https://scilett-fsg.uitm.edu.my/
Vol. 16(1) JANUARY 2022

ISSN: 1675-7785
eISSN: 2682-8626

DOI: 10.24191/sl.v16i1.15582

In Silico Study of the Effectiveness of 16S rRNA Gene as a Universal Genetic Marker to Identify Closely Related *Burkholderia* spp. in Panicle Blight of Rice

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Received: 1 August 2021; Accepted: 29 September 2021; Published: 14 January 2022

ABSTRACT

The 16S rRNA gene is a housekeeping genetic marker that is available in almost all bacterial species and it is used in bacterial phylogeny and taxonomy studies. In many studies, the 16S rRNA gene is used in identification of certain bacterial species. Being a less conserved genetic marker, certain studies found it is a useful tool to infer the genome-wide similarity levels among the closely related prokaryotic organisms. Thus, this study aimed to compare the variation in the 16S rRNA partial region of *Burkholderia* spp. that infect the panicle of rice from eight different geographical areas. 58 sequences with total of 688 base pairs (bp) of 16S rRNA gene in *B. glumae* and *B. gladioli* were retrieved from public database based on several countries namely United State, Panama, Ecuador, Thailand, China, India, Korea and Malaysia. Then, the data sequences were analysed and validated using MEGAX and ABGD software respectively. The result of phylogenetic tree confirmed that *B. glumae* and *B. gladioli* were species that present in the panicle blight of rice. However, Data Analysis in Molecular Biology and Evolution (DAMBE) and Automatic Barcode Gap Discovery (ABGD) software were not able to detect substitution saturation and divergence between *B. glumae* and *B. gladioli* respectively based on the 58 sequences of the 16S rRNA partial region. Hence, it proves that 16S rRNA gene is an ineffective genetic marker to be used to differentiate the closely related species of bacteria from similar genus.

Keywords: *16S rRNA gene, Burkholderia glumae, Burkholderia gladioli, MEGA X, DAMBE, ABGD*

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Vol. 16(1) JANUARY 2022

ISSN: 1675-7785
eISSN: 2682-8626

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INTRODUCTION

The genus *Burkholderia* consists of Gram negative bacteria and the existence of its members are plentiful in ecological niches [1]. Adaptation to different environments by *Burkholderia* is caused by the genome plasticity that may increase the gene mutation and recombination [2,3]. As a result, one species from the genus *Burkholderia* may be found in several different habitats such as soil, plant, animal surfaces, rhizosphere, water [3] clinical specimens and foods [4]. Nevertheless, a growing number of strains and species of *Burkholderia* have also been identified as plant–associated bacteria in recent years [1]. Zhou-qi *et al.* [5] stated that the infection by *Burkholderia glumae* in rice has been found to be the cause of severe loss of yield in fields up to 75% in Louisiana, USA. This statistic of yield loss shows that bacterial panicle blight acts as a potential high-risk bacterial disease of rice [5]. Despite *B. glumae*, the presence of another species in *Burkholderia* genus; *Burkholderia gladioli* was found to be the causal agent of the occurrence of grain rot, seedling rot and panicle blight [6]. However, this species tends to be less frequently isolated from infected rice panicles and they are less virulence than *B. glumae* [7]. In addition, the appearance of *B. gladioli* in Louisiana, Texas and Arkankas was only about 20 percents [5]. According to Aflaha *et al.* [8], the occurrence of bacterial panicle blight was due to the production of toxoflavin by *B. glumae* that is essential for its virulence.

A discovery by Syahri *et al.* [9] and Nandakumar *et al.* [10], established that panicle blight cases were detected when brown discolouration appeared on the lower third to half of the hulls. Despite grain discolouration, the stem of infected rice plant remains green [10, 9]. The contaminated grains will turn the discolouration of grain from green to darker-brown and comprises reddish-brown lines across the floret during the end stage of the season [9; 11]. Moreover, in another study, it proved that spikelet sterility and discolouration of emerging grains, loss weight in grains, floret sterility and inhibition of seed germination also were due to the infection of *B. glumae* [4, 12,9]. Other than that, a study performed by Weny *et al.* [40] concluded that high night temperature and frequent rainfalls were also part of the main important environmental factors initiating the growth of *B. glumae* that lead to appearance of the symptoms of panicle blight which predispose rice to disease outbreak. Bacterial panicle blight (BPB) frequently occur in tropical and semi-tropical countries having the optimal temperature range from 30℃ to 35℃ for bacterial reproduction [14,4].

However, climate changes may cause the formation of new disease because most of the plant pathogens that live in high optimal temperatures will emerge and become more prevalent worldwide [15; 9]. Since 1999, different *Burkholderia* species have been detected in the rhizosphere of various crops around the world [16]. As an example, *B. cepacia* was found in rhizosphere of rice, pea, horsetail, arum lily and wheat in the UK [17]. Meanwhile, a similar species of *Burkholderia* has been isolated from the rhizosphere of maize in the US. Thus, the rhizosphere is considered as a reservoir for the members of genus *Burkholderia* [18]. In a study

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eISSN: 2682-8626

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conducted by Eberl and Vandamme [18], the researchers also stated that plant hosts can be infected or colonized by more than one species of the genus *Burkholderia*. For instance, the first rice rhizosphere detected in Vietnam is *Burkholderia vietnamiensis* which was isolated along with other *Burkholderia* species that come from rhizosphere of maize and coffee. This shows that different type of soil or crop management affect the diversity of *Burkholderia* species [5].

One of the most conserved regions in bacteria is 16S rRNA gene [19] and it is crucial for bacterial phylogeny and taxonomy studies [20]. In addition, 16S rRNA gene also acts as a housekeeping genetic marker because it presents in almost all bacterial cells. Besides, this genetic marker has not changed over time but 16S rRNA gene exhibits considerable variations [13]. It also has larger number of base pairs (1,500 bp) that is suitable to be used or applied in bioinformatics studies [21]. The application of 16S rRNA gene revealed that *Burkholderia glumae* was the first causal agent of emergence of bacterial panicle blight (BPB) reported in North India. Destructive BPB was occurred seriously in basmati and non-basmati rice which led to chaffy grains and the strains from India were shared 99 percent similarity of the nucleotide sequences with *B. glumae* (NR102846) obtained from public database [22]. Moreover, confirmation test for the presence of *B. gladioli* in panicle blight disease in Panama was taken place through BLAST analysis of the 16S rRNA region which resulted in 99 percent similar with the sequence data from Genbank [23]. According to the data above, 16S rRNA gene sequencing can be utilized as a tool to identify bacteria at the species level [24].

