Biological Forum – An International Journal **14(4a): 639-646(2022)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Multilocus Sequence Analysis of *Burkholderia glumae* Strains from Indian Hot Spot Regions causing Bacterial Panicle Blight of Rice (*Oryza sativa* L.)

Sanjeev Kumar[1], Kalyan K. Mondal[1]*, Thungri Ghoshal[1], Aditya Kulshreshtha[2], Sreenayana B.[1], Amrutha Lakshmi M.[1], Mrutyunjaya S.[1], Rashmi E.R.[1], Kalaivanan N.S.[1] and Chandra Mani[1]

[1]Division of Plant Pathology, ICAR–Indian Agricultural Research Institute, New Delhi, India.
[2]Texas A&M AgriLife Research and Extension Center Weslaco, Texas, USA.

(Corresponding author: Kalyan K. Mondal)*
(Received 25 September 2022, Accepted 10 November, 2022)
(Published by Research Trend, Website: www.researchtrend.net)

ABSTRACT: Bacterial panicle blight (BPB) is one of the emerging diseases of paddy in the Tarai region of Uttarakhand and the disease problem is increasing every year. The present investigation was conducted to examine the *Burkholderia glumae* isolates causing bacterial panicle blight (BPB) disease in rice by multilocus sequence analysis (MLSA) of four housekeeping genes *atpD, recA* and *gltB*. The *atpD* gene sequence of Indian strains BG1 and BG2 from UP, BG5 and BG6 from Delhi showed 100% nucleotide homology with NCBI strain *B. glumae* 3BGNY2-4 isolated from rice in China with grouping together in the same clade, however, other *Burkholderia* spp, showed lower nucleotide identity and formed a separate cluster from all the *B. glumae* strains. The *gltB* and *recA* gene sequence of Indian strains showed 100% nucleotide identity with NCBI strain *B. glumae* 336gr-1 and *B. glumae* HN2 respectively, thus confirming the species to be *B. glumae.* Similarly, in the phylogenetic tree of *gltB* and *recA* gene sequence all four Indian isolates clustered together in the phylogenetic tree where BG5 and BG6 strain from Delhi shared the same clade. Thus, based on the grouping of *atpD, gltB* and *recA* gene sequence and phylogenetic analysis of all four Indian isolates of BPB pathogen of rice confirmed the species identity as *B. glumae.* Thus, present study reveals use of multiple genes for species identification and classification of *B. glumae*.

Keywords: MLSA, *atpD, recA, gltB* and *Burkholderia glumae.*

INTRODUCTION

The genus *Burkholderia*, a β-proteobacteria group, was created to accommodate seven species from the *Pseudomonas* ribosomal RNA group II (Palleroni *et al.,* 1973, Yabuuchi *et al.,* 1992). Eventually, many more species were described and included in this new genus. Others were removed or reclassified, such as *Burkholderia pickettii* (now *Ralstonia pickettii*) and *B. solanacearum* (now *Ralstonia solanacearum*), or *B. cocovenenans*, which was a synonym of *B. gladioli* and *B. vandii*, a synonym of *B. plantarii*. Currently, *Burkholderia* comprises more than 60 species, which are distributed in diverse habitats. For example, several species are important components of the rhizosphere (Compant *et al.,* 2008). Others have been found in water, plant roots, or legume nodules, or can be opportunistic pathogens for plants or humans (Vandamme *et al.,* 2007). Among the pathogenic *Burkholderia*, *B. glumae*, *B. gladioli* and *B. plantarii* are extremely important because they are the etiologic agents of many diseases in rice, onion, gladioli and other host plants. The *Burkholderia glumae* (BG) is another important pathogen that causes bacterial panicle blight in rice, spreading rapidly in rice growing regions of North India (Mondal *et al*., 2015).

In 2001, two reports presented a totally different view of *Burkholderia*. First, the genus was rich in nitrogen-fixing species (Estrada-de los Santos *et al.,* 2001), and second, several nitrogen-fixing species were found to nodulate legume plants (Moulin *et al*., 2001). This last feature was striking because previously legumes were thought to be nodulated only by α-proteobacteria. Today, eight *Burkholderia* species that elicit effective nodule formation on legume roots have been reported and more are in the pipeline awaiting description (Gyaneshwar *et al.,* 2011). Recently, strains from *B. fungorum* were found to nodulate *Phaseolus vulgaris*, although ineffectively (Ferreira *et al*., 2012). Payne *et al.* (2005) designed *Burkholderia* genus-specific *recA* PCR that produced a 385-bp amplicon, the sequence of which was also able to discriminate all species examined. Phylogenetic analysis of 188 novel *recA* genes enabled clarification of the taxonomic position of several important *Burkholderia* strains and revealed the presence of four novel *B. cepacia* complex *recA* lineages. The phylogenetic relationships among *B. glumae* strains were carried out using combined nucleotide sequences of the *gyrB* and *rpoD* genes; showed no diversity among 41 strains of *B. glumae* (Maeda *et al.,* 2006). 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 nucleotide. These results indicate that the diversity of nucleotide sequences of *gyrB* and *rpoD*

among *B. glumae* strains is very restricted. The multilocus sequence typing (MLST) was performed on 107 *B. multivorans* isolates to provide a detailed analysis of the global population biology of this species by Baldwin *et al.* (2008). MLST resolved 64 *B. multivorans* sequence types. The MLST concatenated sequence analysis, clearly distinguished the 64 *B. multivorans* subtypes from all other BCC species (100% bootstraps). Ramachandran *et al*. (2021) found that clustering of *B. glumae* isolates were similar, of which the isolates were clustered in the same clade (clade A) with *B. glumae* reference strains with a bootstrap confidence value of 84% for 16S rRNA and 100% for *gyrB*. The isolates were distinctly separated from the *B. gladioli* reference strains in clade B.

