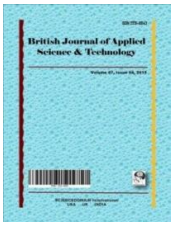

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Classical Identification, 16S rDNA Sequencing, and Molecular Characterization of *Bacillus* species from Convenience Foods

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Identification of microorganisms is central to the study of microbiology at all levels of research. The methods employed are also important. It is however very pertinent that scientists the world over continuously improve on the method of microbial identification for greater efficiency. A study was conducted to isolate and identify *Bacillus* species from some ready-to-eat (RTE)/convenience food samples. The *Bacillus* species isolated were identified by using the classical method. The same isolates were further identified via use of the 16SrDNA sequencing method.The classical method identified all bacilli isolates as members of a precise species in the genus *Bacillus*, but with discrepancies observed in 3 out of 9 identified cases (33.3%) when comparison was made with PCR/sequencing method. PCR/sequencing method provided results which were in accordance with both classical and genotypic identification in more than 70% of cases.This study emphasized the presumptive nature of classical methods in identifying *Bacillus* species/strains, without further recourse to the use of more sensitive and molecular methods. Identities from the PCR method hold greatest sway and are regarded as most reliable as it involves the analysis of genetic sequences of this group of microorganisms.

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Keywords: Classical; identification; Bacillus; convenience foods; PCR.

1. INTRODUCTION

The genus *Bacillus* currently comprises in excess of 60 species, commonly found in the environment and as laboratory contaminants [1]. *Bacillus* species are Gram positive rods often arranged in pairs or chains with rounded or square ends and usually have single endospore. The endospores are generally oval and are very resistant to adverse conditions. Sporulation is not repressed by exposure to air. Many *Bacillus* species are haemolytic, a useful characteristic in differentiating them from *Bacillus anthracis* (which is non-haemolytic). They are aerobic or facultatively anaerobic and most species are motile (a notable exception is *B. anthracis*) by peritrichous flagella. Most species are oxidase positive, which may lead to confusion with *Pseudomonas* species, especially if the *Bacillus* species are poorly stained. They are usually catalase positive and metabolize carbohydrates by fermentation. Significant isolates should usually be referred to a reference laboratory for confirmation of identity and toxin testing [1].

Dairy products, fatty foods, bread, cakes and pastries, seafood can easily be contaminated with *Bacillus* spp*. Bacillus cereus* (*B. cereus*) can cause food poisoning resulting in gastroenteritis. *Bacillus* species are ubiquitous in nature and can be found in soil or in a variety of dried foods such as grains, legumes, starches and spices as vegetative cells and endospores [2]. Based on the report of European Food Safety Agency [3], 1–33% of food borne poisonings is caused by *B. cereus*. Food poisoning is caused by presence of bacteria in food due to improper food preparation or cooking process, and exposure of food to temperatures of 30°C. Common food poisonings are usually mild, but deaths due to food poisoning are also reported. Food poisoning occurs within 48 hours after consumption of contaminated food or drink [4]. Some of *B. cereus* outbreaks are under reported as the illness associated with these bacteria limit itself and do not become severe. A recent survey on culture practices for outbreaks of apparent food borne illness showed that 20% of state public health laboratories do not make *B. cereus* testing routinely available. The survey also found that most of food handlers (in food stalls and restaurants) were unaware that cooked rice was a potentially hazardous food [5].

Gram positive and aerobic spore-forming bacilli belonging to the genus *Bacillus*, and other related species play important roles in food poisoning and spoilage. There is however some difficulty due to the lack of standard methods for identification of members of *Bacillus* species in food testing laboratories [6]. Species differentiation of the genus is complex, and in some instances in routine laboratories, a combination of Gram stain and colonial appearance may be regarded as sufficient indication of a *Bacillus* species being present in a clinical sample. Although the use of morphological and physiological tests have provided the best means available for laboratories to identify microorganisms, these methods have proven to be quite laborious, inconsistent and generally unreliable for this group of microorganisms (ibid). As a result of the absence of reliable and standardized methods for the identification of this group of microorganisms, investigators have generally focused on the isolation and identification of *Bacillus cereus* as a causative agent of food related illnesses. However, numerous other investigations have demonstrated that a considerably larger range of species can cause food related illnesses [7,8].