The 16S rRNA gene sequencing has been used by many researchers to identify *Burkholderia* species present in rice panicle blight. Analysis of 16S rRNA gene is a standard approach of bacterial taxonomy study and species identification based on the detection of sequence differences (polymorphism) in the hypervariable regions that present in all bacteria [25]. Molecular Evolutionary Genetics Analysis (MEGA) is one of the most appropriate software that comprises many features to analyze and store data in many types of files such as FASTA format, MEGA format and Excel Workbook [26]. Kumar *et al.* [27] stated in their review that MEGA helps researches to assemble sequences, align sequences, build phylogenetic or phylogenomic trees, compute genetic distance table and perform BLAST search. The MEGA software provides maximum-likelihood (ML) analysis for constructing the phylogenetic trees [28]. Kumar *et al.* [27] disclosed that a recent MEGA X software had been optimized with 64-bit computing systems which allow larger data analysis by researchers and scientists. Previously, MEGA 6.0 was utilized to build phylogenetic trees and perform comparative genomic analysis of genus *Burkholderia* based on 21 conserved proteins sequences [29]. As the result, they proposed that the genus *Burkholderia* was divided into three clades which contains clinically relevant *Burkhoderia*, pythopathogenic *Burkholderia* and *Paraburkholderia* gen. nov.

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However, the identification of *Burkholderia* spp. based on 16S rRNA gene by Mirghasempour *et al.* [30] only focusing on certain areas in China. Therefore, there is an absence of a data compilation of bacterial panicle blight from Western (United State, Panama and Ecuador) and Asian (Thailand, China, India, Korea and Malaysia) countries.

Thus, the relatedness among closely related species of *B. glumae* and *B. gladioli* from two different continents (Western and Asia) may not be observed. Besides, the study of variability of 16S rRNA gene in *B. glumae* and *B. gladioli* species is also indeterminate. Consequently, the factors of acclimation of *B. glumae* and *B. gladioli* species at different geographical areas may not be well-studied. This study analyzes the compilation of rice panicle blight data between Western (United State, Panama and Ecuador) and Asian (Thailand, China, India, Korea and Malaysia) countries. Moreover, this study is also conducted to compare the variability of 16S rRNA partial region of *Burkholderia* species that infect the panicle of rice from different geographical areas. In addition, Martínez-Porchas *et al.* [31] stated that the application of short 16S rRNA gene led to specificity and sensitivity loss. Hence, the effectiveness of 16S rRNA partial region as a genetic marker in bacterial panicle blight can be observed and evaluated based on the data obtained in this study.

EXPERIMENTAL

Data Retrieval from Databases

Retrieval of the 59 DNA sequences in this study consisted of 58 data of *Burkholderia* spp. and *Hafnia alvei* as an outgroup. All data were retrieved by the accession numbers from several journals stored in the Canadian Journal of Plant Pathology, APS Publications, Journal of Plant Pathology, Agricultural Science Journal and PubMed. All the 59 data sequences in this study were deposited in the National Center for Biotechnology Information (NCBI) database. Data from eight different geographical areas were retrieved and compiled as shown in Table 1.

Table 1: List of *Burkholderia* species used in this study

No.	Accession no.	Species	Geographical areas	Sizes (bp)	References
1.	DQ355164	*B. glumae*	USA	1494	Nandakumar *et al.* [10]
2.	DQ355166	*B. glumae*	USA	1494	Nandakumar *et al.* [10]
3.	DQ355167	*B. glumae*	USA	1494	Nandakumar *et al.* [10]

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4.	DQ355165	*B. glumae*	USA	1494	Nandakumar *et al.* [10]
5.	KP689100	*B. glumae*	India	1521	Mondal *et al.* [22]
6.	KP689101	*B. glumae*	India	1521	Mondal *et al.* [22]
7.	KP689102	*B. glumae*	India	1521	Mondal *et al.* [22]
8.	MN400210	*B. glumae*	China	1525	Hou *et al.* [32]
9.	MN400211	*B. glumae*	China	1525	Hou *et al.* [32]
10.	KF995706	*B. glumae*	China	1440	Hou *et al.* [32]
11.	KF995707	*B. glumae*	China	1445	Riera-Ruiz *et al.* [33]
12.	KF995708	*B. glumae*	China	1421	Riera-Ruiz *et al.* [33]
13.	EF193638	*B. glumae*	Panama	1442	Riera-Ruiz *et al.* [33]
14.	EF193639	*B. glumae*	Panama	1442	Riera-Ruiz *et al.* [33]
15.	EF193640	*B. glumae*	Panama	1442	Riera-Ruiz *et al.* [33]
16.	EF193641	*B. glumae*	Panama	1442	Riera-Ruiz *et al.* [33]
17.	KX638433	*B. glumae*	Korea	1497	Riera-Ruiz *et al.* [33]
18.	MT765056	*B. glumae*	Korea	1455	Choi *et al.* [34]
19.	MT765057	*B. glumae*	Korea	1455	Choi *et al.* [34]
20.	MT765058	*B. glumae*	Korea	1455	Choi *et al.* [34]
21.	MT765059	*B. glumae*	Korea	1455	Choi *et al.* [34]
22.	KY826526	*B. glumae*	Thailand	1318	Choi *et al.* [34]
23.	KY826527	*B. glumae*	Thailand	1318	Choi *et al.* [34]
24.	KY826528	*B. glumae*	Thailand	1318	Choi *et al.* [34]
25.	KY826529	*B. glumae*	Thailand	1318	Pet–amphai *et al.* [35]
26.	KY826530	*B. glumae*	Thailand	1318	Pet–amphai *et al.* [35]
27.	MN164411	*B. glumae*	Malaysia	1309	Unpublished
28.	MN164412	*B. glumae*	Malaysia	1308	Unpublished