As the number of described *Burkholderia* species has increased, 16S rRNA sequence analyses have revealed two sub-lineages within the genus. These two groups have been recovered with different phylogenetic reconstruction methods using 16S rRNA sequences (Payne *et al.,* 2005), multilocus sequence analysis (MLSA) with *gyrB* and *rpoB* genes sequences (Ait-Tayeb *et al.,* 2008) or seven housekeeping genes (Spilker *et al*., 2009), or *recA* gene sequences (Vandamme *et al.,* 2000). Not only MLSA but also multilocus sequence typing (MLST) have been used for epidemiologic and population genetics studies, delineation of species, and assignment of strains to defined bacterial species in *Burkholderia* (Ait-Tayeb *et al*., 2008). Different housekeeping genes have been useful for this purpose and an MLST database exists for BCC members (http://pubmlst.org/bcc/). For phylogenetic analyses based on single gene are not generally recommended for species identification because the effects of gene duplication or loss, horizontal gene transfer and recombination can interfere with the accurate reconstruction of an organism's evolutionary history. Hence the multiple genes may be used for species identification and classification.

In context of earlier reports, few attempts have been made regarding identification of the pathogen at the species level. Thus, we performed MLSA of a set of *Burkholderia* spp. using three housekeeping genes, *atpD* (ATP synthase beta chain), *recA* (recombinase A gene) and *gltB* (glutamate synthase large subunit to explore the positioning of the *Burkholderia* species in a phylogenetic analysis. In this report, we present evidence that the genus *Burkholderia* is composed of distinctly different phylogenetic lineages.

MATERIALS AND METHODS

Bacterial Strains. The diseased samples infested with bacterial panicle blight (BPB) were collected from the various paddy growing locations like Uttar Pradesh, Delhi and NCR regions. A set of 10 isolates of *Burkholderia glumae* was isolated and taken for analysis by MLSA. Several species with sequenced genomes were also chosen from NCBI database for the analysis. A list of strains used in this study is presented in Table 1.

Multilocus sequence analysis assay (MLSA) of BG isolates. Phylogenetic analysis based on a single gene are not generally recommended for species identification because the effects of gene duplication or loss, horizontal gene transfer (HGT) and recombination can interfere with the accurate reconstruction of an organism's evolutionary history (Marrero *et al.,* 2013). Multilocus sequence analysis (MLSA) is a technique which is evaluated as a means for rapid identification of bacterial isolates to species level discrimination and determination of their phylogenetic relationships with intra- and inter-generic relationships. The species level identification of four isolates (BG1, BG2, BG5 and BG6) of *B. glumae* and their phylogenetic relationships were confirmed by using multilocus sequence analysis (MLSA).

Primers and PCR conditions. The partial sequences of housekeeping genes *atpD* (ATP synthase beta chain), *recA* (recombinase A gene) and *gltB* (glutamate synthase large subunit) were randomly selected and the primers were designed to obtain DNA fragments in the range of 737–1467 bp. The MLSA analysis was performed as described previously by Spilker *et al.* (2009); Payne *et al.* (2005) with small modifications. Details of the primers, and expected amplicon size of housekeeping genes used for MLSA analysis are furnished in the Table 2. The housekeeping genes *atpD, recA and gltB* were randomly selected and the following primers were designed to obtain DNA fragments in the range of 737–1467 bp. For the ATP synthase beta chain (*atpD*), the primers atpD-F (ATGAGTACTACTGCTTTGGTAGAAGG) and atpD-R (CCTCGGCCAGCGTCGGCTG) were employed. For the recombinase A (*recA*), the primers recA-F (AGAAGCAGTTCGGCAAGGGCT) and recA-R (GATGTCGAAGATCGACTCGC) primers were used. For the glutamate synthase large subunit (*gltB*), the primers gltB-F (ATGGGCAAGGCAACCGGTTT) and gltB-R (TCAGCGCGGCAGTTCCGAATGCC) were employed. The MLSA analysis was performed as described previously by Spilker *et al.* (2009) with small modifications.

The 25 µl PCR reaction was slightly changed and it consisted 5.0µl of 5× Taq buffer, 1.0µl of 10mM dNTPs, 2.50 µl of 25mM $MgCl_2$, 0.5 µl each of (*gltB*, *atpD*), 1 µl (*recA*) primer. After a 2 min denaturation step at 95°C, 35 PCR cycles were completed, each PCR cycle consisting of 30 s at 94°C, 30 s at the appropriate annealing temperature, and extension for 60 s at 72°C, followed by a final extension step of 5 min at 72°C (Spilker *et al*., 2009).

Gel electrophoresis. PCR amplified fragments were analyzed on 1.5% agarose gel in electrophoresis unit using 1X TAE buffer (1X TAE prepared from a 50X TAE buffer stock solution that includes per liter 18.61g Na_2EDTA, 57.1 ml glacial acetic acid and 242 g Tris-Base). 15 µl of PCR amplified products was loaded in to the wells separately and 1kb ladder included in one of the well. The gel was run in 1X TAE for 2 h at a constant voltage of 85V at room temperature. After the running of gel, the amplified DNA was visualized under UV light and documented by using the gel documentation system (BIO-RAD, GEL DOCTM XR+

with image Lab™ software).

