Discrimination of *Burkholderia gladioli* pv.*alliicola* and *B. cepacia* complex using the *gyr*B gene of *B. gladioli pv. alliicola*

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Abstract. The aim of the present study was to investigate the efficiency of the *gyrB* gene derived from *Burkholderia gladioli* pv.*Alliicola* (Bga) on the identification of Bga from the *B. cepacia* complex (Bcc) based on the COnsensus-DEgenerate Hybrid Oligonucleotide Primer (CODEHOP) strategy. A set of primers used for the specific amplification of the *gyrB* gene in Bga were designed according to the CODEHOP principle. A total of 1,644 bp of the *gyrB* gene sequence of Bga were acquired by CODEHOP amplification. The sequence was blasted in GenBank and it revealed an average of 86% similarity with the *gyrB* gene of nine genomovars of Bcc. A phylogenetic tree was constructed using the *gyrB* gene sequences. The microarray method was adopted to discriminate Bga from Bcc based on the specific probes designed upon the *gyrB* gene, and five genomovars of Bcc demonstrated a good discrimination from Bga on the microarray chip. CODEHOP strategy succeeded in amplification of the *gyrB* gene of Bga, which made it possible for the identification of Bga from five genomovars of Bcc.

Introduction

Burkholderia gladioli pv.*Alliicola* (Bga), one of the four pathovars known in *B. gladioli,* was originally described by Severini in 1913 (1) as a phytopathogen causing rot of *Gladiolus* corms. Initially, it was considered as a synonym of *Pseudomonas marginata* (2), and was then given the present name *B. gladioli* (3). As a gram-negative bacillus, *B. gladioli* had been primarily considered to be a plant pathogen. However, it was also reported to be associated with the onset of pulmonary infections in humans, including cystic fibrosis (CF) and chronic granulomatous disease (CGD) (4-7).

B. cepacia complex (Bcc) is a group of closely related gram-negative bacteria with similar phenotypes and genotypes (8). To date, a total of 17 genomovars have been identified from the environment and are widely used as a reagent for biodegradation and biocontrol, acting as a plant growth-promoting rhizobacterium (9-11). Similar to Bga, Bcc may also induce fatal infections in vulnerable individuals, for example those with CF and CGD (12-15), as well as induce rot of onions (16). These pathogenic similarities suggested a close association between Bga and Bcc.

Currently, extensive studies have been conducted on the taxonomy of Bcc; however, a high incidence of misidentification for Bcc is reported as ~50% of isolated Bcc actually belongs to *B. gladioli* (17). To date, polymerase chain reaction (PCR)-based methods have been commonly used to isolate unknown family members using 16S and 23S ribosomal RNA, and *recA* or *gyrB* (18-24). Among these markers, the *gyrB* gene encoding subunit B of DNA gyrase was demonstrated to be effective in the discrimination of species within Bcc and Bga due to its sufficiently variable rate (25). Although such strategies have been indicated to be successful in isolating closely related sequences, failure is usually encountered when sequences are more distantly related or are in low copy numbers.

In the present study, a novel strategy named after COnsensus-DEgenerate Hybrid Oligonucleotide Primer (CODEHOP) (26) was adopted to test its applicability for PCR amplification of the *gyrB* gene of Bga by CODEHOP primers designed upon related protein sequences of the *gyrB* gene of Bcc. A total of 1,644 base pairs of the *gyrB* gene of Bga were acquired and two probes, designed based on the *gyrB* gene sequences of Bga and Bcc, were used to distinguish between Bga and five genomovars of Bcc. A phylogenetic tree was constructed based on the *gyrB* gene of Bga and nine genomovars of Bcc that demonstrated a divergence between Bga and Bcc. The present study demonstrated the utility of CODEHOP in the study of homologous genes between Bga and Bcc. Furthermore, the results indicated that the *gyrB* gene was a suitable marker in distinguishing Bga and Bcc; however, it was less effective for the genotyping of different genomovars of Bcc. In the future, more effective markers should be developed

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or a combination of different marker genes may be more effective and robust in the genotyping of Bcc.