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29.	MN164413	*B. glumae*	Malaysia	1309	Unpublished
30.	MN164414	*B. glumae*	Malaysia	1308	Unpublished
31.	MN164415	*B. glumae*	Malaysia	1309	Unpublished
32.	JF431409	*B. gladioli*	Korea	1326	Seo *et al.* [36]
33.	JF431410	*B. gladioli*	Korea	1329	Seo *et al.* [36]
34.	DQ355168	*B. gladioli*	USA	1494	Seo *et al.* [36]
35.	DQ355169	*B. gladioli*	USA	1494	Nandakumar *et al.* [10]
36.	EF088208	*B. gladioli*	USA	1492	Nandakumar *et al.* [10]
37.	EF088209	*B. gladioli*	USA	1492	Nandakumar *et al.* [10]
38.	EU090890	*B. gladioli*	China	1525	Mirghasempour *et al.* [30]
39.	MG386180	*B. gladioli*	China	1428	Mirghasempour *et al.* [30]
40.	JX566502	*B. gladioli*	China	1506	Mirghasempour *et al.* [30]
41.	MH281642	*B. gladioli*	China	978	Mirghasempour *et al.* [30]
42.	EF178441	*B. gladioli*	China	1352	Mirghasempour *et al.* [30]
43.	MF536137	*B. gladioli*	India	1260	Unpublished
44.	KT862889	*B. gladioli*	India	1230	Unpublished
45.	MN599032	*B. gladioli*	India	1410	Unpublished
46.	MK183027	*B. gladioli*	India	1000	Unpublished
.47.	KP842828	*B. gladioli*	India	1423	Unpublished
48.	EF193642	*B. gladioli*	Panama	1442	Nandakumar *et al.* [23]
49.	EF193643	*B. gladioli*	Panama	1442	Nandakumar *et al.* [23]
50.	EF193644	*B. gladioli*	Panama	1442	Nandakumar *et al.* [23]
51.	EF193642	*B. gladioli*	Panama	1442	Nandakumar *et al.* [23]
52.	JX566503	*B. gladioli*	Ecuador	1506	Riera-Ruiz *et al.* [33]
53.	KF669880	*B. gladioli*	Ecuador	680	Riera-Ruiz *et al.* [33]

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54.	KF669881	*B. gladioli*	Ecuador	686	Riera-Ruiz *et al.* [33]
55.	KF669882	*B. gladioli*	Ecuador	690	Riera-Ruiz *et al.* [33]
56.	KF669883	*B. gladioli*	Ecuador	698	Riera-Ruiz *et al.* [33]
57.	KF669884	*B. gladioli*	Ecuador	799	Riera-Ruiz *et al.* [33]
58.	KF669885	*B. gladioli*	Ecuador	750	Riera-Ruiz *et al.* [33]
59	M59155.2	*Hafnia alvei*	Outgroup	1488	Woese *et al.* [37]

All the nucleotide sequences of 16SrRNA gene from 7 different geographical areas were deposited in National Center for Biotechnology Information (NCBI).

Geographical Distribution of Bacterial Panicle Blight (BPB)

In this study, data samples from Western countries such as United States, Panama, Ecuador and Asian countries such as, India, China, Thailand, Korea and Malaysia were obtained from National Center for Biotechnology Information (NCBI) database and the distribution areas were plotted in Figure 1.

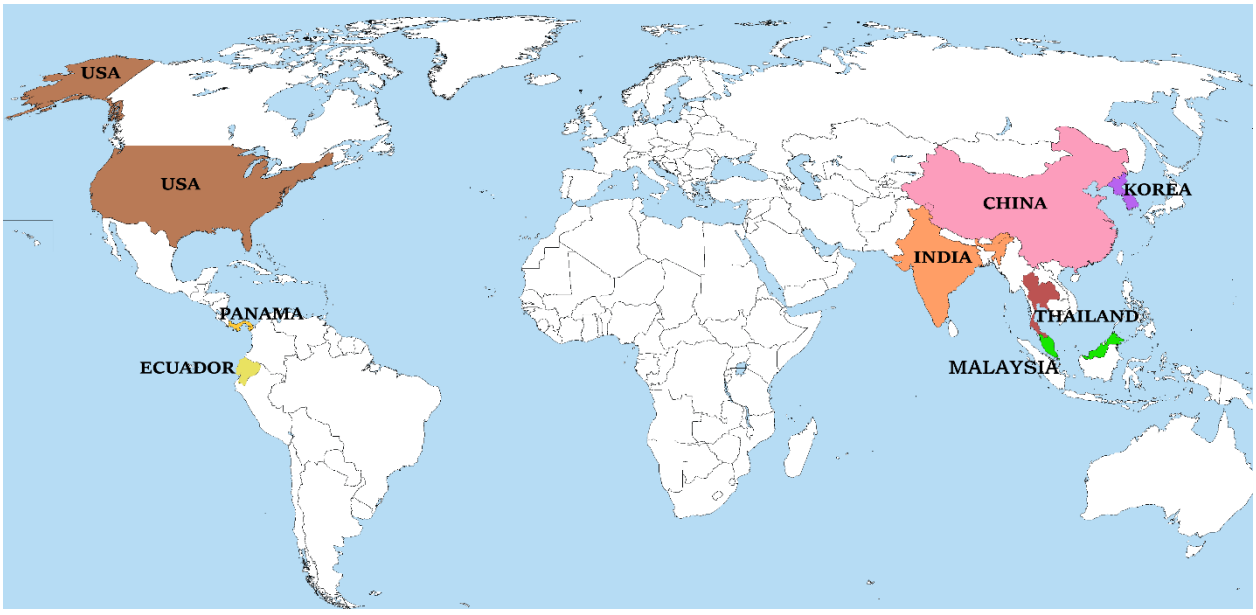

Figure 1: The distribution of bacterial panicle blight (BPB) from two different continents (Western and Asia) in this study

https://scilett-fsg.uitm.edu.my/
Vol. 16(1) JANUARY 2022

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Multiple Sequence Alignment by ClustalW Algorithm

At the beginning of alignment process, the sequences of 16S rRNA partial region were selected and aligned by ClustalW algorithm with the implementation of progressive alignment method [38]. Initial step of the progressive alignment method started with alignment of two closely related sequences of 16S rRNA gene. The next closely related 16S rRNA sequence was added afterwards to the initial paired sequence until a global alignment was produced [38]. On top of that, all the sequences were trimmed to produce similar length of DNA sequences avoiding from misleading analysis of data. The succession of DNA sequences alignment in this study was produced several outputs such as the sites of conserved regions (C), variable regions (V) and parsimony informative sites (P) of the partial region of 16S rRNA gene sequences.