Sequencing and analysis of the sequencing data of three housekeeping genes. An unpurified 50µl PCR amplified product of three housekeeping genes (*atpD, recA and gltB*) products of four BG isolates (BG1, BG2, BG5 and BG6) were sent for bidirectional Sanger sequencing by outsourcing from Agri Genome Labs Private Ltd. Sequences obtained were curated using Finch TV software (https://digitalworldbiology.com/FinchTV). Sequences were end-trimmed, edited, and contigs-assembled using DNA baser (http://www.dnabaser.com/download/DNA-Baser-sequence-assembler). Contigs were assembled in Bio-Edit sequence alignment editor and the gene sequences were compared with other accessions available in GenBank database by BLAST. Nucleotide sequence similarities were determined by using the NCBI database (https://www.ncbi.nlm.nih.gov/search/) and bacterial identity was established by the closest match. First sequence of 10 sequences showing similarity with the query sequence was retrieved from NCBI data base and phylogenetic tree was constructed using MEGA X software.

RESULTS

Multilocus sequence analysis (MLSA) of *B. glumae* isolates. The species identity of four Indian isolates viz., BG1, BG2, BG5 and BG6 was further confirmed by characterization and sequences analysis of three house-keeping genes *viz*., *atp*D, *glt*B and *rec*A.

***atpD* gene sequence and phylogenetic analysis.** All four Indian isolates of *B. glumae* isolated from bacterial panicle blight of rice amplified at 832bp by using atpDF and atpDR primers (Fig. 1). In BLAST analysis the Indian strains BG1 and BG2 from UP, BG5 and BG6 from Delhi showed 100% nucleotide homology with NCBI strain *B. glumae* 3BGNY2-4 isolated from

***glt*B gene sequence and phylogenetic analysis.** Using gltBF and gltBR primers, all four Indian isolates of *B. glumae* amplified at 1,467 bp (Fig. 3). All four BG isolates (BG1, BG2, BG5 and BG6) showed 100% nucleotide identity with USA rice strain *B. glumae* 336gr-1 and *B. glumae* 411gr-6 as well as China rice strain *B. glumae* HN1 of *B. glumae*, respectively, in BLAST analysis of *glt*B gene. Other *Burkholderia* spp. such as *B. plantarii* PG1, *B. perseverans* INN12, *B. gladioli* BK04, *B. pseudomultivorans* SUB-INT23-BP2, *B. vietnamiensis* AU2011, *B. cenocepacia* C6433 and *B. cepacia* showed lower nucleotide identity range from 91.63% to 97.41% with the Indian isolates of BG1, BG2, BG5 and BG6. Whereas, the outgroup *X. vesicatoria* ATCC 35937 LMG911 isolated from tomato in New Zealand showed least nucleotide identity of 69.41 % with the all four Indian isolates (Table 4). The *glt*B gene sequences of four isolates were deposited in GenBank under accession numbers: OQ065126, OQ065127, OQ065128, OQ065129. The phylogentic analysis was done based on 1,467 bp size of *glt*B of four BG isolates and other *Burkholderia* spp. as well as the outgroup *X. vesicatoria* ATCC 35937 LMG911 retrieved from NCBI and showed that all four isolates and other global *B. glumae* strains were rice in China and 99.76% nucleotide homology with strains from USA i.e., *B. glumae* 336gr-1 (CP023203) and *B. glumae* 411gr-6 (CP021157) and South korean strain *B. glumae* BGR13S (CP033656). However, other *Burkholderia* spp. like *B. perseverans* INN12, *B. gladioli* KACC 11889, *B. ubonensis* MSMB0783, *B. plantarii* LMG 9035 and *B. pseudomallei* BD5 showed lower nucleotide identity varying with the range of 93.97 to 97.36% (Table 3) and they formed a separate cluster from all the *B. glumae* strains. The *atp*D gene sequences of four isolates were deposited in GenBank under accession numbers: OQ065134, OQ065135, OQ065136, OQ065137. The phylogeny tree was constructed based on 832 bp sequences of the *atpD* gene of the BG isolates and compared with the 10 isolates of *Burkholderia* spp. and *E. amylovora* CFBP1430 as an outgroup obtained from NCBI database (Fig. 2). The sequences of Indian isolates (BG1, BG2, BG5 and BG6) were grouped with the reference strains of *B. glumae* from the Genbank database. The result of sequence and phylogenetic analysis of *atp*D gene for all four Indian isolates of BPB pathogen of rice confirmed the species identity as *B. glumae.* The similarity matrix of *atp*D gene revealed that all the four India isolates BG1, BG2, BG5 and BG6 exhibited a 100% *atp*D gene similarity identity among themselves, as well as with other *B. glumae* isolates from other countries. However, at the species level, the similarity identity of Indian BG strains ranged from 99.66% to 99.98% with other global strains of *Burkholderia* spp. For the *atp*D gene sequence, Indian BG strains shared the least similarity identity of 99.94% with *B. ubonensis* MSMB0783 from Australia and *B. pseudomallei* BD5 from Singapore. The outgroup *E. amylovora* CFBP1430 almost similar similarity identity of 99.66% to 99.68% with the Indian BG strain as well as other global *Burkholderia* spp.

clustered together. The out-group *X. vesicatoria* ATCC 35937 LMG911 closely differentiated with BG strains from India and other global strains of *Burkholderia* spp. collected from NCBI (Fig. 4). The similarity identity matrix based on *glt*B gene of Indian isolates of *B. glumae* along with other *Burkholderia* species and *X. vesicatoria* ATCC 35937 LMG911 was prepared and revealed that all Indian isolates i.e., BG1, BG2, BG3 and BG4 were identical to each other and had 100% similarity identity matrix with three other global *B. glumae* strains *B. glumae 336gr-1 (USA), B. glumae* 411gr-6 (USA) and *B. glumae* HN1 from China which clearly indicates that these India strains of *B. glumae* isolated from rice belongs to the *B. glumae*. However, among *Burkholderia* spp. *B. cenocepacia* C6433 and *B. cepacia* DDS_7H-2 showed least sequence similarity identity matrix of 99.90% with *B. gladioli* BK04, while the outgroup *X. vesicatoria* ATCC 35937 LMG911 showed sequence similarity of 99.56% with *B. cepacia* DDS_7H-2.