Prevailing neglect of *Bacillus* identification may be attributed to two factors. First is the diagnostic test used. Many of the classical tests for *Bacillus* species described by Gordon et al. [9] required special, selective/differential media. These are very time consuming and expensive to prepare. Many of these media have short shelf lives resulting in considerable wastage if their use is infrequent. The requirement for media containing unusual ingredients increased the familiar problem of test standardization [10]; and inconsistent results may be obtained in consequence. Any new scheme for *Bacillus* identification should therefore use widely available and standardized materials for performing a good number of rapid tests which give reproducible results. The second factor leading to neglect of *Bacillus* identification is the character of the genus. *Bacillus* is an unusually wide taxon which contains most aerobic endospore-forming rods. In terms of deoxyribonucleic acid (DNA) base ratios it is the equivalent of some bacteria families [11]. Furthermore, some species are ill-defined, existing with closely related species as complexes or in which the boundary of a

particular species is difficult or impossible to identify. Members of the *Bacillus cereus* group usually include *B. cereus*, *B. thuringiensis*, *B. mycoides*, and *B. anthracis* [12]. *Bacillus pseudomycoides* and the psychrotolerant *Bacillus weihenstephanensis* have also been included into this group. *B. cereus* and *B. thuringiensis* are closely related and genomic studies have proposed that they should be merged into a single species [13]. On the other hand, *Bacillus subtilis* group usually include *B. subtilis*, *B. licheniformis, B. pumilis* and *B. amyloliquifaciens* [14]. Even in well established species there is considerable variation between strains. Thus, classical test schemes using few characters often do not permit identification of atypical and intermediate strains and in spite of the excellent work of Gordon and her colleagues in 1973, as well as others, it is widely agreed that there is considerable room for improvement in the taxonomy of the genus and that a study of new isolates, particularly, is important.

There has been very little study on the isolation and molecular characterization of *B. cereus* and other *Bacillus* species in ready-to-eat (RTE) rice products and pastries (farinaceous products) in Nigeria. The aim of this study was to isolate and carry out classical identification and molecular characterization [using 16S ribosomal (ribos) universal primers] of *B. cereus* and other *Bacillus* species from some ready-to-eat food samples. Identification and classification of *Bacillus* species is highly presumptive under the classical scheme, and this group of microorganisms usually require more sensitive methods for detection and characterization to strain level.

This paper aimed at providing initial data which would encourage the use of molecular identification methods in the assay for *Bacillus* species especially in developing countries. It is also the hope that test for presence of *Bacillus* species would be included in routine food test for RTEs.

2. METHODOLOGY

2.1 Sample Collection

Sample collection was carried out according to the methods of Cheesbrough [15] and Fawole and Oso [16]. A total of sixty RTE food samples (10 samples each of 3 different types of pastry products – Egg roll, Meat pie, and Buns; 10 each of different rice products – White rice, Jollof rice, and Fried rice) were purchased from different food vending sites and cafeterias within a period of ten weeks. Food samples purchased were appropriately labelled and transferred to the laboratory for immediate analysis.

2.2 Growth and Isolation of Bacterial Cultures

Bacillus bacteria were isolated from convenience food samples using the serial dilution technique with pour plating into Nutrient agar (NA); and also with spread plating unto HiCrome *Bacillus* agar (HiMedia), a selective/differential isolation media, for assessment of *Bacillus* species. The fourth dilution was used for plating unto these media. Culture media were prepared according to manufacturer's specification and materials sterilized in an autoclave at 121°C for 15 minutes.

2.3 Preservation of Bacteria Isolates

Discrete colonies isolated and purified by repeated sub-culturing were preserved according to Olutiola et al. [17], on slants at 4°C for further characterization.

2.4 Classical Identification

Morphological and biochemical tests to identify isolates were carried out using the methods of Fawole and Oso [16] and Bergey's Manual of Systematic Bacteriology [18]. Some of the biochemical tests carried out in the classical/conventional method includes ability of microbial isolates to ferment carbohydrates (glucose, lactose, mannitol, galactose etc.); as well as oxidase, citrate, indole, catalase, motility, and starch hydrolysis tests.