Construction of Phylogenetic Tree

The best model selection for constructing the phylogenetic tree was proposed and suggested in the Best-Fit Substitution Model (ML) table in Appendix A. An automatic Neighbor-joining tree with maximum likelihood estimation method was chosen in the analysis. The analyses were conducted using the Kimura 2-parameter model and the rate variation among sites was modelled with a gamma distribution of five. All the selected 59 nucleotide sequences involved in this analysis was based on the total of 688 bp in the final dataset. Model selection analysis and evolutionary divergence estimation between the 59 sequences were used to infer the phylogenetic tree in MEGA X.

RESULTS AND DISCUSSION

The Pacific Ocean extends from the south of Antarctic zone to the north of Arctic zone and lying between Asian and Western continents on east region [39]. Although continents are separated by the Pacific Ocean, bacterial panicle blight disease is one of the major threats for rice producing countries in Western (5,994,935 km^2) and Asian (44,580,000 km^2) continents. In Indonesia, spread of BPB was stimulated by several factors such as climate, genetic change of the pathogen; *B. glumae* and seeds importing activities. However, seeds importing activities from China, India and Philippines where the BPB outbreaks occurred are the major cause for rapid dissemination of BPB in Indonesia [40]. Moreover, Paz-Carrasco *et al.* [41] confirmed that *B. glumae* became prevalence for BPB disease because they have spread to several rice producing regions and led to severe damage in El Oro, Cañar, Guayas and Los Ríos.

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In this study, 16S rRNA partial region for identification of *B. glumae* and *B. gladioli* were retrieved from public database namely National Centre for Biotechnolgy Information (NCBI). The length of aligned nucleotide sequences of *Burkholderia* spp. and the outgroup were trimmed to 688 bp as the output sequence. Several statistical attributes such as conserved sites (C), variable sites (V), parsimony informative sites (S), percentage of coverage and percentage of conservation of the aligned sequences were generated in the Sequence Data Explorer in MEGA X.

According to the result shown in Table 2, 79.6% of the conserved sites and 18.7% of the variable sites of *Burkholderia* spp. are the most important regions in this analysis because this information help to discover the similarities and differences among *Burkholderia* spp. respectively [26]. Accordingly, the variable sites percentage (18.7%) in the partial region of 16S rRNA gene within *Burkholderia* species proved that abiotic factors in eight different geographical areas may affect the nucleotide sequences in the genetic marker [42]. Parsimony informative sites in *Burkholderia* analysis comprised at least two types of DNA nucleotides and a minimum of two amino acids occurred with the lowest frequency of two [43].

In other word, parsimony in phylogeny is the simplest explanation of the evolutionary relationship among *Burkholderia* species with smallest number of changes in the nucleotide sequences and the lowest changes is presumed to be correct [44]. In studies conducted by Ki *et al.* [45], Dudu *et al.* [44] and Chen *et al.* [46], the percentage of parsimony informative sites are in the range between 8.0% to 44.2%. Unfortunately, only 15 out of 688 sites (2%) were parsimony-informative in this study. This parsimony informative site is the only approach to estimate the construction of an evolutionary tree that minimizes the steps required to produce the observed variations in the 59 data sequences.

Table 2: Percentage of aligned sequences of 16S rRNA gene of *Burkholderia* species

Regions	Percentages (%) of Sequence Data
Conserved (C)	79.6
Variables (V)	18.7
Parsimony informative (S)	2

Elucidation of Best-Fit Substitution Model

Explicit or implicit assumptions regarding to the DNA substitution process are required to perform any phylogenetic methods. Best-Fit Substitution Model was proposed by Posada and Crandall [47[40]] and they stated that it is a simple application or program used to differentiate among the DNA substitution models stored in a hierarchical hypothesis-testing framework by applying the

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likelihood ratio test calculation using X^2 distribution with q degree of freedom method. Besides, model test helps to compute the Akaike information criterion (AIC) values.

The goodness-of-fit of each suggested model is measured by the Bayesian information values (BIC) and improved by AIC values [47]. Generally, each proposed model in MEGA X provides several estimation values such as parameter of Gamma distribution (+*G*), invariant site (+I), Akaike information criterion (AIC) and Bayesian Information Criterion (BIC) [47]. Based on the best fit substitution table shown in Appendix A, the best model suggested for this study is Kimura 2-parameter using Gamma distribution (+*G*) with 5 rate categories. Invariable (+I) in K2+G+I model indicates that certain fraction of the sites is evolutionary invariable. Each proposed model and parameters are depending on the nucleotide data input.

Phylogenetic Relationship and Genetic Distance Evaluation Among and Within the Species of Burkholderia

Phylogenetic tree is a diagram that shows the evolutionary relationships between species from a particular ancestor [26]. The evolutionary history in this study was inferred by using the Neighbor-Joining method. Referring to the result of the analysis of the partial region of 16S rRNA gene of *Burkholderia* genus shown in Figure 2, all the sequences except for the *Hafnia alvei* (outgroup) are emerged from a common ancestor which proved that the 58 sequences are species from *Burkholderia* genus.

All 31 sequences of *B. glumae* species within Clade I in the phylogenetic tree were highly supported (98%) by using bootsrap method. Meanwhile, Clade II showed a cluster of *B. glumae* and *B. gladioli* with 74% bootsrap support. A phylogenetic tree that was constructed by Riera-Ruiz *et al.* [33] using MEGA 7.0 with 1000 bootstraps based on Kimura 2-parameter model depicted higher value of confidence level with *B. gladioli* within Clade II in the phylogenetic tree were species than *B. glumae* due to inhibition of colonization by *B. gladioli* in the rice samples. Besides, production of virulence factor; toxoflavin reduced the growth of the rice panicles, leaves, coleoptile and roots because the roots region composed of various sugar such as galactose, fructose, mannitol, arabitol and ribitol for bacterial growth [3].

