

# A diagnostic multiplex PCR scheme for identification of plant-associated bacteria of the genus *Pantoea*

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## Abstract

**Background:** The genus *Pantoea* forms a complex of more than 25 species, among which several cause diseases of several crop plants, including rice. Notably, strains of *Pantoea ananatis* and *Pantoea stewartii* have been found to cause bacterial leaf blight of rice in Togo and Benin, while other authors have observed that *Pantoea agglomerans* can also cause bacterial leaf blight of rice. The contribution of these and perhaps other species of *Pantoea* to plant diseases and yield losses of crop plants is currently not well documented, partly due to the lack of efficient diagnostic tools.

**Result:** Using 34 whole genome sequences of the three-major plant-pathogenic *Pantoea* species, a set of PCR primers that specifically detect each of the three species, *P. agglomerans*, *P. ananatis*, and *P. stewartii*, was designed. A multiplex PCR protocol which can distinguish these three species and also detects members of other *Pantoea* species was

26 further developed. Upon validation on a set of reference strains, 609 suspected *Pantoea*  
27 strains that were isolated from rice leaves or seeds originating from 11 African countries were  
28 screened. In total, 41 *P. agglomerans* strains from eight countries, 79 *P. ananatis* strains from  
29 nine countries, 269 *P. stewartii* strains from nine countries and 220 unsolved *Pantoea* strains  
30 from ten countries were identified. The PCR protocol allowed detecting *Pantoea* bacteria  
31 grown in vitro, in planta and in rice seeds. The detection threshold was estimated at 5 ng/mL  
32 of total genomic DNA and  $1 \times 10^5$  CFU/mL of heated cells.

33

34 **Conclusion:** This new molecular diagnostic tool will help accurately diagnose major plant-  
35 pathogenic species of *Pantoea*. Due to its robustness, specificity, sensitivity, and cost  
36 efficiency it will be very useful for plant protection services and for the epidemiological  
37 surveillance of these important crop-threatening bacteria.

38

39

40 **Keywords:** Plant pathogen, *Pantoea*, rice, *Oryza sativa*, multiplex PCR, diagnostic tool

41

## 42 **Background**

43

44 The genus *Pantoea* was first described in 1989 and was recently taxonomically classified as a  
45 member of the *Erwiniaceae* family [1]. More than 25 species of this genus have been  
46 described and reported worldwide [2,3]. Etymologically, the genus name *Pantoea* is derived  
47 from the Greek word ‘Pantiois’, which means “of all sorts or sources” and reflects the diverse  
48 geographical and ecological sources from which the bacteria have been isolated. Several  
49 species of the genus are qualified as versatile and ubiquitous bacteria because they have been  
50 isolated from many different ecological niches and hosts [2,4]. Remarkably, some species  
51 have the ability to colonize and interact with members of both the plant and the animal  
52 Kingdom [5]. Among the plant-interacting species, *Pantoea ananatis*, *Pantoea agglomerans*  
53 and *Pantoea stewartii* are well known for their phytopathogenic characteristics. They are  
54 recognized as the causal agent of several diseases, such as leaf blight, spot disease, dieback,  
55 grain discoloration, seed stalk rot, center rot, stem necrosis, palea browning, bulb decay etc.  
56 and affect several economically important crops, including cereals, fruits and vegetables  
57 [2,6,7].

58

59 Bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* is an important disease of rice  
60 and affects rice cultivation in most regions of the world where rice is grown. The bacterium has  
61 been associated with this disease since a very long time [8]. Surveys were conducted from  
62 2010 to 2016 to estimate the extent and importance of the disease and the phytosanitary status  
63 of rice fields in West Africa. While leaves showing bacterial blight (BB)-like symptoms were  
64 frequent, isolation or molecular detection of xanthomonads using the Lang et al diagnostic  
65 tool [9] often failed. Instead, other bacteria forming yellow colonies were observed and turned  
66 out to belong to the species *P. ananatis* or *P. stewartii*, as documented for samples from Togo

67 and Benin [10,11]. Additionally, other cases of BB and grain discoloration caused by  
68 *Sphingomonas* sp. and other undescribed species have been detected in several sub-Saharan  
69 Africa countries [12]. This situation represents an “emerging” bacterial species complex that  
70 may constitute a threat to rice production in Africa. Therefore, a robust, specific, sensitive,  
71 and cost efficient diagnostic tool is of primary importance for accurate pathogen detection.  
72 However, none of the several simplex and multiplex PCR tools [13–18] and other molecular  
73 [19–24], physiological, biochemical [24–28] diagnostic tools available for *Pantoea* allows  
74 accurate simultaneous detection of the three major plant-pathogenic *Pantoea* species. Some of  
75 these methods are poorly reproducible and often limited to a single species while others are  
76 reproducible but again limited to one species or are not suited to doubtlessly detect African  
77 strains.