Materials and methods

Strains and reagents. Bga strain 20157 was kindly provided by Professor Hasan Bolkan from the Campbell's Agricultural Research Centre (Davis, CA, USA). The DNA of Bcc was provided by Dr Guan-Ning Xie of Zhejiang University (Hangzhou, Zhejiang, China). *Taq* DNA polymerase, deoxynucleotide (dNTP) mixture for the PCR reaction, DNA marker DL2000, *Escherichia coli* competent cells (DH5α) for transformation and a MiniBEST plasmid purification kit were purchased from Takara Biotechnology Co., Ltd., (Dalian, China). E.Z.N.A.® Bacterial DNA Kit and E.Z.N.A Gel Extraction Kit was purchased from Omega Bio-Tek, Inc., (Norcross, GA, USA). A T-clone kit (pGEM®-T Vector System) including the pGEM®-T Vector and T4 DNA Ligase was purchased from Promega Corp., (Madison, WI, USA). Ampicillin, isopropyl β-D-1-thiogalactopyranoside and X-gal used for screening of clones were provided by Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Primer synthesis and DNA sequencing were performed by Takara Biotechnology Co., Ltd. The reagents used in the experiments were all of analytical purity.

Design of CODEHOP primers. Clustalx software (http://www.clustal.org/) was utilized to investigate the sequence homology of the *gyrB* gene with Bcc and Bga. The *gyrB* protein sequence of Bcc was retrieved and downloaded from the National Center for Biotechnology Information (NCBI) Gen Bank database (https://www.ncbi.nlm.nih.gov/genbank/). The sequence length was limited between 630 and 655 amino acids, which was close to its full length. Furthermore, alignment of the protein sequence was conducted by the online Block Maker program (http://blocks.fhcrc.org/blockmkr/make_blocks.html) to identify the conservative sequences. The sequences obtained were input into the CODEHOP primers designing tool (https://virology.uvic.ca/virology-ca-tools/j-codehop/) to search for the appropriate primers, and the codon preference was set as '*Burkholderiaglumae*' as it was the only species of *Burkholderia* in the provided codon preference list and was homologous to Bga.

CODEHOP amplification. The CODEHOP amplification reaction was performed in a total volume of 25 µl containing 10X PCR buffer, 25 mmol/ldNTPs, 20 µmol/l of each primer, 5 U/µl*Taq* DNA polymerase and 30 ng Bga genomic DNA. Genomic DNA extraction was performed using the E.Z.N.A.® Bacterial DNA kit(Omega Bio-tek, Inc.). The amplification conditions were as follows: 94˚C for 4 min followed by 45 cycles of 94˚C for 30 sec, 47-49.5˚C for 60 sec and 72˚C for 90 sec, as well as a final extension at 72˚C for 7 min. Following this, the products were electrophoresed on a 1.2% agarose gel and dyed with ethidium bromide.

Verification. To verify the amplified fragment obtained using CODEHOP primers, the verification primers were designed according to the *gyrB* gene of *B. gladioli* downloaded from the NCBI database using Primer Premier 6.0 software (Premier Biosoft International, Palo Alto, California, USA). The primers were then used for amplification of genomic DNA of Bga to test their validity. The PCR reaction was performed in a total volume of 25 µl containing 2.5 µl 10X PCR buffer, 2 µl dNTPs (25 mmol/l), 0.8 µl of each primer (20 µmol/l), 0.2 µl *Taq* DNA polymerase (5 U/µl) and 30 ng Bga genomic DNA template. The amplification conditions were as follows: 94˚C for 4 min followed by 35 cycles of 94˚C for 30 sec, 50˚C for 30 sec, 72˚C for 45 sec and a final extension at 72˚C for 7 min.

Cloning of CODEHOP amplification products. Purification of CODEHOP amplification products was performed with an E.Z.N.A gel extraction kit, according to the manufacturer's instructions. Following purification, the fragments were linked with a T-vector using pGEM®-T Vector Systems kit purchased from Promega Corp., at 4˚C overnight. Subsequently, the products were transformed into *E.coli* competent cells (DH5α) by performing heat-shock, followed by cultivation on a shaker at a speed of 150 RPM for 1 h at 37˚C.Subsequently, the plasmid was extracted using a MiniBEST plasmid purification kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol. Sanger sequencing was then performed to verify the inserted sequence. Sequencing was completed by Sangon Biotech Co., Ltd. (Shanghai, China).