***rec*A gene sequence and phylogenetic analysis.** All four Bacterial panicle blight isolates of *B. glumae* amplified at 737 bp by using *rec*AF and *rec*AR primers (Fig. 5). In BLAST analysis of *rec*A gene, *B. glumae* strains *B. glumae* HN2 from China, *B. glumae* BGR13S

from South Korea and *B. glumae* 2BGCN5-2 from Thailand showed 100% nucleotide identity with all four Indian isolates BG1, BG2, BG5 and BG6 (Table 5). However, at the species level, *B. perseverans* INN12, *B. plantarii* LMG 9035, *B. gladioli* FDAARGOS_389, *B. caryophylli* ATCC 25418, *B. multivorans* FDAARGOS_722, *B. savannae* MSMB0852, *B. vietnamiensis* To17 and *B. cepacia* J2543 from various hosts, showed 93.49-97.56% similarity with Indian *B. glumae* isolates (Table 5). The four Indian isolates' *rec*A gene sequences were deposited in GenBank under the accession numbers: OQ065130, OQ065131, OQ065132, OQ065133. The phylogeny tree was constructed based on a 737 bp sequences of the *rec*A gene of the four Indian isolates and compared with 11 isolates of *Burkholderia* spp. and one isolate of *E. amylovora* CFBP1430 obtained from NCBI Database (Fig. 6). All four Indian isolates were found to be clustered together in the phylogenetic tree where BG5 and BG6 strain from Delhi shared the same clade. Other global *Burkholderia* spp. from the NCBI and the outgroup *E. amylovora* CFBP1430 formed separate grouping from *B. glumae* strains. The similarity identity matrix based on sequence of *rec*A gene of Indian isolates of *B. glumae* and other isolates of *Burkholderia* spp. obtained from NCBI database was prepared. All the four isolates i.e., BG1, BG2, BG5, BG6 and global strains *viz*., *B. glumae* HN2, *B. glumae* BGR13S and *B. glumae* 2BGCN5-2 were identical to each other and had 100% similarity identity matrix. However, these Indian BG strains were 99.93-99.98% identical to other *Burkholderia* spp collected from NCBI database. The other *Burkholderia* spp. were close to each other and had sequence similarity of about >99% among them. In case of similarity identity matrix of outgroup *E. amylovora* CFBP1430 showed least value i.e., 69.50% with the *Burkholderia* species including Indian isolates of *B. glumae*, which indicates that *E. amylovora* CFBP1430 is different and hence can be separated through *rec*A gene analysis.

DISCUSSION

Phylogenetic analysis based on a single gene are not generally recommended for species identification because the effects of gene duplication or loss, horizontal gene transfer (HGT) and recombination can interfere with the accurate reconstruction of an organism's evolutionary history (Marrero *et al.,* 2013). Multilocus sequence analysis (MLSA) technique is evaluated as a means for rapid identification of bacterial isolates to species level discrimination and determination of their phylogenetic relationships with intra- and inter-generic relationships. The species level identification of four isolates (BG1, BG2, BG5 and BG6) of *B. glumae* and their phylogenetic relationships were confirmed by using multilocus sequence analysis (MLSA) of three housekeeping genes. We developed an approach for the genetic identification of *Burkholderia glumae* and to discriminate between members of the closely related *Burkholderia* spp.

We observed that in BLAST analysis the *atpD* gene sequence of Indian strains BG1 and BG2 from UP, BG5 and BG6 from Delhi showed 100% nucleotide homology with NCBI strain *B. glumae* 3BGNY2-4 isolated from rice in China. The similarity matrix of *atp*D gene exhibited a 100% *atp*D gene similarity identity among themselves. Thus, we can say that there is no any difference in *atpD* gene sequence among Indian strains. The Indian BG strains grouped together with other BG strains of NCBI, however, other *Burkholderia* spp, showed lower nucleotide identity varying with the range of 93.97 to 97.36% (Table 3) and they formed a separate cluster from all the *B. glumae* strains. The grouping of *atpD* gene sequence and phylogenetic analysis of all four Indian isolates of BPB pathogen of rice confirms the species identity as *B. glumae*. Vandamme and Dawyndt (2011) came to similar conclusions using ML to examine 100,000 base positions randomly extracted from single-copy core genes of the available *Burkholderia* genomes released up to date.