78  
79 To overcome this unsatisfying situation, a molecular method was set up for detecting in a  
80 single reaction the three major plant-pathogenic *Pantoea* species (*P. ananatis*, *P. stewartii*  
81 and *P. agglomerans*), as well as other members of the genus. A universal multiplex PCR tool  
82 was therefore developed and first tested in silico on available genome sequences and on a set  
83 of reference strains from USA, Brazil, Spain and Japan. Afterwards, 609 suspected *Pantoea*  
84 strains from eleven African countries were evaluated with the newly described diagnostic  
85 tool. *P. agglomerans* was detected in rice leaves from several African countries for the first  
86 time. Finally, the specificity and sensitivity of the multiplex PCR was monitored by analyzing  
87 serial dilutions of genomic DNA, serial dilutions of bacterial cell suspensions and solutions of  
88 ground leaves and seeds that had been artificially or naturally infected. This new diagnostic  
89 tool will prove useful for phytosanitary services in routine diagnostics of *Pantoea* spp in any  
90 type of sample (e.g. leaves, seeds, soil, water).

91

92

## 93 **Materials and Methods**

94

### 95 **Bioinformatics prediction of specific PCR primers**

96 *Pantoea* genome sequences were retrieved from NCBI GenBank (Table 1). Sequences for  
97 housekeeping genes were identified by TBLASTN [29]. Sequences were then aligned with  
98 MUSCLE [30] at EMBL-EBI [31]. Diagnostic primers that can differentiate the three species,  
99 *P. agglomerans*, *P. ananatis* and *P. stewartii*, and one primer pair that would amplify DNA  
100 from the whole *Pantoea* genus were designed manually. The  $T_m$  for PCR primers were  
101 automatically predicted by  $T_m$  calculator tool at  
102 <http://www.thermoscientificbio.com/webtools/multipleprimer/> which was developed based on  
103 the modified nearest-neighbor interaction method [32].

104

### 105 **Optimization of the multiplex PCR**

106 Different types of samples including total genomic DNA, bacterial cells, symptomatic rice  
107 leaves, as well as discolored and apparently healthy rice seeds were analyzed. Plant material  
108 was ground and macerated before use. To develop a multiplex PCR scheme, individual primer  
109 pairs were first tested against the different samples mentioned above, using annealing  
110 temperatures close to the predicted  $T_m$  ( $T_m \pm 5^\circ\text{C}$ ) and with progressive number of PCR  
111 cycles (25 to 35). Primer pairs were then mixed from duplex to quintuplex and PCR  
112 conditions were evaluated, testing annealing temperatures close to the optimal  $T_m$  of the  
113 individual primer pairs ( $T_m \pm 3^\circ\text{C}$ ) and various numbers of PCR cycles. At the end, three  
114 promising combinations of annealing temperatures and numbers of PCR cycles were re-  
115 evaluated in simplex PCR with the samples mentioned above. The best combination with high

116 specificity and without background amplification was selected as the new diagnostic tool  
117 (Tables 2 to 4).

118

119 **Evaluation of the sensitivity of the multiplex PCR scheme using genomics DNA and heat**  
120 **cells**

121 Simplex and multiplex PCR were then used to evaluate the sensitivity of all the species-  
122 specific primer pairs individually or in combination with the genus-specific and the 16 sRNA  
123 primer pairs. Serial dilutions of total genomic DNA and heated bacterial cells were used for  
124 this evaluation. Three *Pantoea* strains, *P. ananatis* strain ARC60, *P. stewartii* strain ARC222,  
125 and *P. agglomerans* strain CFBP 3615, were used and distilled sterilized served as a negative  
126 control.

127

128 To evaluate the PCR scheme on live plant material, leaves and seeds were artificially infected  
129 with strains of the three *Pantoea* species. Rice leaves of the cultivar Azucena were inoculated  
130 as described previously [10,11]. To produce contaminated seeds, early maturity panicles of  
131 the Azucena rice cultivar were spray-inoculated with a 5%-gelatinized bacterial solution ( $10^6$   
132 CFU/mL). Distillated and gelatinized (5%) sterile water served as a negative control. Three  
133 weeks post inoculation, approximately 40% of the grains in the panicles exhibited  
134 discolorations. Panicles inoculated with sterile distilled water showed no symptoms. A total of  
135 five grains whose surface was first treated with a solution of hypochlorite (10%) and ethanol  
136 (70%) and then rinsed with sterile distilled water were ground in 100 mL of sterile distilled  
137 water. After centrifugation, the supernatant was used for PCR.

138

139 **Evaluation of the multiplex PCR scheme on a large collection of African *Pantoea* strains**

140 Bacterial strains used in this study are listed in Additional file 1. In total, 615 *Pantoea* strains

141 from eleven African countries (Benin, Burkina Faso, Burundi, Ghana, Ivory Coast, Mali,  
142 Niger, Nigeria, Senegal, Tanzania, Togo) and seven reference strains from USA, Brazil, Spain  
143 and Japan were analysed by the new diagnostic tool. The African strains were isolated from  
144 rice leaves with BB symptoms, and from discolored and apparently healthy rice seeds. The  
145 samples had been collected from 2008 to 2016 in the main rice-growing areas of the  
146 countries. Other bacteria, including *Xanthomonas* spp, *Sphingomonas* spp, *Escherichia coli*,  
147 *Erwinia* spp, *Burkholderia* spp, and *Pseudomonas* spp, were used as controls. The strains  
148 were purified as single colonies, individually grown and preserved as pure cultures following  
149 routine methods [33]. Bacterial colonies were grown for 24 to 48 h on PSA plates containing  
150 10 g peptone, 10 g sucrose, 16 g agar and 1 g glutamic acid per liter. Total genomic DNA was  
151 extracted using the Wizard genomic DNA purification kit (Promega) according to the  
152 manufacturer's instructions. DNA quality and quantity were evaluated by agarose gel  
153 electrophoresis and spectrophotometry (Nanodrop Technologies, Wilmington, DE).