Phylogenetic analysis. The *gyrB* gene sequences were compared with known sequences deposited in the NCBI database using the BlastN program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). For each genomovar of Bcc, the strain with the most similar sequence of the *gyrB* gene of Bga was selected. Phylogenetic analysis was performed using the free tool, MEGA 5(https://www.megasoftware.net/) with the Neighbor-Joining algorithm, and the topological accuracy of the trees was evaluated with 1,000 bootstrap replicates.

Microarray analysis. A universal primer of the *gyrB* gene was designed according to the *gyrB* gene sequence obtained from Bga and sequences of Bcc downloaded from GenBank. However, two probes identifying Bga and several probes identifying Bcc and its genomovar were designed using AlleleID software 7.60(PremierBiosoft International, Palo Alto, CA, USA). Probes, with a 15-bp poly (dT) and an amino group at the 5' terminal were conjugated to the glass substrate modified by an aldehyde group. Fluorescent labeling was incorporated in the amplification products by cy3-dCTP within the PCR procedure. PCR reaction was performed in a total volume of 10 µl containing 10X PCR buffer, 25 mmol/ldNTPs, 250 nmol Cy™3-dCTP, 20 µmol/l of each primer, 5 U/µl *Taq* DNA polymerase and 0.5 µl Bga/Bcc genomic DNA. The amplification instructions were as follows: 94˚C for 4 min followed by 35 cycles of 94˚C for 30 sec, 56˚C for 1 min, 72˚C for 45 sec and 72˚C for 7 min. Amplification products were initially mixed with the hybridization buffer (6X Saline Sodium Citrate, 0.5% SDS, 100 µg/ml, Salmon DNA (Sigma-Aldrich; Merck KGaA) preheated to 50˚C, followed by heating at 95˚C for 5 min. Subsequently, the mixture was incubated on ice and water for 5 min. After a 2-h hybridization, the chip was washed twice with washing buffer (20 mM Tris-HCl, 150 mM NaCl,

Table I. Consensus-degenerate hybrid oligonucleotide primers.

Name	Sequence (5'-3')	Degeneracy	Tm value, ˚C
BgyrbF	GACGGCAAGAAGCGCttyatggartt	4	60.2
BgyrbR	CACGGACACGCGCacrttnccrtg	16	60.6
BgyrbR'	TGGTAGTCGGCGGTGtgytgraaytc	8	60.3

Y represents T or C; r represents A or G; n represents T, A, G or C; uppercase letters represent the consensus clamp; lowercase letters represent the degenerate core.

Table II. Verification primers.

Name	Sequence (5'-3')	Length of amplicon, bp
bgrybseq1F	CGAGTATCACTACGACATCC	442
bgrybseq1R	CACCTTCACCGACAACAC	
bgrybseq2F	TCCGACGATCTTCCACAT	271
bgrybseq2R	CACCTTCACCGACAACAC	

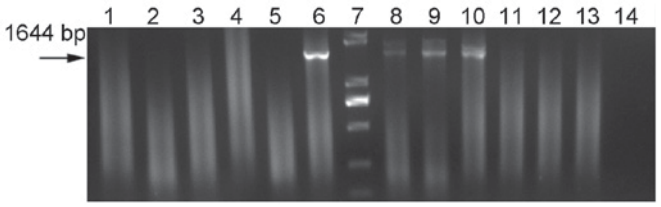

Figure 1. COnsensus-DEgenerate Hybrid Oligonucleotide Primer polymerase chain reaction results at different annealing temperatures. Annealing temperatures: Lanes 1 and 2, 49.5˚C; lanes 3 and 4, 49˚C; lanes 5 and 6, 48.5˚C; lane 7, marker (100, 250, 500, 750, 1,000 and 2,000 bp); lanes 8 and 9, 48˚C; lanes 10 and 11, 47.5˚C; lanes 12 and 13, 47˚C; lane 14, blank control. The template used was *Burkholderia gladioli* pv.*alliicola*20157 DNA.