However, partial sequence of 16S rRNA gene in this study may affect the construction of phylogenetic tree because Martínez-Porchas *et al.* [31] concluded that larger fragment of 16S rRNA gene led to higher sensitivity and sensibility as it was found to be a massive high throughput in the study. Although both species are emerged into several subclades in Figure 2, *B. glumae* and *B. gladioli* are still deposited into a similar major clade of *Burkholderia* genus. All ambiguous positions were removed for each sequence pair.

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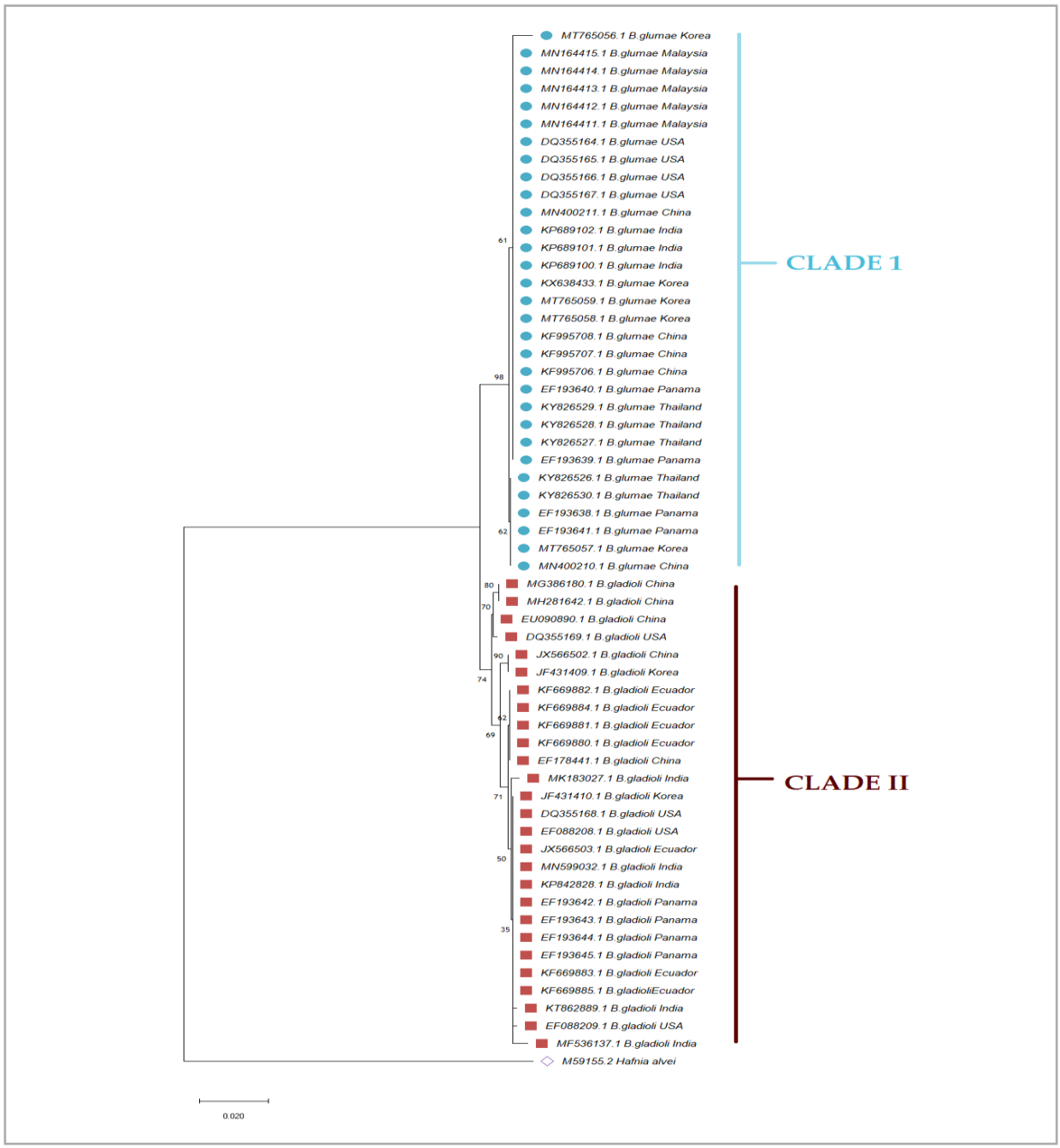

Figure 2: Phylogenetic analysis of 16S rRNA partial region in *B.glumae* and *B.gladioli* by Maximum Likelihood method to construct Neighbour-joining tree based on the Kimura 2-parameters model. A discrete Gamma distribution with five rate categories (parameter = 0.05) was used.

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The genetic diversity between *B. glumae* and *B. gladioli* can be observed in Table 3. According to the Table 3, the genetic diversity of *B. glumae* are in the range between 0.000-0.007. Meanwhile, ranges of genetic diversity of *B. gladioli* species are between 0.000-0.010. This small range of genetic diversity or genetic variation in *B. glumae* and *B. gladioli* may be only due to different abiotic factors of a specific habitat or living environment [10] as these pathogenic bacteria need to grow rapidly by adjusting their enzyme level in order to utilize any available nutrients in the surrounding to survive [48]. Bena *et al.* [49] found that dissemination of bacterial panicle blight from Asia to America caused by *B. glumae* was unrelated to the major loss of genetic diversity.

However, a genome sequence analysis was performed by Cui *et al.* [50] and they disclosed that horizontal gene transfer (HGT) by *B. glumae* plays a part in adaptation at different geographical areas. The comparative study also revealed that different niches increase diversity in *B. glumae* for bacterial survival [50]. Table 3 also depicts that within *B. gladioli* species, they are more likely to diverse than *B. glumae* species because different geographical areas affect the acclimation of bacterial adaptation for new environment [51]. A comparison of KEGG pathways was conducted by Lee *et al.* [52] in the pan-genomic study to differentiate the biological capabilities and niche adaptation by *B. gladioli* strains. As the result, they discovered that selenium from the soil was absorbed and assimilated by the plants led to homeostasis of important nutrients, photosynthesis, stress tolerance and stimulation of plant growth.