154

155

## 156 **Results**

157

### 158 **Development of a diagnostic PCR scheme for plant-associated *Pantoea***

159 We aimed at designing diagnostic PCR primers that would target conserved housekeeping  
160 genes. The rationale behind was that these genes should be present in all strains, including  
161 genetic lineages that have not yet been discovered and would not be present in any strain  
162 collection. At the same time, we knew from previous work that sequences of housekeeping  
163 genes are divergent enough to doubtlessly distinguish and identify *Pantoea* strains at the  
164 species level.

165

166 A diagnostic *Pantoea* multiplex PCR method was developed in two steps. First, a complete  
167 inventory of publicly available *Pantoea* genome sequences was compiled, consisting of nine  
168 *P. agglomerans*, 14 *P. ananatis*, and three *P. stewartii* sequences, totaling to 26 whole  
169 genome sequences (Table 1). Complete coding sequences of four housekeeping genes that  
170 have previously been used for multilocus sequence analyses (MLSA) of *Pantoea* species [2],  
171 *atpD*, *gyrB*, *infB*, and *rpoB*, were then extracted and aligned. Sequence regions that were  
172 conserved in all strains of one species but were significantly different in the other two species  
173 were identified manually and chosen to design PCR primers (Table 2). To allow multiplexing,  
174 we made sure that the amplicon sizes would be between 400 and 750 bp and different enough  
175 to be easily distinguishable from each other upon gel electrophoresis (Fig. 1). As a positive  
176 control for the PCR reaction, one primer pair was included that would amplify DNA from all  
177 bacteria belonging to the *Pantoea* genus, resulting in a smaller amplicon of less than 400 bp.  
178 Finally, as a second control, a primer pair was included that targets the ribosomal 16S rRNA  
179 gene and leads to an amplicon that is larger than the four *Pantoea*-specific amplicons.

180

181 In the second step, all primer pairs (Table 2) were evaluated, first by simplex PCR and then  
182 by multiplex PCR, with increasing number of primer pairs, as explained in Material and  
183 Methods. Three *Pantoea* reference strains were used to develop the PCR scheme using  
184 genomic DNA and heat-inactivated bacteria: *P. agglomerans* strain CFBP 3615, *P. ananatis*  
185 strain ARC60 and *P. stewartii* strain ARC222 (Fig. 2). Agarose gel electrophoresis  
186 demonstrated that the multiplex PCR was able to detect and distinguish all three *Pantoea*  
187 species. Notably, the multiplex PCR scheme was also able to detect two or three *Pantoea*  
188 species when the corresponding species were present in the same template DNA, as  
189 demonstrated by PCR reactions containing equal amounts of DNA of the different species  
190 (Fig. 2).



191

192 To simplify the analyses and to avoid isolation of bacteria from plant samples, thus reducing  
193 the costs per sample, the PCR scheme was also evaluated on infected leaf material and  
194 contaminated seeds. As shown in Fig. 3, the multiplex PCR was able to doubtlessly detect all  
195 three *Pantoea* species in both types of plant samples, as demonstrated for the strains CFBP  
196 3615 (*P. agglomerans*), ARC60 (*P. ananatis*), and ARC222 (*P. stewartii*). At the end, a  
197 robust PCR protocol was available that was able to amplify DNA from total genomic DNA,  
198 bacterial cells, symptomatic rice leaves and from infected rice seeds.

199

#### 200 **Evaluation of the sensitivity of the multiplex PCR scheme using genomic DNA and** 201 **heated cell suspensions**

202 The evaluation by simplex and multiplex PCR showed that all the species-specific primers  
203 were very sensitive individually or in combination with the genus-specific and the 16 sRNA  
204 universal primers (Fig. 4). The most sensitive primer pair in simplex PCR was the one  
205 targeting *P. stewartii* with a detection limit of 5 pg under our experimental conditions,  
206 followed by the *P. agglomerans*-specific primer pair (detection limit of 50 pg) and the  
207 *P. ananatis*-specific primer pair (detection limit of 0.5 ng). A similar trend was observed in  
208 the multiplex PCR on genomic DNA, with the same detection limit as in simplex PCR for  
209 *P. stewartii* and *P. ananatis* and a tenfold less sensitivity for *P. agglomerans*.

210

211 When heated bacterial cell suspensions were used as template, the *P. ananatis*-specific primer  
212 pair was the most sensitive allowing detection of  $10^3$  CFU/mL, while the other two primer  
213 pairs were able to detect  $10^4$  CFU/mL. However, when all five primers pairs were used in  
214 multiplex, the sensitivity was very similar for all three species with a detection limit of  
215 approximately  $10^4$  CFU/mL.

