 ^c	Switzerland	Cystic fibrosis patient	L29	AB239178	AB239184
R-15279 ^c	Germany	Cystic fibrosis patient	L30	AB239179	AB239185
R-20202 ^c	France	Cystic fibrosis patient	L31	AB239180	AB239186
R-20904 ^c	Belgium	Cystic fibrosis patient	L32	AB239181	AB239187
LMG 18920	Ghana	Mealybug	L33	AB220897	AB220908
LMG 11626	Java	Poisoned bongkreik	L34	AB220900	AB220911
GTC 1088		Corn flour	L35	AB220901	AB220912
<i>Burkholderia gladioli</i> pv. <i>agaricicola</i>					
GTC 1730	UK	White mushroom	L36	AB220902	B220913
<i>Burkholderia gladioli</i> pv. <i>alliicola</i>					
ATCC 19302 ^T	USA	<i>Allium cepa</i>	L37	AB190640	AB190737
<i>Burkholderia plantarii</i>					
MAFF 302381	Chiba, Japan	Soil used for cultivating rice seedlings	P1	AB190644	AB190741
MAFF 302387	Chiba, Japan	<i>Oryza sativa</i>	P2	AB190645	AB190742
MAFF 302388	Chiba, Japan	<i>Oryza sativa</i>	P3	AB190646	AB190743
MAFF 302391	Chiba, Japan	<i>Oryza sativa</i>	P4	AB190647	AB190744
MAFF 302412	Chiba, Japan	<i>Oryza sativa</i>	P5	AB190648	AB190745
MAFF 302413	Chiba, Japan	<i>Oryza sativa</i>	P6	AB190649	AB190746
MAFF 302466	Chiba, Japan	<i>Oryza sativa</i>	P7	AB190650	AB190747

Table 1. cont.

Strain	Geographical origin	Species name of host plant	Code no.	DDBJ accession no.	
				<i>gyrB</i>	<i>rpoD</i>
MAFF 302470	Chiba, Japan	<i>Oryza sativa</i>	P8	AB190651	AB190748
MAFF 302476	Chiba, Japan	<i>Oryza sativa</i>	P9	AB190652	AB190749
MAFF 302479	Chiba, Japan	Soil used for cultivating rice seedlings	P10	AB190653	AB190750
MAFF 302482	Chiba, Japan	Soil used for cultivating rice seedlings	P11	AB190654	AB190751
MAFF 302484	Chiba, Japan	<i>Oryza sativa</i>	P12	AB190655	AB190752
MAFF 302485	Miyagi, Japan	<i>Oryza sativa</i>	P13	AB190656	AB190753
MAFF 302907	Yamagata, Japan	<i>Oryza sativa</i>	P14	AB190657	AB190754
MAFF 302909	Yamagata, Japan	<i>Oryza sativa</i>	P15	AB190658	AB190755
MAFF 302916	Yamagata, Japan	<i>Oryza sativa</i>	P16	AB190659	AB190756
MAFF 302924	Iwate, Japan	<i>Oryza sativa</i>	P17	AB190660	AB190757
MAFF 302936	Miyagi, Japan	<i>Oryza sativa</i>	P18	AB190661	AB190758
MAFF 311029	Hokkaido, Japan	<i>Oryza sativa</i>	P19	AB190662	AB190759
MAFF 311030	Hokkaido, Japan	<i>Oryza sativa</i>	P20	AB190663	AB190760
MAFF 311031	Hokkaido, Japan	<i>Oryza sativa</i>	P21	AB190664	AB190761
MAFF 311032	Hokkaido, Japan	<i>Oryza sativa</i>	P22	AB190665	AB190762
MAFF 301723 ^T	Chiba, Japan	<i>Oryza sativa</i>	P23	AB190666	AB190763
LMG 16020 ^d	Japan	<i>Vanda</i> sp.	P24	AB220903	AB220914

*Source of strain: a, KUCS; b, KU; c, LMG; d, ATCC.

Phylogenetic relationships among *B. plantarii* strains

Twenty-three of 24 *B. plantarii* isolates from Japan were from rice seedlings and soil used for cultivating rice seedlings in Chiba, Miyagi, Iwate and Yamagata (located in the northern part of the main island of Japan) and in Hokkaido (Fig. 2). The remaining strain, LMG 16020, is the type strain of *B. vandii* that is now classified as a strain of *B. plantarii* (Coenye *et al.*, 1999). Whereas the *Vanda* strain LMG 16020

occupied a distinct position in the tree, the 23 rice strains were divided into three subgroups (Fig. 1). Nucleotide sequences of the *gyrB* PCR products from strains in subgroup I showed one restriction site for both *Hae*II and *Sac*I (data not shown). One restriction site for *Hae*II was located in the *gyrB* PCR products from strains in subgroup II. No *Hae*II and *Sac*I restriction sites were present in the *gyrB* PCR products from strains in subgroup III. *Sac*I and *Hae*II digestion of the *gyrB* PCR products allowed discrimination of strains among subgroups I, II and III (Fig. 2).