2.5 Molecular Characterization of Bacilli Isolates

2.5.1 Isolation of genomic DNA from bacteria

DNA was extracted from 1 ml of bacterial culture. The culture was pelleted by centrifuging at 12,000 rpm for 5 min. Pellets were then treated with lysis buffer and protease enzyme and incubated at 65°C for 1 hr. Nucleic acids were precipitated with isopropanol by centrifuging at 10,000 rpm for 10 min, washed with 1 ml of 70% ethanol solution and dissolved in 0.1 ml of TE buffer. The purity and quantity of DNA were examined by recording its UV absorption spectrum and running on 1% agarose gel electrophoresis.

2.5.2 Sequence determination of 16S rDNA

The DNA isolated was amplified using 16S rDNA universal ribose primers and sequenced for the identification of *Bacillus* species at molecular level. Amplification of the PCR products of expected size was confirmed by electrophoresis. The sequence of the 16S rDNA was determined with a Dye terminator sequencing kit (Applied Biosystems), and the product was analyzed with an ABI Prism DNA sequencer. The gene sequences obtained in this study were compared with known 16SrDNA gene sequences in the National Centre for Biotechnology Information (NCBI) GenBank database.

3. RESULTS

Plate 1 showed the electrophorogram from separated and amplified DNA bands from DNA extracts from RTE *Bacillus* isolates. The morphological and biochemical characteristics of *Bacillus* isolates under the classical identification scheme were shown in Table 1. Table 2 showed the bacilli microorganisms identified using the classical method of identification. A comparison of microbial isolates identified by this method and the PCR/sequencing method was also depicted. Only the Ribos (ribosomal) C microorganism was not recognised as a member of the *Bacillus* genus via the PCR method. Figs. 1-8 showed the relationship between the different species of the *Bacillus* genus using the BioPython programming language. Fig. 7 showed the closest relationship between the genomic relations, while Fig. 2 showed the least relationship. Fig. 9 showed the genetic relatedness of *Bacillus* species isolated from ready-to-eat (RTE) foods, while Fig. 10 better depicted the phyletic relationships between isolated *Bacillus* species and NCBI database closely related sequences.

4. DISCUSSION

In the classical identification approach; appropriate growth characteristics, colonial appearance and Gram stain of the culture, if demonstrated, covers for presumptive identification. However, it had been recommended that confirmation of identity requires that more specific tests be done and commercial identification kit results and/or the reference laboratory reports be cross referenced [1]. HiCrome *Bacillus* agar is a chromogenic medium used in this study, and is useful for detection of β-glucosidase activity of *Bacillus* microorganisms [19]. Tallent et al. [20] also looked at the characteristic appearance of these *Bacillus* microbes on this and other chromogenic media. It is worthy of note that while the classical/conventional method identified all microbial isolates as a member of a particular species in the *Bacillus* genus, in about 33% (3 of 9 identified cases, that is with ribos B, C and G) a discordance was observed. The ribos C microbe was identified as *B. subtilis* in the classical method, but as a member of the *Comamonas* sp. using the PCR method. Also, the ribos B microorganism was identified as *B. cereus* under the classical scheme, but as *B. licheniformis* strain under the PCR. This once again reiterates the presumptive nature of identifying members of this genus using morphological and biochemical characteristics only, and without recourse to the utilization of more recent, advanced and sensitive molecular methods [21,1]. However, the PCR method provided results in agreement with the phenotypic, classical and genotypic identification more than 70% of the time. This method seemed to be more sensitive and precise in the identification of *Bacillus* species/strains. This aligns with the submission of Manzano et al. [22].

The *Bacillus* genus comprises a heterogeneous group whose taxonomic rearrangement at the generic level have led to splitting of the genus, a process that is still on-going. This makes the genus an interesting group of bacteria for taxonomic and identification studies. According to the 'List of Prokaryotic Names with Standing in Nomenclature' 143 unique and valid *Bacillus* species are currently described [23]. As approved by the ad hoc committee for the re-evaluation of the species definition in bacteriology, the current definition for a bacterial species is 'a category that circumscribes a (preferably) genomically coherent group of individual isolates/strains sharing a high degree of similarity in (many) independent features, comparatively tested under highly standardized conditions'. The committee concluded that the standard for species delineation is a DNA-relatedness as measured by DNA-DNA hybridization of 70% or more [24]. It should however be noted that species with a DNA-relatedness of more than 70% usually also have a 16S rRNA sequence similarity of more than 97%. Nonetheless, researchers have stated that bacterial taxa should be delineated polyphasically through a consensus based on both phenotypic and genotypic information.