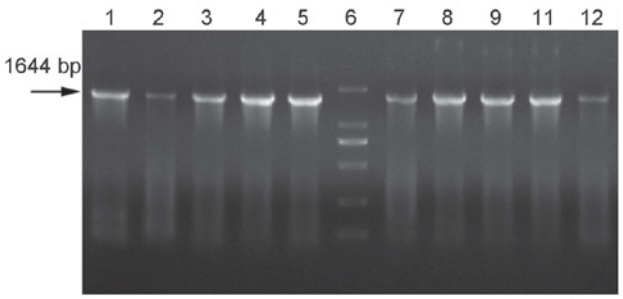

Figure 2. Amplification repeatability of BgyrbF/BgyrbR. Lanes 1-5 and 7-12, PCR results of *Burkholderia gladioli* pv. *alliicola*20157 DNA amplified by BgyrbF/BgyrbR; lane 6, marker DL2000.

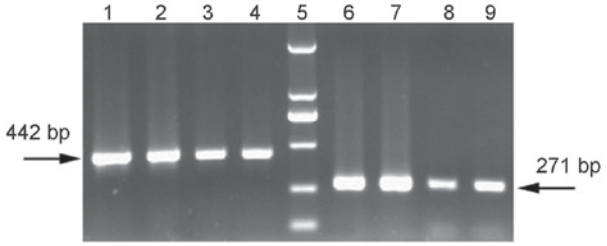

Figure 3. Verification of CODEHOP polymerase chain reaction amplicon. Lanes 1 and 2, CODEHOP amplicon of Bga 20157 *gyrB* gene amplified by bgrybseq1F/bgrybseq1R; lanes 3 and 4, Bga 20157 genomic DNA amplified by bgrybseq1F/bgrybseq1R; lanes 6 and 7, CODEHOP amplicon of the Bga 20157 *gyr*B gene amplified by bgrybseq2F/bgrybseq2R; lanes 8 and 9, *Bga* 20157 genomic DNA amplified by bgrybseq2F/bgrybseq2R; lane 5, marker DL2000.CODEHOP, COnsensus-DEgenerate Hybrid Oligonucleotide Primer; Bga, *Burkholderia gladioli* pv.*Alliicola*.

0.05% Tween-20) and air-dried. Finally, a GenePix 4200A (Molecular Devices, LLC, Sunnyvale, CA, USA) scanner was used for the results.

Results

CODEHOP primers and verification primers. Two sets of primer pairs were eventually selected (Table I), in which Y, R, N in the sequence represented the degenerate bases. The bases in the capital form represented the consensus clamp and those in the lowercase form represented the degenerate core according to the principal of CODEHOP primers. Two pairs of verification primers were designed based on the *gyrB* gene sequence of *B. gladioli* (Table II).

CODEHOP PCR amplification and verification. CODEHOP PCR was performed in a gradient pattern with a decrease in the annealing temperature of 0.5˚C (from 49.5-47˚C) using the BgyrbF/BgyrbR primers. As demonstrated in Fig. 1, positive bands were obtained using such primers at a temperature between 47.5 and 48.5˚C. On this basis, CODEHOP PCR was repeated using an annealing temperature of 48˚C. The repeatability of the amplification results by BgyrbF/BgyrbR are indicated in Fig. 2.

Two verification primers were used to verify the amplified products of CODEHOP PCR. As demonstrated in Fig. 3, two pairs of primers (bgrybseq1F/bgrybseq1R and bgrybseq2F/bgrybseq2R) were valid for amplification of the CODEHOP product, and two amplification fragments with lengths of 442 and 271 bp were obtained, respectively.

Once the verification process was complete, DNA sequencing was performed on the CODEHOP products. The full length of the sequence was 1,644 bp, which was close to the full length of the deposited *gyrB* gene of *B. gladioli*. Sequence comparison was performed by the BlastN program, which revealed a maximum identity of 87% between the *gyrB* gene of Bga and that of the *B. multivorans* strain,

Table III. Strains used for phylogenetic analysis.