Table 2. PCR and sequencing primers used in this study

N, any; R, A or G; S, C or G; Y, C or T; M, A or C.

Primer	Target gene	Sequence (5'–3')	Length (nt)	Reference
UP-1E	<i>gyrB</i>	CAGGAAACAGCTATGACCAAYGSNGGNGNAARTTYRA	37	Yamamoto <i>et al.</i> (2000)
AprU		TGTAAAACGACGGCCAGTGCNNGRTCYTTYTCYTGRCA	38	Yamamoto <i>et al.</i> (2000)
70F2	<i>rpoD</i>	ACGACTGACCCGGTACGCATGTAYATGMNGARATGGG	38	This study
70R2		ATAGAAATAACCAGACGTAAGTTNGTRTAYTTYTTNGCDAT	41	This study
glu-FW	<i>gyrB</i> of <i>B. glumae</i>	GAAGTGTCGCCGATGGAG	18	This study
glu-RV		CCTTCACCGACAGCAGCAT	20	This study
pla-FW	<i>gyrB</i> of <i>B. plantarii</i>	TCGAGCTGGCTGCGCCTC	18	This study
pla-RV		GTCGTCGCCCCGAGGTCTCG	19	This study
gla-FW	<i>gyrB</i> of <i>B. gladioli</i>	CTGCGCCTGGTGGTGAAG	18	This study
gla-RV		CCGTCCCCTGCGGAATA	18	This study

Fig. 2. Ethidium bromide-stained gel of *Haell*- and *SacI*-digested partial fragments of *gyrB* amplified by PCR from *B. plantarii* strains using primers UP-1E and AprU. See Table 1 and Fig. 1, respectively, for strain numbers and subgroups.

Phylogenetic relationships among *B. glumae* strains

Analysis of combined nucleotide sequences of the *gyrB* and *rpoD* genes showed no diversity among 41 strains of *B. glumae*. The nucleotide sequences of *gyrB* in all 41 strains were identical and those of *rpoD* in six Japanese strains and two Indonesian strains differed by only one and two nucleotides, respectively, compared with those of the other 33 strains (data not shown). These results indicate that the diversity of nucleotide sequences of *gyrB* and *rpoD* among *B. glumae* strains is very restricted.

Phylogenetic relationships among *B. gladioli* strains

B. gladioli R-20202, which was obtained from a specimen from a cystic fibrosis patient in France, occupied a unique position among the 37 *B. gladioli* strains examined (Fig. 1). All other isolates formed a rather loose assemblage without subdivision supported by high bootstrap values. The combined nucleotide sequences of *gyrB* and *rpoD* of two rice strains, MAFF 302544 and MAFF 302914, were identical to those of the mung bean strain MAFF 302409 and the *Cymbidium* strain MAFF 302424. Furthermore, those of strain T-1 were identical to those of the Tulip strain MAFF 302515. Moreover, the combined translated amino acid sequences of *gyrB* and *rpoD* of the rice strains MAFF 302543, E-14 and H-1 were identical not only to those of the corn

Fig. 1. Phylogenetic tree of *Burkholderia* species based on the combined nucleotide sequences of *gyrB* and *rpoD* genes constructed with CLUSTAL W (DNA Database of Japan; <http://www.ddbj.nig.ac.jp/search/clustalw-j.html>) using the neighbour-joining method (Saitou & Nei, 1987). Bar, 0.1 substitutions per position. Numbers at nodes represent percentage bootstrap values of 1000 resamplings. Sequences from *E. coli* K-12 were used as the outgroup.

flour strain GTC 1088, *B. gladioli* pv. *agaricola* GTC 1730, the gladiolus strain ATCC 10248^T and soil strains MAFF 302533 and MAFF 302534, but also to the cystic fibrosis patient strains LMG 18157 (USA) and R-15279 (Germany). These results indicate that the pathovar, host plant and geographical origin of the strains correlate poorly with the phylogenetic diversity among the *B. gladioli* strains.