216

## 217 **Evaluation of the multiplex PCR scheme on a large collection of African *Pantoea* strains**

218 Because recent surveys had indicated that *Pantoea* species could be responsible for many  
219 unsolved infections of rice fields in sub-Saharan Africa [10,11], we screened a large  
220 collection of isolates. We first re-evaluated a few African strains that had been identified as  
221 *P. ananatis* (ARC22, ARC60, ARC651) and *P. stewartii* (ARC229, ARC570, ARC646),  
222 using species-specific and the genus-specific PCR primers [10,11]. The multiplex PCR  
223 scheme confirmed their previous taxonomic classification. Next, we screened a large  
224 collection of African bacterial isolates from rice samples (>1000 strains) among which 609  
225 strains were found to belong to the genus *Pantoea* (Additional file 1). Specifically, this work  
226 diagnosed 41 *P. agglomerans* strains from eight countries (Benin, Ghana, Mali, Niger,  
227 Nigeria, Senegal, Tanzania, Togo), 79 *P. ananatis* strains from nine countries (Benin, Burkina  
228 Faso, Burundi, Mali, Niger, Nigeria, Senegal, Tanzania, Togo), 269 *P. stewartii* strains from  
229 nine countries (Benin, Burkina Faso, Ivory Coast, Mali, Niger, Nigeria, Senegal, Tanzania,  
230 Togo) and 220 *Pantoea* sp. strains from ten countries (Benin, Burundi, Ghana, Ivory Coast,  
231 Mali, Niger, Nigeria, Senegal, Tanzania, Togo) (Additional file 1). This result provided first  
232 insights on the presence and prevalence of three important *Pantoea* species in these eleven  
233 African countries.

234

235

## 236 **Discussion**

237

238 Bacterial infections by *Pantoea* spp. are recognized as being responsible for several diseases  
239 of plants, including important crop plants such as rice, maize, sorghum, onion and melon [34–

240 43]. BB of rice caused by species of *Pantoea* were reported in several countries and include  
241 Benin, Togo, Korea, India, Australia, China, Italy, Venezuela, and Russia [10,11,40,44–49].

242

243 Given the fact that more than 25 species of *Pantoea* are currently known and among them  
244 several species can infect plants, efficient diagnostic tools are highly demanded by plant  
245 pathologists and extension workers. Some plant diseases were attributed to only three species  
246 of *Pantoea*, namely *P. agglomerans*, *P. ananatis* and *P. stewartii*, which can therefore be  
247 considered as the major *Pantoea* species infecting plants. For their diagnosis, several PCR  
248 methods are available and have been used but some of them produced amplicons with others  
249 species as well [14,50,51], while others are not well reproducible or are inaccessible in typical  
250 sub-Saharan laboratory due to specific equipment requirements and/or high costs of some  
251 reagents [14,17,18]. Notably, most assays target only one *Pantoea* species or subspecies. For  
252 instance, being of major concern, *P. stewartii* subsp. *stewartii* causing Stewart's bacterial wilt  
253 can be detected by several methods but none of them can at the same time identify other  
254 bacteria of the genus *Pantoea* [14,16,18,52,53]. To the best of our knowledge, no robust  
255 diagnostic scheme exists that can specifically detect all three major *Pantoea* species that  
256 infect plants.

257

258 Based on whole genome sequences, we developed a new multiplex PCR scheme that can  
259 specifically detect the three major species of plant-pathogenic *Pantoea*, *P. agglomerans*,  
260 *P. ananatis* and *P. stewartii*. Different strategies can be followed when developing such a  
261 multiplex scheme using available whole-genome sequences. One possibility is to automatize  
262 the procedure by identifying genomic regions that are shared among a set of strains (e.g. the  
263 target species) and which are absent in another set of strains (non-target species). For  
264 instance, such an approach was used for the development of a *Xanthomonas oryzae*-specific

265 multiplex PCR scheme that can differentiate the two pathovars *oryzae* and *oryzicola* [9]. The  
266 problem with this approach is that it might identify non-essential, often hypothetical genes as  
267 targets for the primer design. While present in the training set, it is hard to predict if these  
268 non-essential genes will be present and conserved in other, hitherto uncharacterized strains,  
269 especially when they originate from other geographical zones and/or belong to more distant  
270 genetic lineages.

271  
272 Here, we targeted housekeeping genes, which are conserved throughout the genus, and relied  
273 on lineage (species)-specific sequence polymorphisms. This approach is considered as very  
274 robust but it cannot be ruled out that recombination events among strains from different  
275 species could undermine the universality of these primer pairs. Yet, we did not find any  
276 evidence for such events in any of the sequenced *Pantoea* strains that were analysed,  
277 including environmental isolates and strains isolated from human and plant samples.  
278 Nevertheless, because this study was focused on isolates from African rice leaves and seeds  
279 and only included a few reference strains from other continents (Additional file 1), it might be  
280 of interest to evaluate the new multiplex PCR tool on *Pantoea* strains isolated from other  
281 organisms (others plants, insects, other animals, humans) and from environmental samples.