Strain	Accession no.	Coverage, %	Similarity (nucleotide, %)	Similarity (protein, %)
Burkholderiacepacia strain LMG 18821	EU240562	99	86	89
Burkholderiamultivorans strain FCF11	AY996876	100	87	87
Burkholderiacenocepacia strain MVPC1_73_gvrIIIb	AY996886	99	86	90
*Burkholderiastabilis*strain LMG 18888	EU240566	99	86	89
Burkholderiavietnamiensis strain LMG 18836	EU240564	98	86	89
Burkholderiadolosa strain LMG 18942	AY987924	100	87	88
Burkholderiaambifaria strain LMG 19182	EU240568	99	86	90
Burkholderiaanthina strain LMG 20980	AY987928	98	85	89
Burkholderiapyrrocinia strain FCF46	AY987932	100	86	89
*Bordetella pertuss*is CS	CP002695	71	80	-

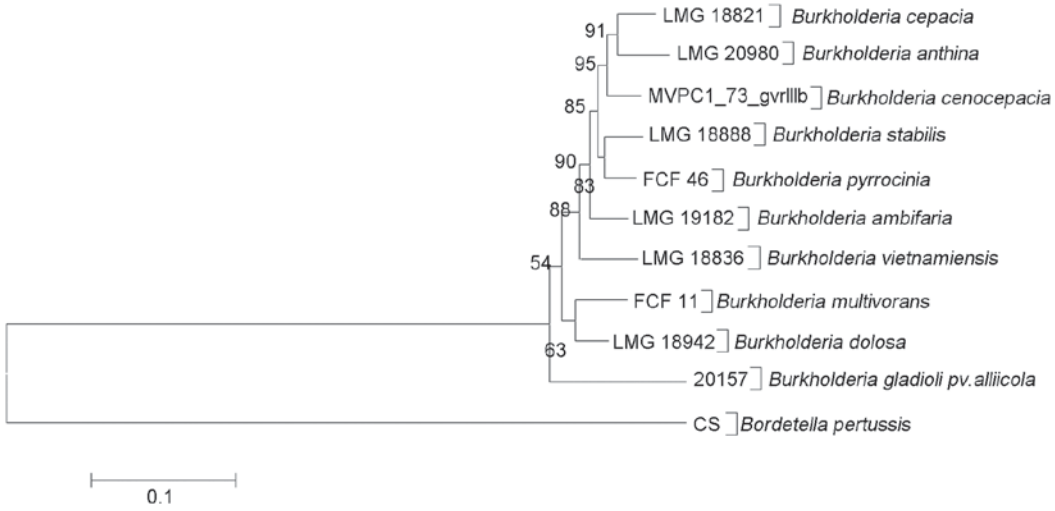

Figure 4. Phylogenetic tree constructed based on the *gyrB*gene sequence.

FCF11. However, according to the sequencing results, the non-degenerate sequences of CODEHOP primers were BgyrbF (5'-GACGGCAAGAAGCGCTTTATGGAATT-3') and BgyrbR (5'-CACGGACACGCGCACATTTCCATG-3').

Phylogenetic analysis. Strains of Bcc selected for the phylogenetic analysis are listed in Table III. Multi-alignments including nine genomovar *gyrB* sequences of Bcc and the *gyrB* sequence of *Bordetella pertussis* CS as outgroup deposited in GenBank were used to construct the phylogenetic tree. As demonstrated in Fig. 4, Bga formed a single cluster separated from Bcc.

Results of the microarray assay. The primers and probes designed for the discrimination of Bga and Bcc are summarized in Table IV. Universal primer pairs designated as 20157F/20157R for the amplification of the *gyrB* gene of Bga and Bcc were designed. A weak, non-specific band was observed in *B. cenocepacia*, *B. stabilis*, *B. anthina* and *B. pyrrocinia* (Figs. 5 and 6). After raising the annealing temperature to 60˚C, the non-specific bands in *B. cenocepacia* and *B. anthina* were eliminated (data not shown). For *B. vietnamiensis*, amplification was effective by decreasing the annealing temperature to 50˚C, as well as for *B. cepacia*. No positive bands were obtained in the amplification performed using the template obtained from *B. andropogonis* and *B. caryohpy*, respectively. On the contrary, a weak, non-specific band was obtained in *B. glumae*; however, the non-specific positive bands were not observed after raising the annealing temperature. A total of five genomovars and Bga amplified by 20157F/20157R were tested on the microchip assay, as demonstrated in Fig. 7. Between the two probes, primer Bga-P1 (Fig. 7A) was more effective for the recognition of Bga. Among the probes designed for identification of Bcc and its genomovars, the probe Geno5-P2 (Fig. 7B-F) designed based on the sequence of *gyrB* gene of *B. vietnamiensis* demonstrated the best recognition rate over all test genomovars, although it was originally designed for the recognition of *B. vietnamiensis*. Geno5-P2 revealed a weak, non-specific recognition rate with that of the amplification product of Bga, which could be easily eliminated following an optimized hybridization procedure. However, all the specific probes failed to recognize the target genomovar, which was speculated to be associated with the high identity between the *gyrB* gene of different genomovars of Bcc.