The *gltB* and *recA* gene sequence of Indian strains showed 100% nucleotide identity with NCBI strain *B. glumae* 336gr-1 and *B. glumae* HN2 respectively, thus confirming the species to be *B. glumae*. Similarly, in the phylogenetic tree of *gltB* and *recA* gene sequence all four Indian isolates clustered together in the phylogenetic tree where BG5 and BG6 strain from Delhi shared the same clade. Estrada-de los *et al*. (2013) observed similar grouping based on distinct lineages in *Burkholderia* spp. All the four isolates *i.e*., BG1, BG2, BG5, BG6 had 100% similarity identity matrix and were identical to each other. In a similar study ,the phylogenetic analysis and phylogenomic analysis were performed based on a multilocus sequence of the housekeeping genes, viz., *atpD*, *gltB*, *gyrB*, *lepA*, *phaC*, *recA*, and *trpB* (Jungkhun *et al.,* 2022). Similarly, Payne *et al*. (2005) designed *Burkholderia* genus-specific *recA* PCR that produced a 385-bp amplicon, the sequence of which was also able to discriminate all species examined. Phylogenetic analysis of 188 novel *recA* genes enabled clarification of the taxonomic position of several important *Burkholderia* strains and revealed the presence of four novel *B. cepacia* complex *recA* lineages. They revealed the genotypic relationships among the strains of *B. glumae* and the other rice-pathogenic species of *Burkholderia* with all the strains isolated from japonica rice in Thailand grouping in the *B. glumae* clade. The strain from the species *B. gladioli* and *B. plantarii* formed clades corresponding to each species. This clearly indicates that multilocus gene analysis using housekeeping genes *atp*D, *glt*B and *rec*A developed in this study provides molecular tools for the identification of *Burkholderia* species that will help to enable researchers to keep pace with the ever-increasing ecological, pathogenic, and genomic interest in the genus.

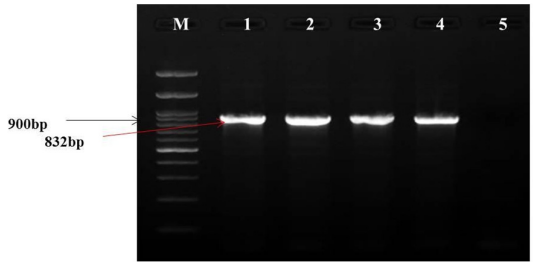

Fig. 1. Amplification of *atp*D gene fragments from representative *B. glumae* isolates (Lane 1: BG1; Lane 2: BG2; Lane 3: BG5; Lane 4: BG6 and Lane 5: Blank; M-100bp ladder (Gene Ruler).

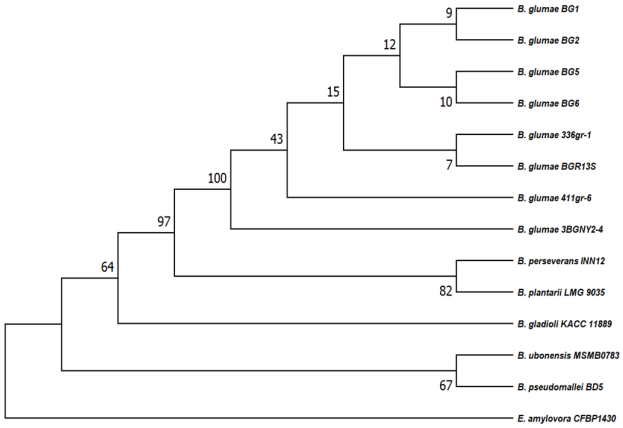

Fig. 2. Phylogenetic tree using Maximum Likelihood method based on Tamura-Nei model(1993) with 1000 Bootstrap replications for *B. glumae* isolates (BG1, BG2, BG5 and BG6) derived from Muscle alignment of *atp*D partial sequences of 832 bp.

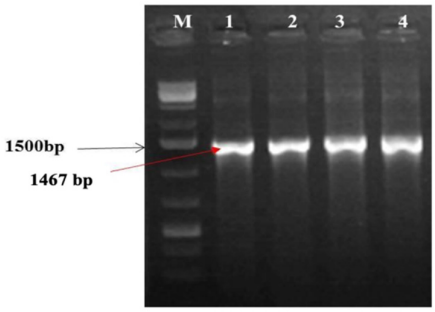

Fig. 3. Amplification of *glt*B gene fragments from representative *B. glumae* isolates (Lane 1: BG1; Lane 2: BG2; Lane 3: BG5; Lane 4: BG6 and Lane 5: Blank; M-1kb ladder (Gene Ruler).

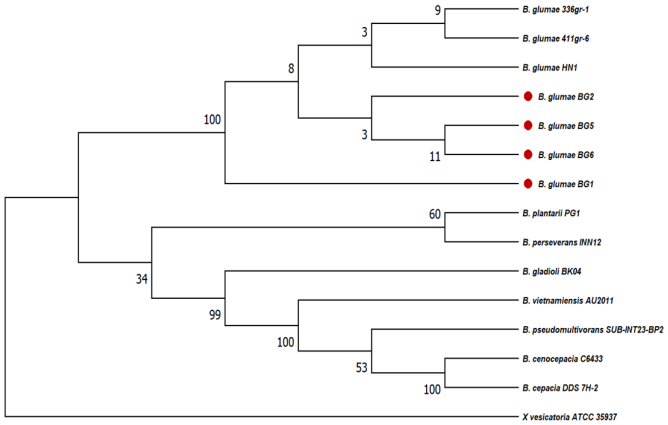

Fig. 4. Phylogenetic tree using Maximum Likelihood method based on Tamura-Nei model(1993) with 1000 Bootstrap replications for *B. glumae* isolates (BG1, BG2, BG5 and BG6) derived from Muscle alignment of *glt*B partial sequences of 1467 bp.