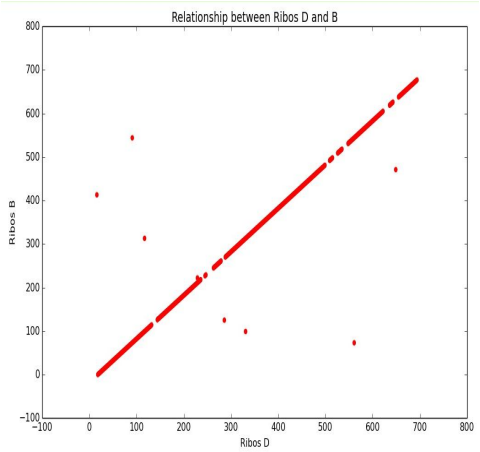

Fig. 1. DNA sequence relations between Ribos D and B isolates

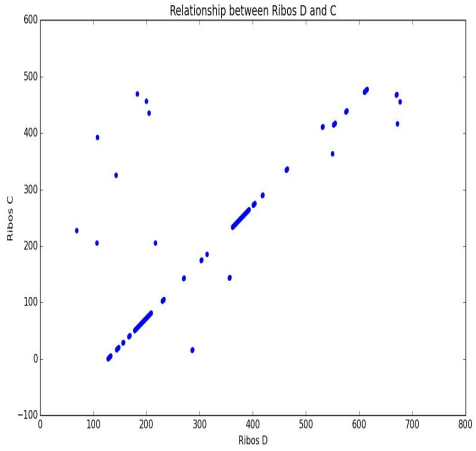

Fig. 2. DNA sequence relations between Ribos D and C isolates

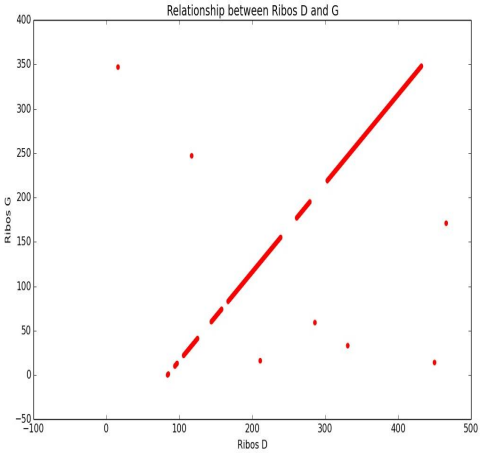

Fig. 3. DNA sequence relations between Ribos D and G isolates

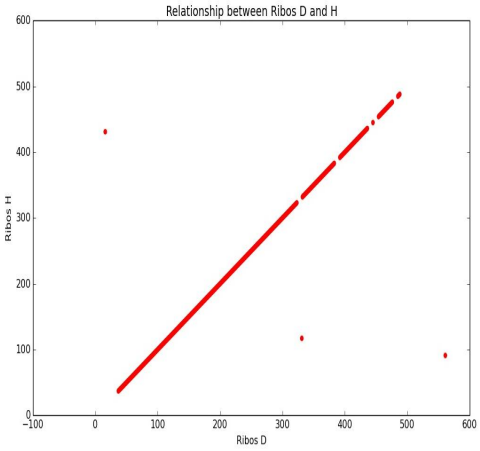

Fig. 4. DNA sequence relations between Ribos D and H isolates

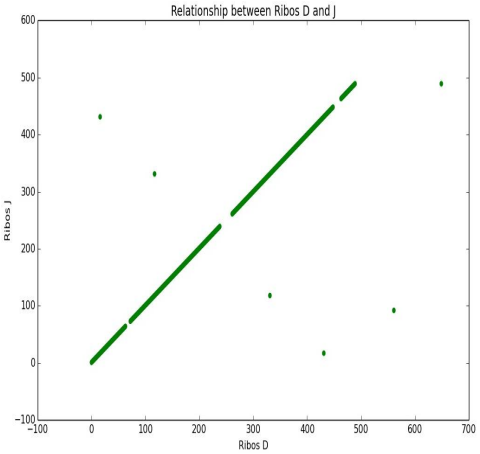

Fig. 5. DNA sequence relations between Ribos D and J isolates