Detection of *B. gladioli*, *B. glumae* and *B. plantarii* in infected rice seeds by multiplex PCR

A multiplex PCR-based detection method for *B. gladioli*, *B. glumae* and *B. plantarii* was developed using the *gyrB* nucleotide sequences (Table 2). A 479 bp fragment specific for *B. gladioli* was amplified from the genomic DNA of all *B. gladioli* strains used in this study (Fig. 3). From the genomic DNA of all *B. glumae* strains tested, a 529 bp DNA fragment was specifically amplified in the multiplex PCR. A 597 bp DNA fragment was specifically amplified from the genomic DNA of all *B. plantarii* strains tested, including LMG 16020 from *Vanda* sp. No DNA fragments were amplified from the genomic DNA of *B. cepacia* ATCC 25416^T. Sequencing of the PCR products confirmed the specificity of the multiplex PCR (data not shown).

To assess the usefulness of multiplex PCR in discriminatory detection of rice seeds infected with *B. gladioli*, *B. glumae* and *B. plantarii*, DNA isolated from pathogen-infected rice seeds was used as a template. A 479 bp fragment specific for *B. gladioli* was only amplified from DNA of rice seeds from plants inoculated with *B. gladioli* MAFF 302386, MAFF 302543, MAFF 302918, MAFF 302919 and T-1 (Fig. 4a). One fragment of 529 bp in length was amplified from DNA of rice seeds from plants inoculated with *B. glumae* MAFF 301169^T. A 597 bp DNA fragment was amplified from DNA of rice seeds from plants inoculated with *B. plantarii* MAFF 302391, MAFF 302907, MAFF 302924, MAFF 311030 and MAFF 301723^T. Furthermore, two fragments of 529 and 597 bp in length were amplified from DNA of rice seeds naturally infected with *B. glumae* and *B. plantarii*, respectively (Fig. 4b). Sequencing of the PCR products

Fig. 3. Sensitive detection of *B. plantarii*, *B. glumae* and *B. gladioli* by multiplex PCR. The expected sizes of partial fragments of *gyrB* amplified by PCR from genomic DNA of *B. plantarii*, *B. glumae* and *B. gladioli* were 597, 529 and 479 bp, respectively. See Table 1 for strain numbers. BC, *B. cepacia* ATCC 25416^T.

Fig. 4. Detection of *B. plantarii*, *B. glumae* and *B. gladioli* in rice seeds by multiplex PCR. The expected sizes of partial fragments of *gyrB* amplified by PCR from DNA isolated from rice seeds infected with *B. plantarii*, *B. glumae* and *B. gladioli* were 597, 529 and 479 bp, respectively. (a) PCR products from DNA of rice seeds produced from rice plants inoculated with *B. plantarii*, *B. glumae* or *B. gladioli*. Lanes: 1–5, *B. gladioli* MAFF 302386, MAFF 302543, MAFF 302918, MAFF 302919 and T-1, respectively; 6, *B. glumae* MAFF 301169^T; 7–11, *B. plantarii* MAFF 302391, MAFF 302907, MAFF 302924, MAFF 311030 and MAFF 301723^T, respectively. (b) Lanes: 1 and 2, PCR products from DNA of rice seeds naturally infected with *B. glumae* and *B. plantarii*, respectively; 3 and 4, PCR products from genomic DNA of *B. glumae* MAFF 301169^T and *B. plantarii* MAFF 301723^T, respectively; 5, PCR products from DNA of non-infected rice seeds.

confirmed the specificity obtained by PCR (data not shown). No products were obtained from DNA from non-infected rice seeds (Fig. 4b). These results show that the multiplex-PCR protocol facilitates specific detection and discrimination of rice seeds infected with *B. plantarii*, *B. glumae* and *B. gladioli*.

Many ecological studies on *B. glumae* and *B. plantarii* have been reported, leading to the development of systems to control the diseases caused by these two *Burkholderia* species. However, the ecology and pathogenicity mechanism of *B. gladioli* on rice plants remain unclear. Moreover, the antagonistic activity of *B. gladioli* against *B. glumae* and *B. plantarii* in rice plants (Miyagawa, 2000) shows that an understanding of the ecology of *B. gladioli* on rice plants would facilitate not only the development of control systems for *B. gladioli*-induced disease, but also an understanding of the ecology of *B. glumae* and *B. plantarii* on rice plants. In the present study, we have designed a multiplex PCR that can be used for the simultaneous detection of *B. plantarii*, *B. glumae* and *B. gladioli* in rice seeds infected with these *Burkholderia* species. Therefore, this assay might facilitate an understanding of the ecological significance of *Burkholderia* species, especially the interactions that control their survival fitness on rice plants, thus leading to the development of relevant control systems.

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