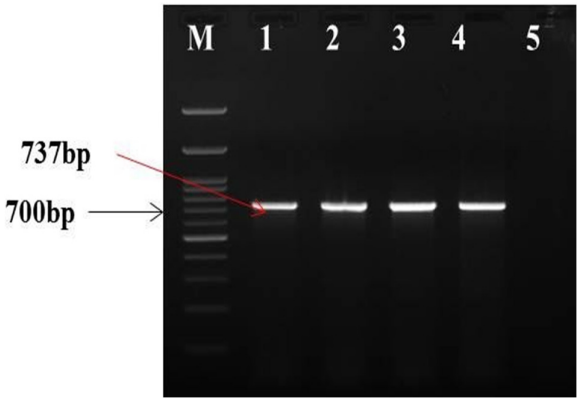

Fig. 5. Amplification of *rec*A gene fragments from representative *B. glumae* isolates (Lane 1: BG1; Lane 2: BG2; Lane 3: BG5; Lane 4: BG6 and Lane 5: Blank; M-100bp ladder (Gene Ruler).

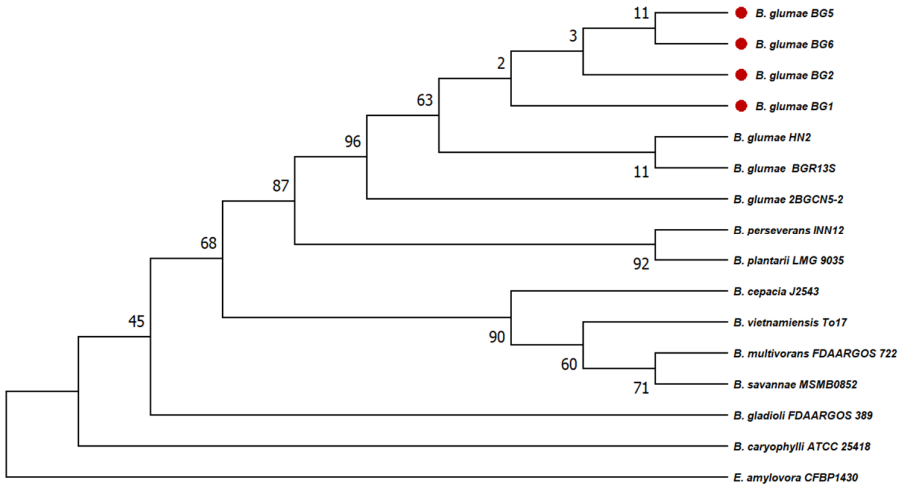

Fig. 6. Phylogenetic tree using Maximum Likelihood method based on Tamura-Nei model (1993) with 1000 Bootstrap replications for *B. glumae* isolates (BG1, BG2, BG5 and BG6) derived from Muscle alignment of *rec*A partial sequences of 737 bp.

Table 1: List of isolates of *Burkholderia glumae* isolated from bacterial panicle blight infected samples collected from different locations.

Isolate Code	Place of Sample Collection
BG1	Sambal, Uttar Pradesh
BG2	Budaun, Uttar Pradesh
BG3	Budaun, Uttar Pradesh
BG4	Sambal, Uttar Pradesh
BG5	New Delhi
BG6	New Delhi
BG7	Hapur, Uttar Pradesh
BG8	Sambal, Uttar Pradesh
BG9	Chandoshi, Uttar Pradesh
BG10	Hapur, Uttar Pradesh

Table 2: Oligonucleotide primer sequences and annealing temperatures for the amplification and sequencing of three MLSA loci for *Burkholderia glumae.*

Gene	Amplicon size (bp)	Primer sequence (5'→3')	Annealing temp (°C)
atpD	832	ATGAGTACTACTGCTTTGGTAGAAGGCCTCGGCCAGCGTCGGCTG	58
recA	737	AGAAGCAGTTCGGCAAGGGCT GATGTCGAAGATCGACTCGC	60
gltB	1467	ATGGGCAAGGCAACCGGTTT TCAGCGCGGCAGTTCCGAATGCC	58

Table 3: *atpD* sequence identity of *B. glumae* isolates causing Panicle blight of rice within groups and out group representing diverse hosts.

***Burkholderia* species (NCBI Accession number)**	**Country**	**Host plant**	**Nucleotide identity(%) of *B. glumae* isolates**			
			***B. glumae* BG1 (OQ065134)**	***B. glumae* BG2 (OQ065135)**	***B. glumae* BG5 (OQ065136)**	***B. glumae* BG6 (OQ065137)**
B. glumae 336gr-1 (CP023203)	USA	Rice	99.76%	99.76%	99.76%	99.76%
B. glumae 411gr-6 (CP021157)	USA	Rice	99.76%	99.76%	99.76%	99.76%
B. glumae BGR13S (CP033656)	South Korea	Rice	99.76%	99.76%	99.76%	99.76%
B. perseverans INN12 (CP045094)	Brazil	Leaf litter	97.36 %	97.36 %	97.36 %	97.36 %
B. gladioli KACC 11889 (CP022005)	South Korea	Gladiolus	95.68 %	95.68 %	95.68 %	95.68 %
B. glumae 3BGNY2-4 (KY826540)	China	Rice	100%	100%	100%	100%
B. ubonensis MSMB0783 (CP013420)	Australia	Rice	94.12%	94.12%	94.12%	94.12%
B. plantarii LMG 9035 (HQ398425)	Germany	NA	97.30%	97.30%	97.30%	97.30%
B. pseudomallei BD5 (CP073728)	Singapore	Water	93.97%	93.97%	93.97%	93.97%
E. amylovora CFBP1430(NC_013961)	Germany	Apple	73.94%	73.94%	73.94%	73.94%

Percent identity score between two each accession number was calculated usinghttps://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=MegaBlast &PROGRAM=blastn&PAGE_TYPE=BlastSearch&BLAST_SPEC=blast2seq
*NCBI accession number of isolates used in this study in parenthesis.