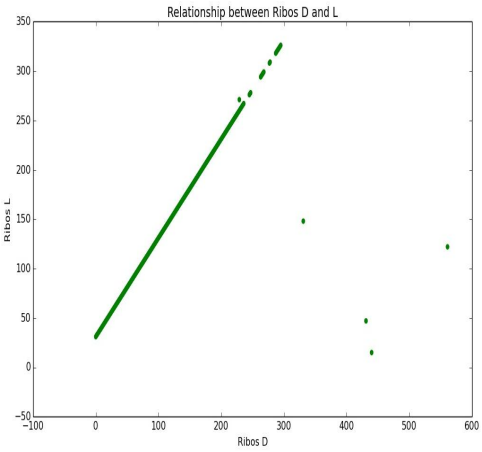

Fig. 6. DNA sequence relations between Ribos D and L isolates

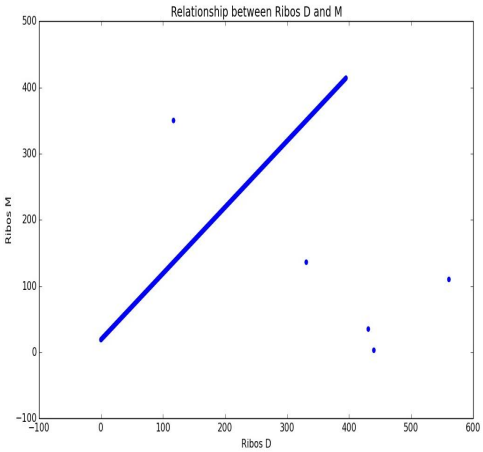

Fig. 7. DNA sequence relations between Ribos D and M isolates

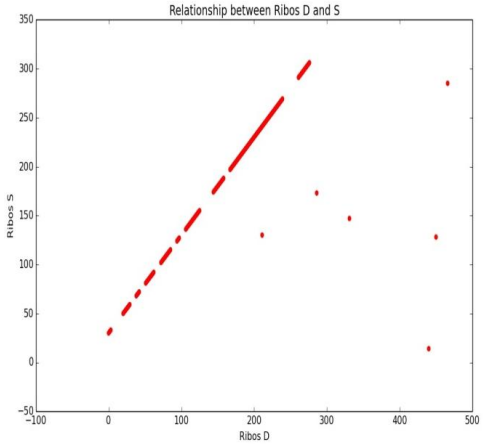

Fig. 8. DNA sequence relations between Ribos D and S isolates

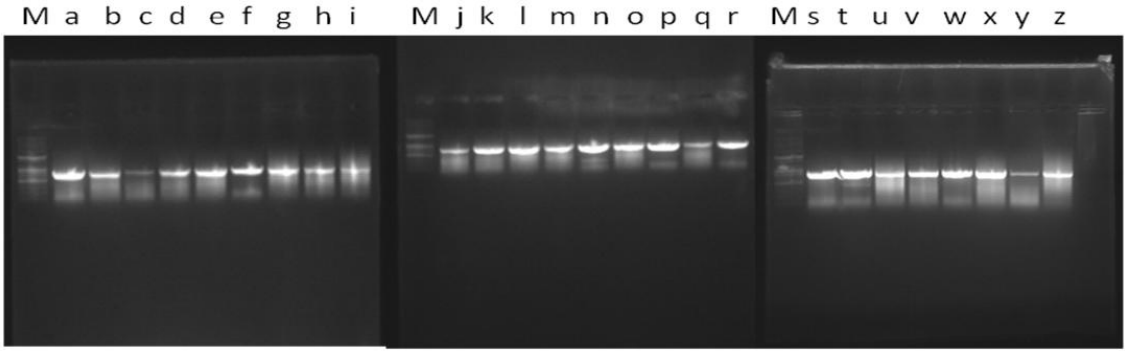

Plate 1. Separated and amplified DNA bands from gel electrophoresis
***Key**: M = 1kilobase pair (1 Kbp) molecular marker; a – z = separated DNA bands for Bacillus isolates*