Table IV. Sequence of primers and probes.

Name	Sequence (5'-3')
20157F	TCCTCCTTGCCGATCCCGCA
20157R	AGAACCGCGGCACCGAAGTG
Bga-P1	NH_2-d$(T)_{15}$-CTTCACCGACAACACGCAG
Geno5-P2	NH_2-d$(T)_{15}$-AAGGTGCTCAACGTCGA

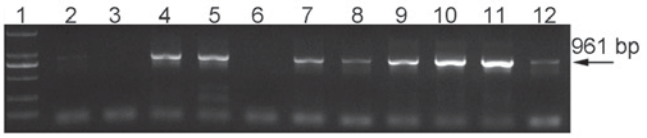

Figure 5. Polymerase chain reaction results of 20157F/20157R. Lane 1, marker DL2000; lane 2, *Burkholderiacepacia* Y3; lane 3, *B. multivorans* PW99; lane 4, *B. cenocepacia* Y10; lane 5, *B. cenocepacia* 317; lane 6, *B. stabilis* J100; lane 7, *B. vietnamiensis* 419; lane 8, *B. anthina* YP46; lane 9, *B. pyrrocinia* 301; lane 10, *B. arboris* HT1; lane 11, *B. seminalis* R45; lane 12, *B. contaminans* Y4.

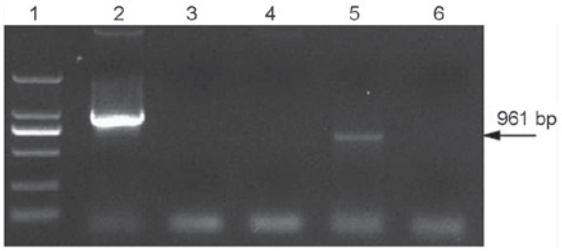

Figure 6. Polymerase chain reaction results of 20157F/20157R. Lane 1, marker; lane 2, *Burkholderia gladioli* pv.*alliicola* 20157; lane 3, *B. andropogonis* ATCC 23060; lane 4, *B. caryohpy*ATCC 11441; lane 5, *B. glumae* ATCC 33617; lane 6, blank control.

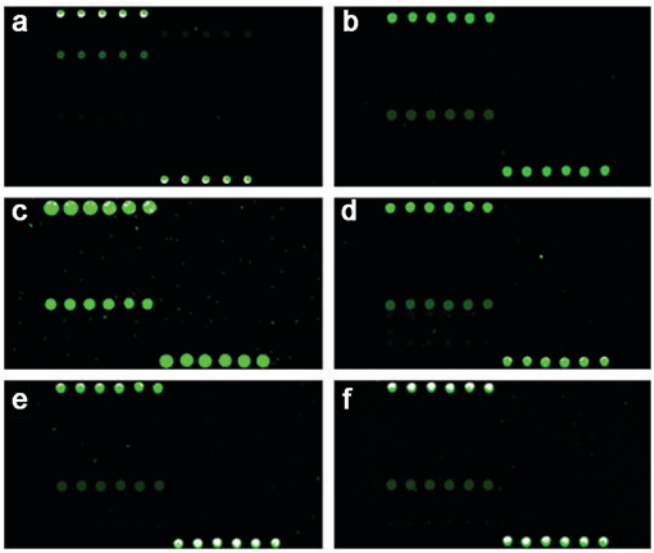

Figure 7. Hybridization results on the microarray. (a) *Burkholderia gladioli* pv.*alliicola* 20157; (b) *B. cepacia* Y3; (c) *B. vietnamiensis* 419; (d) *B. arboris* HT1; (e) *B. seminalis* R45 and (f) *B. contaminans* Y4.