However, selenium and selenoamino acids molecules were required as the substrate for enzymatic activities in plants and pathogenic *B. gladioli* as well. Therefore, a competition between rice plants and pathogen for the limited source of selenium was taken place by two organisms for their survival. Consequently, the pan-genome study provides the illustration on the incidence of gene content versatility in *B.gladioli* due to adaptation at different geographical areas [52]. Besides, endophytic bacteria also able to manipulate the functional traits in their genome enabling for an interaction with the plant host which help the pathogen to mitigate from the adverse condition [53]. Moreover, a comparative genomic study performed by Seo *et al.* [54] shows that two different strains of *B. glumae* from different geographical areas led to genomic variation and genetic difference among the pathogenic *B. glumae* and *B. gladioli*. On top of that, the rearrangement and development of a particular system in *B. glumae* due to evolutionary changes were determined as a virulence-related feature in the pathogenic *B. glumae* which allows the bacteria broaden their host range and produce interaction with the host [55].

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ISSN: 1675-7785
eISSN: 2682-8626

DOI: 10.24191/sl.v16i1.15582

Table 3: Pairwise comparison between individual of 16S rRNA gene based on Kimura-2-parameter among *B. glumae* and *B. gladioli*

No	Species	1	2	3
1	*B. glumae*	0.000-0.007		
2	*B. gladioli*	0.015-0.024	0.000-0.010	
3	*Hafnia alvei*	0.196-0.204	0.192-0.200	0.000-0.000

Results Validation

The result validation was conducted to evaluate the accuracy of the phylogenetic tree which mainly depends on the quality of aligned sequence, consistency and efficiency of preferred method in phylogenetic reconstruction and the genetic diversity in this study. Thus, DAMBE software aids in plotting the transition and transversion number of the observed aligned sequence versus the divergence [56]. As referring to the Figure 3, all the 59 sequences do not experience any substitution saturation due to the highly conserved region of 16S rRNA gene. Besides, the directly proportional graph proves that the input nucleotide sequences data are appropriately aligned. In general, if there is presence of significant saturated nucleotide sequence, it will be excluded from further analysis to avoid from any incorrect phylogenetic tree's reconstruction [56].

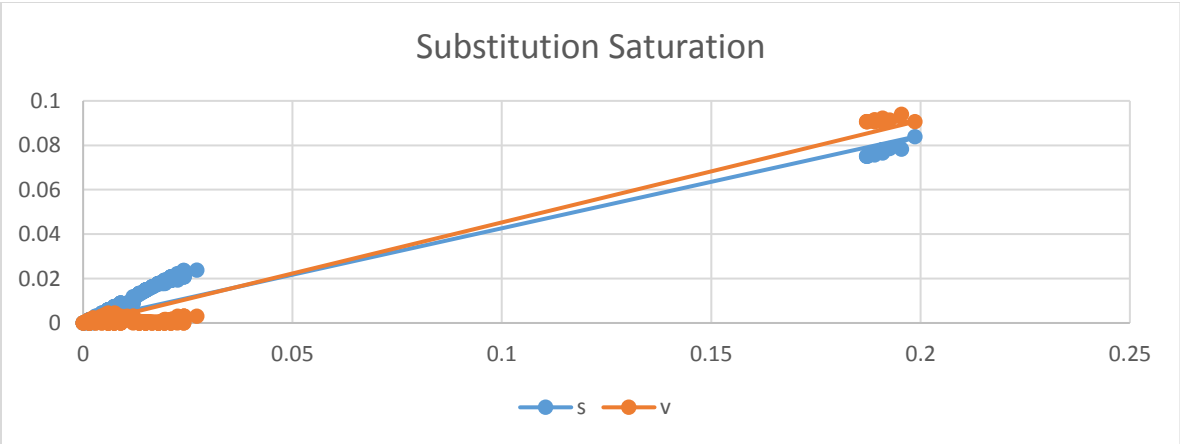

Figure 3: Saturation substitution graph indicates the transversion and transmission in the sequence data

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Vol. 16(1) JANUARY 2022

ISSN: 1675-7785
eISSN: 2682-8626

DOI: 10.24191/sl.v16i1.15582

In addition, Automatic Barcode Gap Discovery (ABGD) software was utilized as a secondary tool to validate data in this study. ABGD was introduced with an automatic barcode gap detection procedure to perform partition on the candidates of the data. Initially, the software works by running the data to produce a model-based one-sided confidence limit for detection of intraspecific divergence. After that, barcode gap is identified as the initial significant gap to perform partition to the data sequence. The gap and inference of the limit are detected, and these data are recursively applied to the data sequence to obtain more accurate partitions until no further partitioning step can be performed [57].

Result from ABGD software is depicted in Figure 4 to observe the intraspecific divergence. In a study conducted by Puillandre *et al.* [57], they stated that the ranges of intraspecific divergence for species delimitation is between one percent until three percent. Unfortunately, the divergence of *B. glumae* and *B. gladioli* based on the partial region of 16S rRNA gene are undisclosed as the result of ABGD software shows only two groups are present in the analysis and it is contradicted with the result from previous phylogenetic tree which depicts the presence of three species in the study. This is because the highly conserved region of 16S rRNA gene causes only a slightly diverged regions in both *Burkholderia* species and eventually this small divergence is undetected in the ABGD software [58,59].

The discovery of this study will redound to the advantages of community considering that identification of *Burkholderia* spp. plays a vital role in managing the rice crop. Rice is a staple food for million peoples in the world particularly in Asian countries. The evolution of a species and identification of the ancestor for a newly-found species can be determined by researcher based on the phylogenetic trees. On top of that, phylogenetic tree also provides the general principle of species evolution which allows scientist to predict the transformation or changes of a particular species in future. Therefore, this approach helps researches to further their study in a new-upcoming pathogen outbreak. As the consequence, government can recommend the amendment of public health policy to avoid any outbreaks occurrence.