In this study, we have been able to establish some genomic relations between a genus of different species of microorganisms using the BioPython programming language. Having compared the sequences of the species, it was illustrated that a close relationship exists between the different species of the *Bacillus* genus (Figs. 1-8). Comparison of 16S rDNA sequences between the isolated bacteria showed that species were very homogeneous, with very few nucleotide differences. This finding suggests that colonization of sample sites were most probably from a common source. This may have occurred from the food handlers or as environmental contaminants from where these foods were prepared. This also points to the ubiquity of members of this genus and their ability to survive in a wide range of environments. Phylogenetic analysis based on 16S rDNA sequences indicated that most of the isolated bacteria belonged to the genus *Bacillus*. Ribos D (*Bacillus atrophaeus*) cluster with Ribos M (Uncultured *Bacillus* sp.) isolate, indicating a close genotypic relationship between the two *Bacillus* isolates. Ribos S (*Bacillus cereus*) and Ribos C (*Comamonas* sp.) isolates branched off farthest, with least genetic relatedness. Both microorganisms were unrelated, isolated from different sources and belong to different genus. All bacilli isolates branch of separately from a single taxa as different species of different strains within a group of the *Bacillus* genus, indicating singularity in identities which may be based on difference(s) on only one or more characteristics biochemically and/or genetically as opined by Khataminezhad et al. [25,26], and Amin et al. [27]. The NCBI database sequence that clustered most closely to the original isolate nucleotide sequence was taken as the best identity for the RTE food isolates as extracted in Table 2 from Fig. 10.

Table 1. Morphological and biochemical characteristics of *Bacilli* isolates

S/N	Gram Rxn	Morph.	Catalase	Oxidase	Indole	Motility	MR.	VP.	Citrate	Starch	Gelatin	Spore	Glucose	Lactose	Sucrose	Maltose	Mannitol	Galactose	Probable identity
1	+	R	+	+	-	+	-	+	+	+	+	+	+	+	+	+	-	+	*Bacillus cereus*
2	+	R	+	+	-	+	-	+	+	+	+	+	+	+	+	-	+	+	*Bacillus subtilis*
3	+	R	+	+	-	+	-	+	-	-	+	+	+	-	-	-	+	-	*Bacillus sphaericus*
4	+	R	+	-	-	+	-	+	+	+	+	+	+	-	+	+	+	-	*Bacillus licheniformis*
5	+	R	+	+	-	+	-	+	+	+	+	+	+	+	+	-	-	+	*Bacillus amyloliquefaciens*
6	+	R	+	+	-	-	-	+	-	+	+	+	+	+	+	-	+	+	*Bacillus mycoides*

***Key**: - = Negative reaction, + = Positive reaction, Gram Rxn= Gram reaction, Morph. = cell morphology, Methyl = Methyl red, VP = Voges Proskauer, R = Rods*

Table 2. Identification of *Bacillus* species by classical method and 16S rDNA sequencing

Isolate label	Sample source	Identity by classical	Identity by 16S sequencing
Ribos B	Buns	*Bacillus cereus*	*Bacillus licheniformis*
Ribos C	White rice	*Bacillus subtilis*	*Comamonas* sp.
Ribos D	Egg roll	*Bacillus sphaericus*	*Bacillus atrophaeus*
Ribos G	Meat pie	*Bacillus licheniformis*	*Bacillus thuringiensis*
Ribos H	Egg roll	*Bacillus subtilis*	*Bacillus amyloliquefaciens*
Ribos J	Fried rice	*Bacillus subtilis*	*Bacillus safensis*
Ribos L	Meat pie	*Bacillus amyloliquefaciens*	*Bacillus subtilis*
Ribos M	Buns	*Bacillus mycoides*	Uncultured *Bacillus* sp. clone
Ribos S	Jollof rice	*Bacillus cereus*	*Bacillus cereus*