282  
283 To reduce the costs and handling time, we generated a multiplex PCR scheme that can work  
284 with both purified genomic DNA or with bacterial lysates. In both cases, sufficient specificity  
285 and sensitivity were obtained allowing detection of as low as 0.5 ng of DNA or  $10^4$  CFU/mL  
286 for all three *Pantoea* species. Such a simple scheme will be of specific interest to  
287 phytopathologists, especially in Africa and other less-developed regions. Indeed, diseases due  
288 to infections by *Pantoea* appear to emerge in Africa as recently documented for Benin and  
289 Togo [10,11]. In this study, the presence of the three major plant-pathogenic *Pantoea* species

290 has been demonstrated for eleven African countries. The fact that most of the BB-like  
291 symptomatic rice samples proved to contain a high number of *Pantoea* bacteria suggests that  
292 infection by *Pantoea* is an underestimated source for BB symptoms and might be widespread  
293 in Africa. However, more rigorous sampling schemes are required to determine the incidence  
294 and prevalence of *Pantoea* in various rice-growing areas in Africa.

295

296 Among the 609 *Pantoea* isolates, we detected 220 strains (36.1%; additional file 1) of  
297 *Pantoea* sp. that could not be assigned to any of the three species that are specifically targeted  
298 by the multiplex PCR scheme. This is an interesting observation that shows that the genus-  
299 specific primer pair does not only serve as an internal positive control of the multiplex  
300 scheme but that it has its own diagnostic value. Obviously, other species of *Pantoea* are  
301 present in Africa and are likely to cause disease of rice plants as well. Yet, it is still unknown  
302 whether or not this group of isolates contains other rice pathogenic species. Pathogenicity  
303 assays need to confirm or disprove their status as novel pathogens. Future work will address  
304 these isolates, using MLSA and whole genome sequencing.

305

306 While screening a large collection of bacterial isolates from rice samples, we also found  
307 strains that neither belonged to *Pantoea* nor to *Xanthomonas* (data not shown). Some of them  
308 were *Sphingomonas* strains [12], while others may represent new species and genera, which  
309 have so far not been connected to rice diseases. These isolates will be further studied by 16S  
310 rRNA analysis. From this study, it was concluded that the number of bacterial species that  
311 affect rice plants in Africa is certainly larger than previously thought.

312

313

314 **Conclusion**

315

316 A new multiplex PCR scheme was developed to diagnose plant-pathogenic *Pantoea* spp. This  
317 tool enabled the efficient confirmation of the presence of *Pantoea* species (*P. ananatis* and  
318 *P. stewartii*) in Benin and Togo, as reported previously, and in several other African countries  
319 (Burkina Faso, Burundi, Ghana, Ivory Coast, Mali, Niger, Nigeria, Senegal, Tanzania).  
320 Moreover, we found evidence for the presence of *P. agglomerans* and other species of  
321 *Pantoea* on rice samples from several African countries. This new diagnostic tool will be very  
322 useful for crop protection services.

323

324

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- 556

557

558 **Declarations**

559

560 **Ethics approval and consent to participate**

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562

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565

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570 The authors declare that they have no competing interests.

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580 **Authors' contributions**



581 KK and RK conceived and designed the experiments. KK, SD, RA, RD evaluated the primers  
582 and multiplex PCR scheme by screening African strains. KK, RK and DS wrote the  
583 manuscript. All authors read and approved the final manuscript.

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600

601

602 **Table 1:** List of *Pantoea* genome sequences used for primers design.

603

Species	Strain	Origin	Country	Year	Accession number	Reference
<i>P. agglomerans</i>	4	Wheat seed	Canada	2012	JPOT01000005	[54]
<i>P. agglomerans</i>	190	Soil	South Korea	2005	JNGC01000002	[55]
<i>P. agglomerans</i>	DAPP- PG734	Olive knot	Italy	2008	JNVA01000008	[56]
<i>P. agglomerans</i>	Eh318	Stem of apple	USA		AXOF01000028	[57]
<i>P. agglomerans</i>	IG1				BAEF01000016	[58]
<i>P. agglomerans</i>	LMAE-2	Sediment	Chile	2010	JWLQ01000032	[59]
<i>P. agglomerans</i>	MP2	Termites	South Africa	2009	JPKQ01000009	[60]
<i>P. agglomerans</i>	RIT273	Willow ( <i>Salix</i> sp.)	USA	2013	ASJI01000010	[61]
<i>P. agglomerans</i>	Tx10	Sputum of cystic fibrosis	USA	2011	ASJI01000010	[62]
<i>P. ananatis</i>	AJ13355		Japan		AP012032	[63]
<i>P. ananatis</i>	B1-9				CAEJ01000016	[64]
<i>P. ananatis</i>	BD442	Maize stalk rot	South Africa	2004	JMJL01000008	[65]
<i>P. ananatis</i>	BRT175	Strawberry epiphye			ASJH01000041	[62]
<i>P. ananatis</i>	CFH 7-1	Cotton	USA	2011	LFLX01000002	[66]