Other than that, study of variation of *Burkholderi*a species in rice panicle blight allows the scientists to figure out the ability of another bacteria that is non-pathogenic to plants, environments and humans that act as biological agents against the pathogenic *B. glumae* and *B. gladioli*. Finding an appropriate biological agent against *Burkholderia* pathogens will result in reduced chemical agents' dependence and minimize the farmers from exposure to hazardous chemical agents. Eventually, water pollution also can be reduced. Moreover, this research may help the scientists to unclose the resistance gene in the rice towards pathogenic *Burkholderia* and increase rice crop productivity. The improvement in rice crop productivity leads to variation in rice production. Thus, demand for good quality of rice crop will also rise. As the result, government can ensure the continuance of economic growth as the demand for good quality of rice crop elevates.

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Vol. 16(1) JANUARY 2022
ISSN: 1675-7785
eISSN: 2682-8626

DOI: 10.24191/sl.v16i1.15582

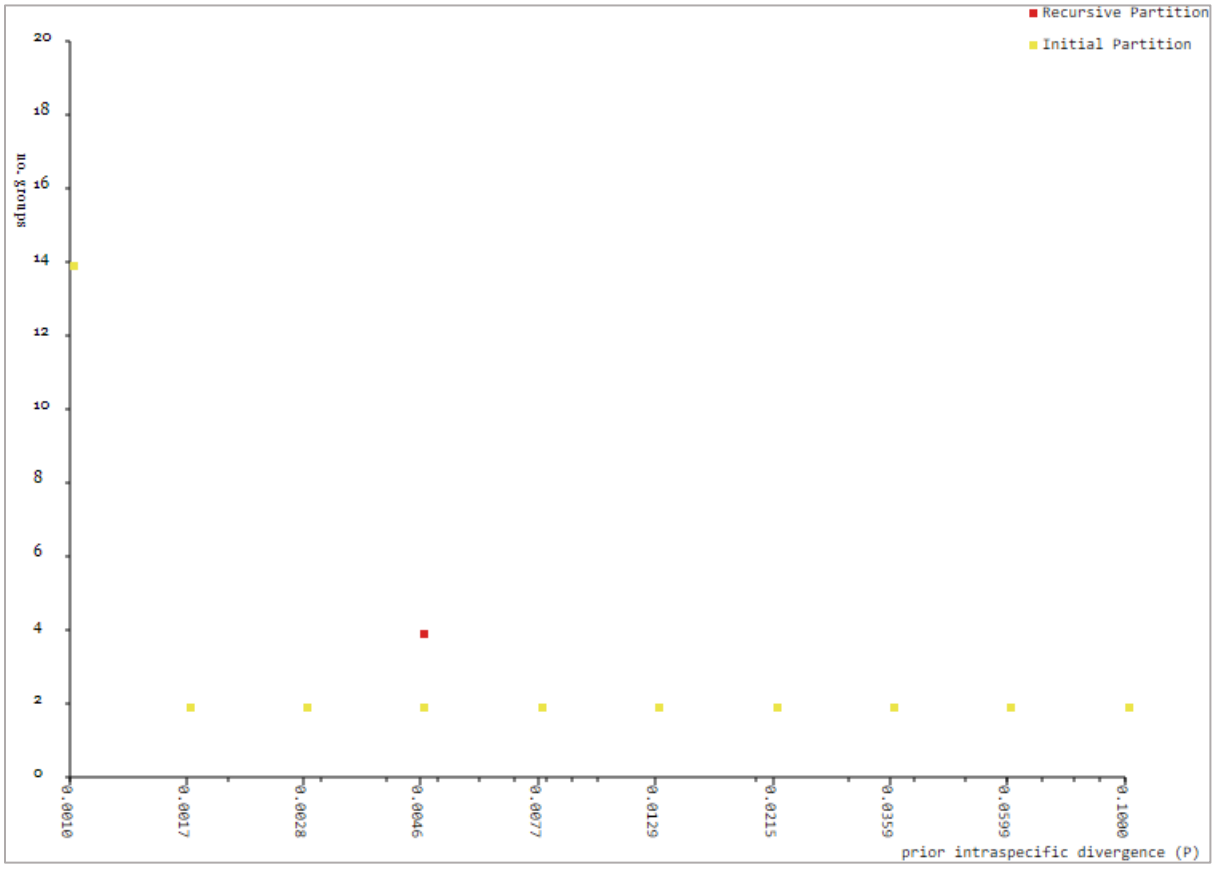

Figure 4: Intraspecific divergence based on the partial region of 16S rRNA gene in *B. glumae* and *B. gladioli* were unable to be detected in the ABGD software

CONCLUSION

16S rRNA gene is a highly conserved region in prokaryotic organisms and this genetic marker is a very good recommendation to be used in distinguishing the non-closely related bacterial species. A good genetic marker should produce similar data output as evidence for a particular study when it is performed with several different bioinformatics software. Unfortunately, this study shows that 16S rRNA gene is an inappropriate genetic marker to differentiate the closely related species bacteria from similar genus. Based on the results obtained in above, the divergence between both *B. glumae* and *B. gladioli* can only be observed through the phylogenetic tree and the genetic distance table obtained from MEGAX. However, the data sequences were unpredicted in ABGD software. *B. glumae* species within Clade I in the phylogenetic tree were highly supported (98%)

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Vol. 16(1) JANUARY 2022

ISSN: 1675-7785
eISSN: 2682-8626

DOI: 10.24191/sl.v16i1.15582

and it depicts that 16S rRNA gene could be used as a good genetic marker to differentiate among the individuals. Whereas, a new genetic marker to differentiate the *B. gladioli* species needs to be identified because *B. gladioli* are grouped within Clade II in the phylogenetic tree with only 74% of the confidence level. The limitation of this study is the 16S rRNA gene is not workable for *Burkholderia plantarii* that can also act as rice pathogen. We recommend the 16S – 23S rDNA internal transcribed spacer (ITS) is another good genetic marker that can be used to differentiate closely-related species bacteria as its efficiency to identify *B. glumae* and *B. gladioli* was proven in few studies.

ACKNOWLEDGMENTS

We would like to acknowledge the support of Faculty of Applied Sciences, Universiti Teknologi MARA, Shah Alam, Selangor, Malaysia for providing the facilities support on this research.