Key: Ribos = relates to the type of primer (ribosomal) used in DNA amplification

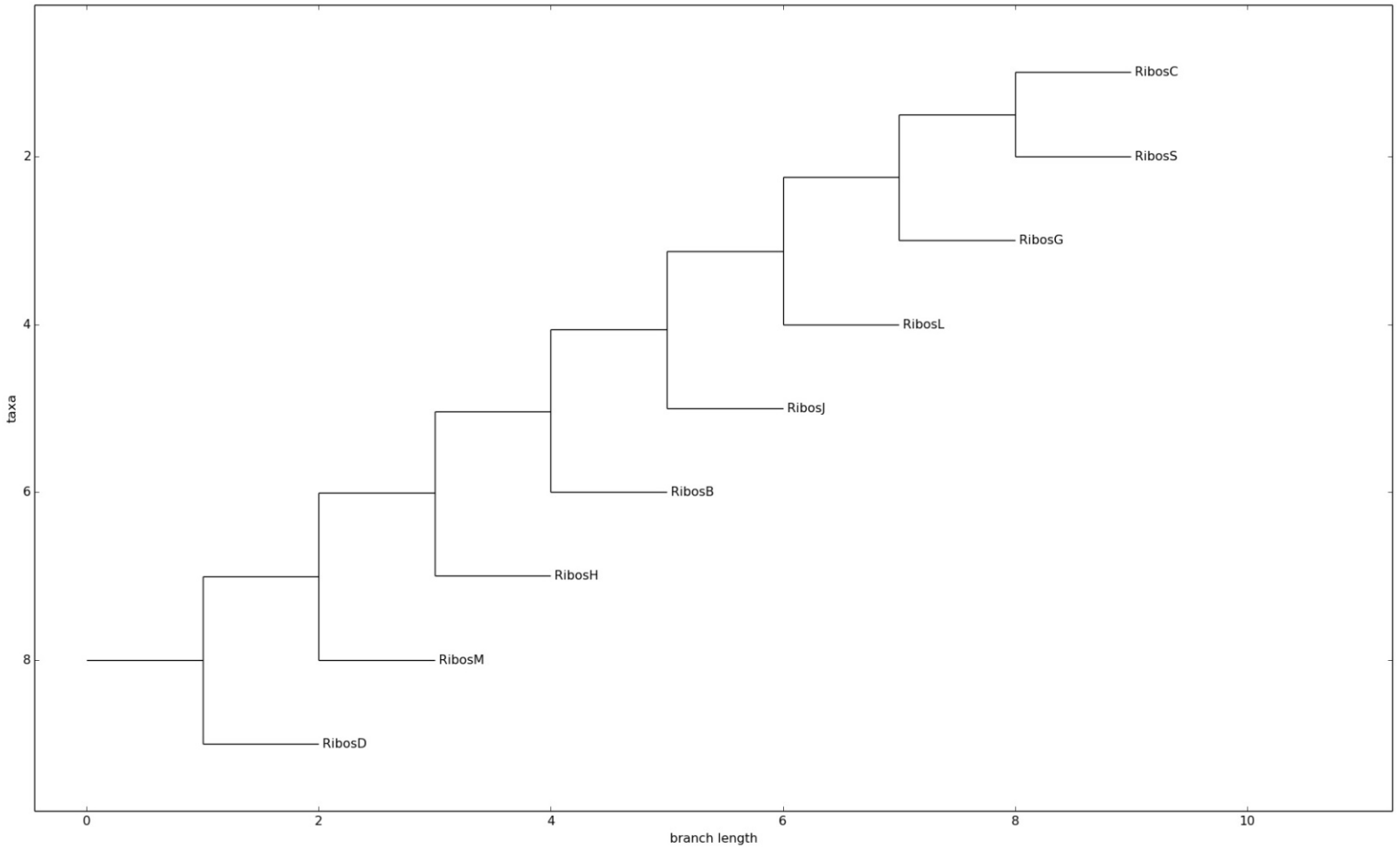

Fig. 9. Genetic relatedness of *Bacillus* species isolated from ready-to-eat (RTE) foods