			boll				
			disease				
<i>P. ananatis</i>	LMG	Blight and	South		CP001875	[67]	
	20103	dieback of	Africa				
		Eucalyptus					
<i>P. ananatis</i>	LMG	Pineapple	Philippines	1928	JMJJ01000009	[68]	
	2665	soft rot					
<i>P. ananatis</i>	LMG	Human	Phillipines	1928	HE617160	[69]	
	5342	wound					
<i>P. ananatis</i>	PA13	Rice grain	Korea		CP003085	[70]	
<i>P. ananatis</i>	PA4	Onion	South	2004	JMJK01000009	[65]	
		seed	Africa				
<i>P. ananatis</i>	S6	Maize			CVNF01000001	[71]	
		seed					
<i>P. ananatis</i>	S7	Maize			CVNG01000001	[71]	
		seed					
<i>P. ananatis</i>	S8	Maize			CVNH01000001	[71]	
		seed					
<i>P. ananatis</i>	Sd-1	Rice seed	China		AZTE01000008	[72]	
<i>P. stewartii</i>	DC283	Maize	USA	1967	AHIE01000032	[73]	
<i>P. stewartii</i>	M009	Waterfall	Malaysia	2013	JRWI01000004	[74]	
<i>P. stewartii</i>	M073a	Waterfall	Malaysia	2013	JSXF01000010	[75]	

604

605

606 **Table 2:** List of PCR primers developed for the *Pantoea* mPCR along with the sequences of  
 607 the GenBank accessions and the corresponding strains.

608

Primer name	Target species	Sequence	Size (bp)	Strain
PANAG_infB_fwd	<i>P. agglomerans</i>	5'-GATGACGARGCCATGCTGC	730	<i>P. agglomerans</i>
PANAG_infB_rev		5'-TGTCCGGCGTGCCGGCTG		(CFBP 3615)
PANAN_gyrB_fwd	<i>P. ananatis</i>	5'-GATGACGARGCCATGCTGC	423	<i>P. ananatis</i>
PANAN_gyrB_rev		5'-GATCTTGCGGTATTCGCCAC		(ARC195)
PANST_rpoB_fwd	<i>P. stewartii</i>	5'-CACCGGTGAACTGATTATCG	539	<i>P. stewartii</i>
PANST_rpoB_rev		5'-GTCCTGAGGCATCAATGTGT		(ARC204)
PANsp_atpD_fwd	<i>Pantoea</i> sp.	5'-GAGGGTAACGACTTCTACCAC	330	<i>P. stewartii</i>
PANsp_atpD_rev		5'-CTGTACGGAGGTGATTGAAC		(ARC222)
				<i>P. agglomerans</i>
				(CFBP 3615)
				<i>P. ananatis</i>
				(ARC235)
16S_27F	Eubacteria	5'-AGAGTTTGATCMTGGCTCAG	920	Eubacteria
16S_907R		5'-CCGTCAATTCMTTTRAGTTT		

609

610

611 **Table 3:** Composition of the multiplex polymerase chain reaction.

612

<b>PCR component</b>	<b>Volume per reaction (<math>\mu\text{L}</math>)</b>		<b>Final concentration</b>
Type of template	Purified DNA	Bacterial cells	
Buffer (5x)	5.0	5.0	1x
dNTPs (2.5 mM each)	0.5	0.5	50 $\mu\text{M}$ each
Oligonucleotides (10 $\mu\text{M}$ )	0.4	0.4	0.16 $\mu\text{M}$ each
Takara ExTaq <sup>TM</sup> (5 units/ $\mu\text{L}$ )	0.1	0.1	0.5 U
Template	2.0	5.0	
Sterile nanopure water (Promega)	13.4	10.4	
Total	25.0	25.0	

613

614

615 **Table 4:** Reaction parameters of the multiplex PCR thermocycler program.

616

<b>Step</b>	<b>Phase</b>	<b>Time</b>	<b>Temperature (°C)</b>
1	Initial denaturation	3 min	94
2	Denaturation	30 sec	94
3	Annealing	30 sec	58
4	Extension	2 min	72
5	Cycling (steps 2-4)	30 cycles	
6	Final extension	10 min	72
7	Soak/hold	$\infty$	4-10
8	End		

617

618

619 **Legends to figures**

620

621 **Figure 1:** Schematic representation of the multiplex PCR scheme. Sizes of the five expected  
622 PCR amplicons are indicated in the middle and their expected migration in a 1.5% TBE  
623 agarose gel is shown on the left side. Diagnostic band patterns for the three plant-associated  
624 *Pantoea* species are shown on the right side.

625

626 **Figure 2:** Detection of three *Pantoea* species by multiplex PCR, using heated cell suspensions  
627 or genomic DNA as template. Three reference strains were used as representatives for the  
628 three *Pantoea* species, *P. ananatis* strain ARC60, *P. stewartii* strain ARC222, and  
629 *P. agglomerans* strain CFBP 3615.