Table 4: *gltB* sequence identity of *B. glumae* isolates causing Panicle blight of rice within groups and out group representing diverse hosts.

***Burkholderia* species (NCBI Accession number)**	**Country**	**Host plant**	**Nucleotide identity(%)of *B. glumae* isolates**			
			***B. glumae* BG1 (OQ065126)**	***B. glumae* BG2 (OQ065127)**	***B. glumae* BG5 (OQ065128)**	***B. glumae* BG6 (OQ065129)**
B. glumae 336gr-1 (CP023203)	USA	Rice	100 %	100 %	100 %	100 %
B. glumae 411gr-6 (CP021157)	USA	Rice	100 %	100 %	100 %	100 %
B. glumae HN1 (CP052867)	China	Rice	100 %	100 %	100 %	100 %
B. plantarii PG1 (CP002580)	Germany	NA	97.41 %	97.41 %	97.41 %	97.41 %
B. perseverans INN12 (CP045094)	Brazil	Leaf litter	97.00 %	97.00 %	97.00 %	97.00 %
B. gladioli BK04 (CP098321)	China	Soil	94.15 %	94.15 %	94.15 %	94.15 %
B. pseudomultivorans SUB-INT23-BP2 (CP013378)	Thailand	Soil	91.90 %	91.90 %	91.90 %	91.90 %
B. vietnamiensis AU2011 (CP072318)	Nigeria	Soybean rhizosphere	91.83 %	91.83 %	91.83 %	91.83 %
B. cenocepacia C6433 (CP098497)	Human	Respiratory isolate	91.63 %	91.63 %	91.63 %	91.63 %
B. cepacia DDS 7H-2 (CP007787)	USA	NA	91.63 %	91.63 %	91.63 %	91.63 %
X. vesicatoria ATCC 35937 LMG911 (NZ_CP018725)	New Zealand	Tomato	69.41 %	69.41 %	69.41 %	69.41 %

Percent identity score between two each accession number was calculated using
https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=MegaBlast&PROGRAM=blastn&PAGE_TYPE=BlastSearch&BLAST_SPEC=blast2seq
*NCBI accession number of isolates used in this study in parenthesis.

Table 5: *rec*A sequence identity of *B. glumae* isolates causing Panicle blight of rice within groups and out group representing diverse hosts.

***Burkholderia* species (NCBI Accession number)**	**Country**	**Host plant**	**Nucleotide identity(%) of *B. glumae* isolates**			
			***B. glumae* BG1 (OQ065130)**	***B. glumae* BG2 (OQ065131)**	***B. glumae* BG5 (OQ065132)**	***B. glumae* BG6 (OQ065133)**
B. glumae HN2 (CP052132)	China	Rice	100 %	100 %	100 %	100 %
B. glumae BGR13S (CP033656)	South Korea	Rice	100 %	100 %	100 %	100 %
B. glumae 2BGCN5-2 (KY826565)	Thailand	Rice	100 %	100 %	100 %	100 %
B. perseverans INN12 (CP045094)	Brazil	Leaf Litter	97.56 %	97.56 %	97.56 %	97.56 %
B. plantarii LMG 9035 (AJ551323)	Germany	NA	97.23 %	97.23 %	97.23 %	97.23 %
B. gladioli FDAARGOS_389 (CP023522)	USA	Onion	95.79 %	95.79 %	95.79 %	95.79 %
B. caryophylli ATCC 25418 (HQ398562)	USA	Culture Collection	95.47 %	95.47 %	95.47 %	95.47 %
B. multivorans FDAARGOS_722 (CP046346)	USA	Homo sapiens	93.63 %	93.63 %	93.63 %	93.63 %
B. savannae MSMB0852 (CP013424)	Australia	Soil	93.49 %	93.49 %	93.49 %	93.49 %
B. vietnamiensis To17 (AY951908)	Italy	NA	93.62 %	93.62 %	93.62 %	93.62 %
B. cepacia J2543 (AF456057)	UK	Onion	93.62 %	93.62 %	93.62 %	93.62 %
E. amylovora CFBP1430 (NC_013961)	Germany	Apple	69.50 %	69.50 %	69.50 %	69.50 %

Percent identity score between two each accession number was calculated using
https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=MegaBlast&PROGRAM=blastn&PAGE_TYPE=BlastSearch&BLAST_SPEC=blast2seq
*NCBI accession number of isolates used in this study in parenthesis.

CONCLUSION

Our study brings an overview on using different housekeeping genes for characterising isolates of *B. glumae* using the sequences of internal fragments of three house-keeping genes *atp*D, *glt*B and *rec*A. It can be used for bacterial identification and classification as it offers the opportunity for provision of a more in depth view of the phylogenetic relationships of *Burkholderia* spp. As the pathogen is of quarantine importance, this knowledge can be used for the monitoring the disease globally.

FUTURE SCOPE

The comparative genomics approaches with whole genome sequence information would provide better resolution in corroborating the identification and variations among the isolates.

Acknowledgements. The authors are grateful to the Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi for providing all the necessary infrastructure and materials so that the study could be conducted. No external financial assistance was provided for the study.
Conflict of Interest. None.

REFERENCES

Ait-Tayeb, L., Lefevre, M., Passet, V., Diancourt, L., Brisse, S. and Grimont, PAD. (2008). Comparative phylogenies of *Burkholderia*, *Ralstonia*, *Comamonas*, *Brevundimonas* and related organism derived from *rpoB*, *gyrB* and *rrs* gene sequences. *Research Microbiology, 159,* 169–177.