AUTHOR'S CONTRIBUTION

All the authors have reviewed the research articles, analyzed the data, wrote, and revised the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors agree that this research was conducted in the absence of any self-benefits, commercial or financial conflicts and declare absence of conflicting interests with the funders.

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eISSN: 2682-8626

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eISSN: 2682-8626

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APPENDIX

I. Appendix A – Best-Fit Substitution Model; Kimura 2-parameter model with Gamma distribution with 5 rate categories was chosen.

Table. Maximum Likelihood fits of 24 different nucleotide substitution models

Model	Parameters	BIC	AICc	*lnL*	(+*I*)	(+*G*)	*R*	*f*(A)	*f*(T)	*f*(C)	*f*(G)	*r*(AT)	*r*(AC)	*r*(AG)	*r*(TA)	*r*(TC)	*r*(TG)	*r*(CA)	*r*(CT)	*r*(CG)	*r*(GA)	*r*(GT)	*r*(GC)
K2	116	4378.919	3382.557	-1574.938	n/a	n/a	1.22	0.250	0.250	0.250	0.250	0.056	0.056	0.137	0.056	0.137	0.056	0.056	0.137	0.056	0.137	0.056	0.056
K2+I	117	4383.692	3378.746	-1572.026	0.57	n/a	1.49	0.250	0.250	0.250	0.250	0.050	0.050	0.150	0.050	0.150	0.050	0.050	0.150	0.050	0.150	0.050	0.050
K2+G	117	4386.177	3381.231	-1573.269	n/a	0.38	1.55	0.250	0.250	0.250	0.250	0.049	0.049	0.152	0.049	0.152	0.049	0.049	0.152	0.049	0.152	0.049	0.049
T92	117	4387.227	3382.281	-1573.794	n/a	n/a	1.22	0.231	0.231	0.269	0.269	0.052	0.060	0.148	0.052	0.148	0.060	0.052	0.127	0.060	0.127	0.052	0.060
JC	115	4391.561	3403.782	-1586.556	n/a	n/a	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
T92+I	118	4392.301	3378.772	-1571.033	0.57	n/a	1.49	0.231	0.231	0.269	0.269	0.046	0.054	0.161	0.046	0.161	0.054	0.046	0.139	0.054	0.139	0.046	0.054
K2+G+I	118	4393.175	3379.646	-1571.470	0.30	0.69	1.60	0.250	0.250	0.250	0.250	0.048	0.048	0.154	0.048	0.154	0.048	0.048	0.154	0.048	0.154	0.048	0.048
T92+G	118	4394.766	3381.237	-1572.266	n/a	0.40	1.52	0.231	0.231	0.269	0.269	0.046	0.053	0.162	0.046	0.162	0.053	0.046	0.140	0.053	0.140	0.046	0.053
HKY	119	4398.836	3376.723	-1569.003	n/a	n/a	1.21	0.204	0.259	0.312	0.225	0.059	0.072	0.122	0.047	0.168	0.052	0.047	0.140	0.052	0.110	0.059	0.072
JC+I	116	4399.247	3402.884	-1585.101	0.44	n/a	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
HKY+I	120	4400.133	3369.437	-1564.354	0.58	n/a	1.53	0.204	0.259	0.312	0.225	0.052	0.063	0.135	0.041	0.186	0.045	0.041	0.154	0.045	0.122	0.052	0.063
T92+G+I	119	4401.810	3379.698	-1570.490	0.29	0.67	1.61	0.231	0.231	0.269	0.269	0.044	0.051	0.166	0.044	0.166	0.051	0.044	0.143	0.051	0.143	0.044	0.051
JC+G	116	4402.145	3405.782	-1586.550	n/a	0.77	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
HKY+G	120	4402.635	3371.939	-1565.605	n/a	0.37	1.57	0.204	0.259	0.312	0.225	0.051	0.062	0.136	0.041	0.188	0.045	0.041	0.156	0.045	0.123	0.051	0.062
TN93	120	4406.408	3375.712	-1567.491	n/a	n/a	1.22	0.204	0.259	0.312	0.225	0.058	0.070	0.155	0.046	0.138	0.051	0.046	0.115	0.051	0.140	0.058	0.070
TN93+I	121	4408.005	3368.726	-1562.992	0.57	n/a	1.49	0.204	0.259	0.312	0.225	0.052	0.062	0.176	0.041	0.144	0.045	0.041	0.119	0.045	0.160	0.052	0.062
JC+G+I	117	4409.232	3404.286	-1584.796	0.02	0.81	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
HKY+G+I	121	4409.747	3370.468	-1563.863	0.34	0.64	1.72	0.204	0.259	0.312	0.225	0.049	0.058	0.141	0.038	0.195	0.042	0.038	0.161	0.042	0.128	0.049	0.058
TN93+G	121	4410.579	3371.300	-1564.279	n/a	0.40	1.52	0.204	0.259	0.312	0.225	0.051	0.062	0.177	0.040	0.146	0.045	0.040	0.121	0.045	0.160	0.051	0.062
TN93+G+I	122	4417.630	3369.768	-1562.507	0.25	0.68	1.55	0.204	0.259	0.312	0.225	0.051	0.061	0.178	0.040	0.147	0.044	0.040	0.122	0.044	0.161	0.051	0.061
GTR	123	4432.672	3376.228	-1564.731	n/a	n/a	1.22	0.204	0.259	0.312	0.225	0.062	0.091	0.155	0.049	0.138	0.048	0.060	0.114	0.037	0.140	0.055	0.052
GTR+G	124	4437.469	3372.442	-1561.832	n/a	0.47	1.47	0.204	0.259	0.312	0.225	0.055	0.084	0.173	0.044	0.144	0.042	0.055	0.120	0.032	0.157	0.048	0.044
GTR+I	124	4438.337	3373.309	-1562.265	0.53	n/a	1.42	0.204	0.259	0.312	0.225	0.055	0.087	0.171	0.044	0.142	0.044	0.057	0.118	0.032	0.155	0.051	0.045
GTR+G+I	125	4448.028	3374.418	-1561.813	0.19	0.70	1.49	0.204	0.259	0.312	0.225	0.055	0.084	0.174	0.044	0.145	0.042	0.055	0.120	0.032	0.158	0.048	0.044