630 Lanes 1 & 5, pool of heated cells of the three *Pantoea* species; lane 2, *P. ananatis*; lane 3,  
631 *P. stewartii*; lane 4, pool of genomic DNA from *P. ananatis* and *P. agglomerans*;

632

633 **Figure 3:** Detection of three *Pantoea* species in artificially infected rice leaves and in  
634 contaminated seeds. The following *Pantoea* strains were used: *P. ananatis* strain ARC60,  
635 *P. stewartii* strain ARC222, and *P. agglomerans* strain CFBP 3615.

636 Lane 1, *P. ananatis* (leaf sample); lane 2, *P. ananatis* (seed); lane 3, *P. stewartii* (leaf); lane 4,  
637 *P. stewartii* (seed); lane 5, *P. agglomerans* (leaf); lane 6, *P. agglomerans* (seed); lane 7, A  
638 yellow bacterial colony isolated from rice seeds; lane 8, water.

639

640 **Figure 4:** Sensitivity of PCR amplification in simplex and multiplex PCR. Serial dilutions of  
641 total genomic DNA and heated bacterial cells were evaluated. I, simplex PCR with bacterial  
642 cells; II, multiplex PCR with bacterial cells; III, simplex PCR with genomic DNA; IV  
643 multiplex PCR with genomic DNA. Three *Pantoea* strains were used: *P. ananatis* strain

644 ARC60 (A), *P. stewartii* strain ARC222 (B), and *P. agglomerans* strain CFBP 3615 (C).

645 Simplex PCR were performed with the corresponding, species-specific primer pairs,

646 PANAN\_gyrB for *P. ananatis*, PANST\_rpoB for *P. stewartii*, and PANAG\_infB for

647 *P. agglomerans*. The multiplex PCR included all five primer pairs.

648 The following amounts of bacteria or genomic DNA were used as templates for the PCR,

649 corresponding to 10-fold serial dilutions: Lanes 1 to 12  $10^6$  CFU/mL,  $10^5$  CFU/mL,  $10^4$

650 CFU/mL,  $10^3$  CFU/mL,  $10^2$  CFU/mL,  $10^1$  CFU/mL,  $10^0$  CFU/mL,  $10^{-1}$  CFU/mL,  $10^{-2}$

651 CFU/ml,  $10^{-3}$  CFU/ml,  $10^{-4}$  CFU/mL and water; lanes 12 to 24, 50 ng, 5 ng, 0.5 ng, 50 pg, 5

652 pg, 0.5 pg, 50 fg, 5 fg, 0.5 fg, 50 ag, 5 ag and water; M, molecular size marker (1 kb DNA

653 ladder, Promega).

654

655 **Additional files**

656

657 **Additional file 1:** List of bacterial strains used to evaluate the multiplex PCR scheme.