Discussion

CODEHOP strategy, a modified method of the ordinary degenerate PCR, is more effective than the ordinary degenerate, PCR particularly between distantly related species (26). A CODEHOP primer with a 5' non-degenerate consensus sequence and a 3' degenerate core guarantees the stringency and degeneracy in the amplification process.

In the present study, CODEHOP strategy was successfully performed to amplify the sequence of the *gyrB* gene of Bga based on the protein sequences of Bcc. One forward primer and two reverse primers were designed (one of the reverse primers failed to work). The amplification of the primer set BgyrbF/BgyrbR was not as stable as expected during the amplification performed on different strains of Bga. Thus, the most stable annealing temperature was 48˚C. Furthermore, the strategy was more effective than the ordinary degenerate PCR, which failed to work for the amplification of the *gryB* gene (data not shown).

To obtain a magnified replication of CODEHOP amplification products, the products were purified and linked with a T-vector, and were transformed into *E. coli* competent cells (DH5α). Sequencing results revealed that the gene obtained showed an average similarity of 86% with the *gyrB* gene of nine genomovars of Bcc and a maximum identity of 87% with that of *B. multivorans*. In order to investigate the sequence homology of the *gyrB* gene between Bcc and Bga, a comparison was performed using Clustalx software, which revealed no evident variable region in these sequences. Subsequently, the nucleotide sequence was translated into a protein sequence, followed by querying using the BlastX program in GenBank. The results revealed an averaged similarity of 89% with the protein sequences of the nine genomovars of Bcc. Additionally, the revolutionary rate of the *gyrB* gene was faster at the nucleic acid level than at the protein level in related species, thus the nucleic acid sequence was employed to construct the phylogenetic tree.

A phylogenetic tree was constructed based on the single *gyrB* gene sequence of Bga and Bcc, which revealed a revolution divergence on the *gyrB* gene between these two categories. The discrimination utility of the *gyrB* gene between the two categories was then tested using microarray methods. To the best of our knowledge, it is difficult to find a well-performed universal primer for the *gyrB* gene of Bga and Bcc due to sequence variation. Mass sequence data was analyzed to find appropriate probes. In the present study, two probes for recognition of *gyrB* of Bga, three universal probes for recognition of that in Bcc, together with six probes specific for recognition of five different genomovars were initially designed. All the specific probes revealed cross-hybridization between different genomovars on the chip. Two probes, *Bga*-P1 and Geno5-P2, for recognition of Bgaand Bcc, respectively, demonstrated the best hybridization result. Furthermore, sequence analysis revealed that a difference was widely observed in the *gyrB* gene within the strains of the same genomovar. A noteworthy discovery was identified in *B. multivoran*; two obvious groups existed in the *gyrB*gene of *B. multivoran*based on the similarity of gene sequences. This may explain the failure in the amplification using a universal primer, although it demonstrated a close similarity with the *gyrB* gene of Bga.

It remains a challenge to discriminate Bga from Bcc in clinical practice, and several molecular methods have been developed for this based on the 16S and 23S rRNA genes (17,18). In addition, a multiplex-PCR protocol has also been developed for the specific detection of *B. plantarii*, *B. glumae* and, *B. gladioli* in rice seeds based on the *gyrB* sequences (23). In the present study, CODEHOP strategy was used for amplification of the *gyrB* gene of Bga based on the protein sequence of the *gyrB* gene of Bcc. As demonstrated in the results, the strategy was practical, which makes it a utility tool for homologous gene study or marker gene selection between Bga and Bcc even within Bcc. In conclusion, the *gyrB* gene was a useful marker gene in discriminating Bga from Bcc based on the phylogenetic tree. Due to the high similarity of the *gyrB* gene sequence of Bga and Bcc, a single probe was not adequate to distinguish Bga from Bcc as revealed by the microarray results. Therefore, more specific probes based on discriminative genes that recognize different species would be required.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LZ designed the experimental protocol, performed the experiments, collected and analyzed raw data and wrote the manuscript. WG modified the hybridization protocol of the microchip array. YY modified the protocol of CODEHOP PCR amplification. ZW provided the concept of the current study, reviewed all methods and results, revised the manuscript and approved publication of the final version of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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