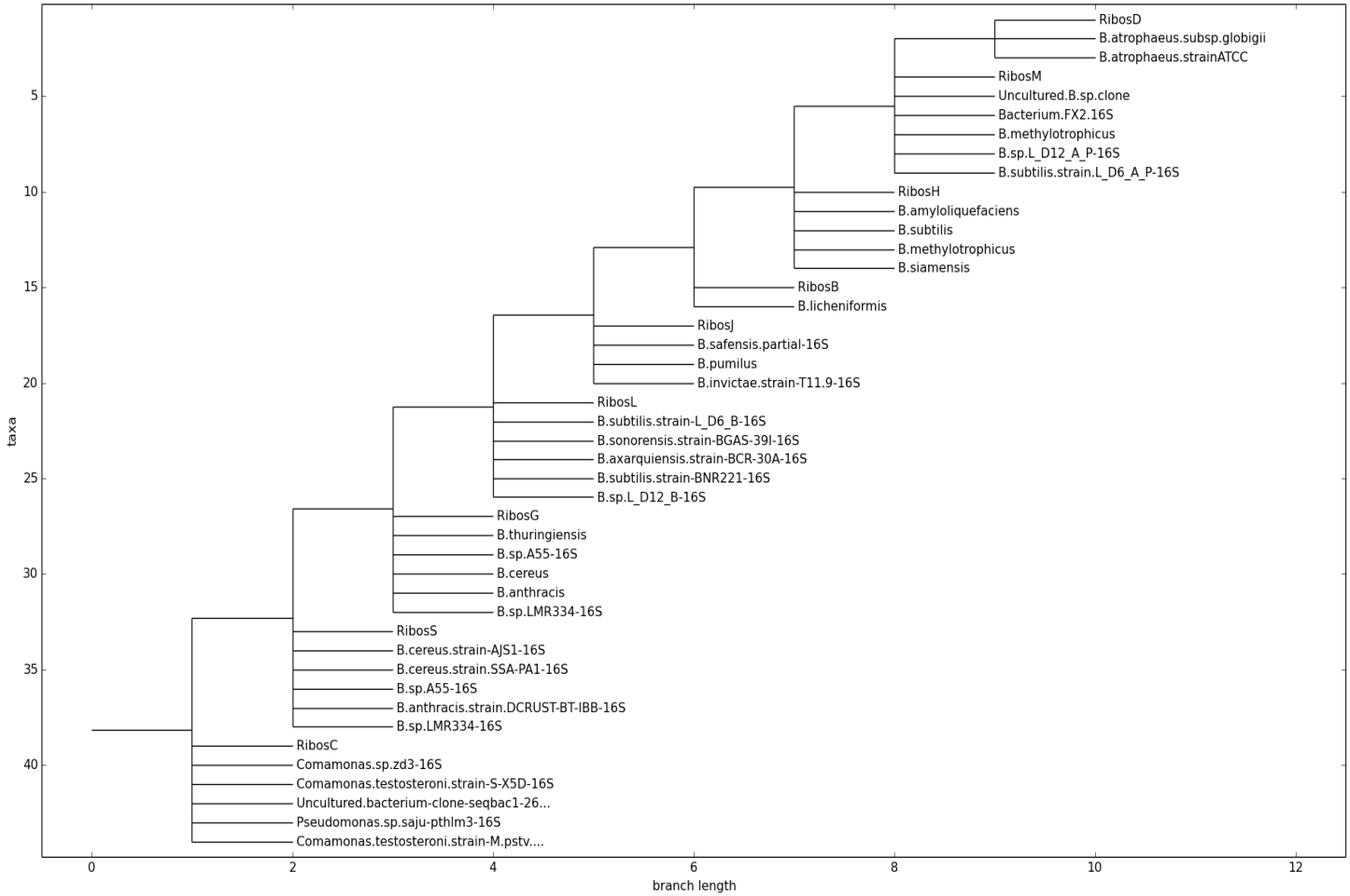

Fig. 10. Phyletic relatedness of isolated *Bacillus* species and database closely related sequences

5. CONCLUSION

This study has demonstrated that some of the popular types of ready-to-eat foods (RTEs) that are sold on the streets are contaminated with *Bacillus cereus* and other *Bacillus* species. This work puts forward the need to make test for *Bacillus* species part of normal routine checks and analyses for ready-to-eat foods. It also reiterates the need for use of more sensitive and precise methods of identification for this group of microorganisms. Members within the *Bacillus* genus are closely related molecularly. While classical methods are fast becoming obsolete, the use of methods such as the analytical profile index (API) kit and the PCR/sequencing method, among others, are highly recommended for the identification of *Bacillus* species. Hence, we join in reaffirming a stance as submitted by earlier researchers that classical schemes often do not allow the precise identification of atypical and intermediate strains. It is widely agreed that there is considerable room for improvement in the taxonomy of the genus, *Bacillus*. It is particularly important that study of new *Bacillus* isolates would require more sensitive and efficient methods of identification for greater precision in their classification. In the future, species/strain-specific methods should be developed and tailored towards the identification of particular strains within the *Bacillus* genus.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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