658



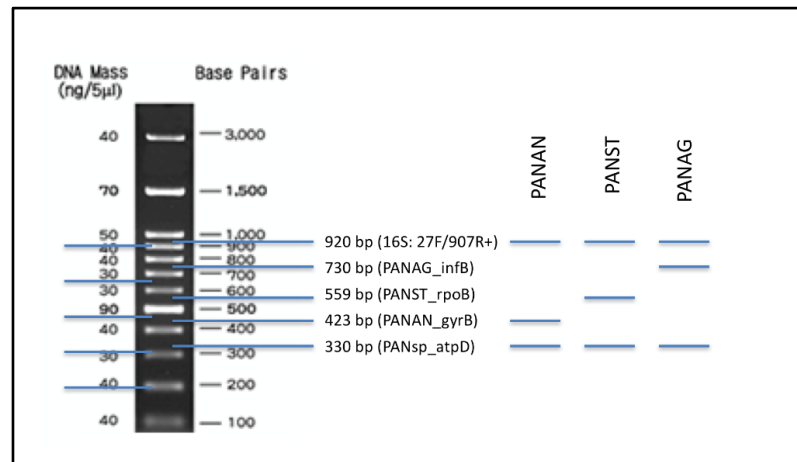


Figure 1

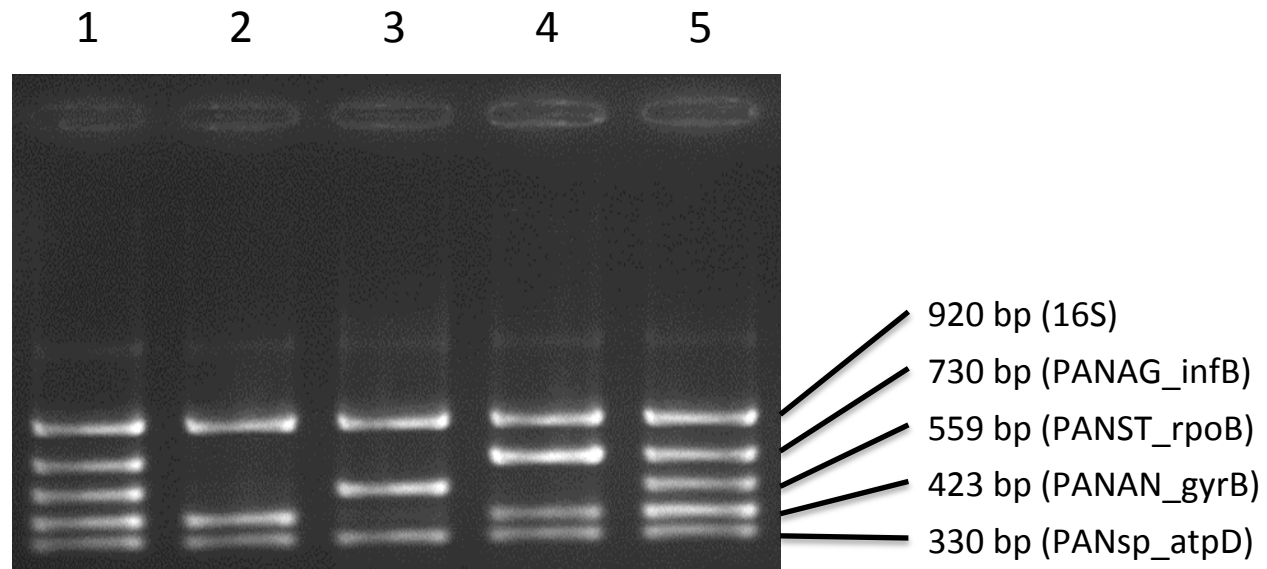


Figure 2

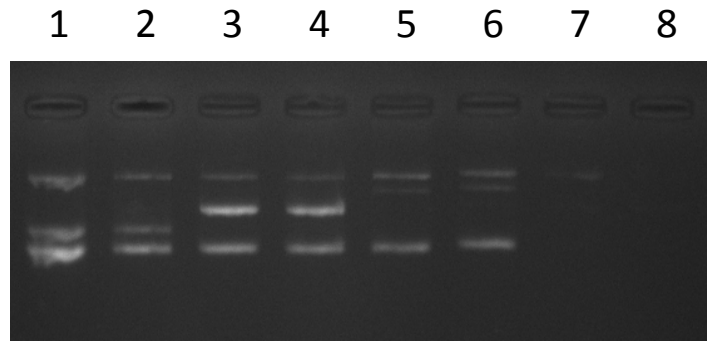


Figure 3

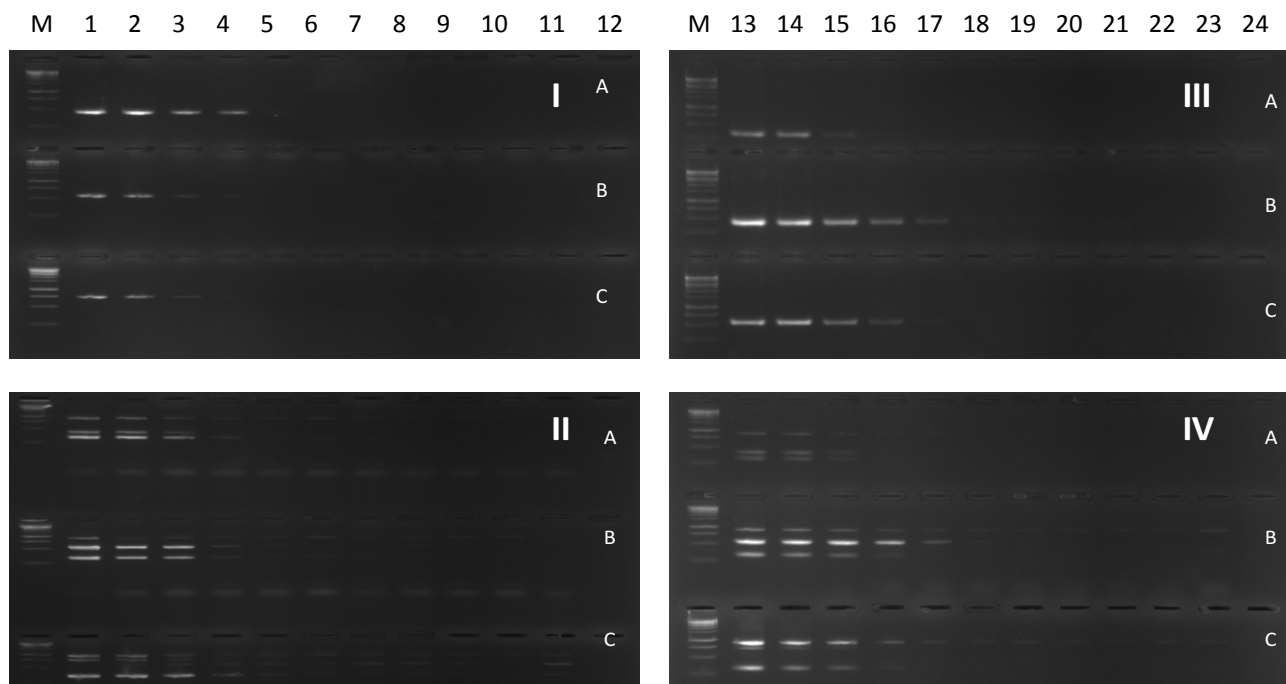


Figure 4