Baldwin, A., Mahenthiralingam, E., Drevinek, P., Pope, C., Waine, D. J., Henry, D. A., Speert, D. P., Carter, P., Vandamme, P., LiPuma, J. J. and Dowson, C. G. (2008). Elucidating global epidemiology of *Burkholderia multivorans* in cases of cystic fibrosis by multilocus sequence typing. *Journal of clinical microbiology*, *46*(1), 290–295.

Compant S., Nowak J., Coenye T., Clement C. and Barka E. A. (2008). Diversity and occurrence of *Burkholderia* spp. in the natural environment. *FEMS Microbiological Reviews, 32,* 607–626.

Estrada-de los Santos, P., Bustillos-Cristales, R. and Caballero-Mellado, J. (2001). *Burkholderia*, a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. *Applied Environmental Microbiology, 67,* 2790–2798.

Estrada-de los Santos, P., Vinuesa, P., Martínez-Aguilar, L., Hirsch, A. M. and Caballero-Mellado, J. (2013). Phylogenetic analysis of *Burkholderia* species by multilocus sequence analysis. *Current microbiology, 67*(1), 51–60.

Ferreira, P. A. A., Bomfeti, C. A., Soares, B. L., Moreira, F. M. S. (2012). Efficient nitrogen-fixing *Rhizobium* strains isolated from Amazonian soils are highly tolerant to acidity and aluminum. *World Journal of Microbiology and Biotechnology, 28*, 1947–1959.

Gyaneshwar, P., Hirsch, A. M., Chen, W. M., Elliott, G. N., Bontemps, C., Gross, E., dos Reis Junior, F. B., Sprent, J. I., Young, J. P. W. and James, E. K. (2011) Legume nodulating β-proteobacteria: diversity, host range and future prospects. *Molecular Plant Microbe Interaction, 24,* 1276–1288.

Jungkhun, N., Gomes de Farias, A. R., Watcharachaiyakup, J., Kositcharoenkul, N., Ham, J. H., and Patarapuwadol, S. (2022). Phylogenetic characterization and genome sequence analysis of *Burkholderia glumae* strains isolated in Thailand as the causal agent of rice bacterial panicle blight. *Pathogens*, *11*(6), 676.

Marrero, G., Schneider, K. L., Jenkins, D. M. and Alvarez, A. M. (2013). Phylogeny and classification of *Dickeya* based on multilocus sequence analysis. *International journal of systematic and evolutionary microbiology, 63*(9), 3524–3539.

Mondal, K. K., Mani, C. and Verma, G. (2015). Emergence of Bacterial Panicle Blight Caused by *Burkholderia glumae* in North India. *Plant Disease, 99*(9), 1268-1268.

Moulin, L., Munive, A., Dreyfus, B. and Boivin-Masson, C. (2001). Nodulation of legumes by members of the beta-subclass of Proteobacteria. *Nature, 411*, 948–950.

Palleroni, N. J., Kunisawa, R., Contopoulou, R. and Doudoroff, M. (1973). Nucleic acid homologies in the genus *Pseudomonas*. *International Journal of Systematic Bacteriology, 23,* 333–339.

Payne, G. W., Vandamme, P., Morgan S. H., LiPuma, J. J., Coenye, T., Weightman, A. J., Jones, T. H. and Mahenthiralingam, E. (2005). Development of a *recA* gene-based identification approach for the entire *Burkholderia* genus. *Applied Environmental Microbiology, 71*, 3917–3927.

Ramachandran, K., Vijaya, S. I. and Ahmad, F. N. (2021). Characterization and identification of *Burkholderia glumae* as the causal pathogen of bacterial panicle blight of rice (*Oryza sativa* L.) in Malaysian rice granaries. *Journal of General Plant Pathol*ogy, *87,* 164–169.

Spilker, T., Baldwin, A., Bumford, A., Dowson, C. G., Mahenthiralingam, E. and LiPuma, J. J. (2009). Expanded multilocus sequence typing for *Burkholderia* Species. *Journal of Clinical Microbiology, 47,* 8.

Vandamme, P. and Dawyndt, P. (2011). Classification and identification of the *Burkholderia cepacia* complex: past, present and future. *Systematic Applied Microbiology, 34*, 87–95.

Vandamme, P., Govan, J. and LiPuma, J. (2007). Diversity and role of *Burkholderia* spp. In: Coenye T, Vandamme P (eds) *Burkholderia* molecular microbiology and genomics. *Horizon Bioscience Norfolk,* 1–28.

Vandamme, P., Mahenthiralingam, E., Holmes, B., Coenye, T., Hoste, B., De Vos P., Henry, D. and Speert, D. P. (2000). Identification and population structure of *Burkholderia stabilis* sp. nov. (formerly *Burkholderia cepacia* genomovar IV). *Journal of Clinical Microbiology, 38,* 1042–1047.

Yabuuchi, E., Yoshimasa, K., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T. and Arakawa, M. (1992). Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. *Microbiology and Immunology, 36*, 1251–1275.

How to cite this article: Sanjeev Kumar, Kalyan K. Mondal, Thungri Ghoshal, Aditya Kulshreshtha, Sreenayana B., Amrutha Lakshmi M., Mrutyunjaya S., Rashmi E.R., Kalaivanan N.S. and Chandra Mani (2022). Multilocus Sequence Analysis of *Burkholderia glumae* Strains from Indian Hot Spot Regions causing Bacterial Panicle Blight of Rice (*Oryza sativa* L.). *Biological Forum – An International Journal, 14*(4a): 639-646.