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Research Article

Molecular Identification of Microbes Associated with Kola nut (*Cola nitida)* in Ibadan, Nigeria

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Abstract
Kola nut is an important dehiscent fruit but its supply fails to meet the demand due to limitations such as inadequate post-harvest practices leading to spoilage during transportation and storage. Identification of microbial contaminants is a necessary step in reducing the loss. Hence, this work aimed at characterizing the genome of dominant fungus and bacteria associated with kola nut (*Cola nitida*). Samples of kola nuts were collected from Oje and Oja-oba markets in Ibadan, Nigeria. Isolates were extracted from the lesion region of the samples using the standard method. Macro-morphological and micro-morphological observations of the pure culture of the isolates were conducted. After genomic DNA extraction of fungal and bacterial isolates, Polymerase Chain Reaction (PCR) was carried out using Internal Transcribed Sequence (ITS 1 and ITS 4) and 27F primers for fungal and bacterial genomes respectively. Sanger sequencing of the purified DNA fragments was conducted utilizing the Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000. Molecular Evolutionary Genetics Analysis (MEGA version 6) was used for phylogenetic analysis. The result revealed that one fungus and two bacteria were associated with kola nuts. The fungal isolate showed 100% identity with *Aspergillus wentii* from the National Center for Biotechnology Information data base. The fungus showed a good relationship with *A. dimorphicus* and *A. europaeus*. The two bacterial isolates were also identified through the phylogenic analysis as *Bacillus cereus* and *B. subtilis. B. cereus* is closely related to *B. thuringiensis*. This research provides background information on the Kola nut microbial contaminant.

Keywords: *Bacteria, Contaminant, Fungus, Identification, Primers, Sequencing*

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Introduction
Cola species are evergreen, formerly placed in the cocoa family (Sterculiaceae) but now in Malvaceae (Oyedeji *et al*., 2013). The most common species are C. verticillata (Thonn.) starf, C. acuminata and C. nitida (vent.) Schott and Endlicher, with the last two being the most significant from an economic standpoint. (Lovejoy, 1980). In Nigeria, *C. nitida* is commonly known as *gbanja* and *goro* among Yoruba and Hausa tribes respectively (Asogwa *et al*., 2006). This fruit that dehisces is nutrient-rich, and its extract is now widely used as a substitute medication to treat whooping cough and asthma (Adedayo *et al*., 2019; Aduama-Larbi *et al*., 2022). It is being processed industrially for the production of wines, soft drinks, drugs and animal feeds. The caffeine in the nut dilates the bronchial air passage enabling it to be used in managing respiratory diseases such as whooping cough or pertussis and bronchial asthma (Oduwaye *et al*., 2018). The value of kola nut is traditionally recognized among people who chew it raw and also use it as a symbol of authority and for other traditional rites. These nuts are also used during weddings, rituals, funerals and naming ceremonies. People chew kola nuts as a nervous system stimulant to reduce sensations of hunger and suppress sleep (Aduama-Larbi *et al*., 2022). Unfortunately, kola nuts do not meet the demand

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because of constraints such as poor postharvest techniques that facilitate spoilage during transportation and storage.

To minimize postharvest loss of kola nuts as a result of microbial invasion, the identification of common microbes becomes necessary. The most prevalent moulds linked to kola are typically those belonging to the *Aspergillus, Fusarium* and *Penicillium* genera. (Daouda *et al*., 2013; Terna *et al*., 2017). Fungi such as *Aspergillus niger, A. flavus, A. fumigatus, F. oxysporum, Lasiodiplodia theobromae. theobromea, R. stolonifer, Botrytis cinerea, Colletotrichum gloeosporioides, C. lindemuthianum, Fusarium solani* and *Penicillium expansum* had been identified to be associated with kola nut in storage (Oduwaye *et al*., 2018). Most of these moulds produce life-threatening mycotoxins that cause health issues.

Fruits and vegetables are an indispensable part of human nutrition. These kinds of food are usually eaten fresh or with minimal processing that does not rid of the pathogens. Consuming contaminated fruits can lead to serious disorders such as gastrointestinal diseases. Kola nut is a fruit that is consumed raw. The risk of contamination is very high. This makes it necessary to examine the potential risk evaluation of kola nut by identifying microbial contaminants that are associated with it. Hence, the aim of this study is to identify microbes associated with kola nut obtained from local markets in Ibadan, Nigeria using molecular tools

Materials and Methods

Cola nitida were randomly purchased from local markets in Oje market (Latitude: 7.3881 Longitutde: 3.9069) and Oja Oba market (Latitude: 7.3743 Longitude: 38949), Ibadan North, Oyo State, Nigeria because the fruits are always available and commonly sold in those markets. The *C. nitida* samples were sorted, labelled and seemingly healthy ones were wrapped in banana leaves and stored in a sterile polythene bags for seven (7) days at 25±2 ^{o}C.

Lesion portions of the samples were cut into small pieces of about 2 mm using a sterile blade. After being surface sterilized for two minutes with 1% sodium hypochlorite, the pieces were rinsed five times with distilled water and dried on sterile filter paper. They were picked with sterile inoculating needle and inoculated on Potato Dextrose Agar (PDA) plates using direct plating methods (Garuba *et al*., 2022). The plates were labelled and incubated at 25±2 ^{o}C. Then, sub-culturing of frequently occurring microbial colonies was carried out to obtain pure culture for each isolate.

Macro-morphological and micro-morphological observations of the pure culture of the isolates were conducted. The macroscopic characteristics observed were the size of the colony, the colony's surface colour and the colour of the plate's back side. Using a light microscope (binocular compound Olympus microscope model (CH)), the following features were observed under magnification: the type and nature of the reproductive apparatus (sporangiophore or conidiophore), the shape and colour of the spore, the presence or absence of spores, the colour of the hyphae, and the presence or absence of septa (Fawole and Oso, 2007). Manuals and atlas were checked for the exactitude in morphological identification (Barnett and Hunter, 2010; Cambell and Stewart, 1980). Biochemical tests were conducted for bacterial isolates as described by Fawole and Oso (2007).

Extraction of Bacterial genomic DNA was conducted following an established protocol of Trindade *et al*. (2007). Cultures of single colonies grown on a liquid medium were centrifuged at 4600x g for 5 min. After that, the pellets were again suspended in 520 microliters of TE buffer (pH 8.0, 10 mM Tris-HCl, and 1 mM EDTA). Three microliters of Proteinase K (20 mg/ml) and fifteen microliters of 20% SDS were added. After the mixture was incubated for an hour at 37 ^{o}C, 80 µL of a 10% CTAB solution in 0.7 M NaCl and 100 µl of 5 M NaCl were added and mixed. The suspension was placed on ice for fifteen minutes after being incubated for ten minutes at 65 ^{o}C. Equal parts chloroform and isoamyl alcohol (24:1) were added, and the mixture went through centrifugation for 20 minutes at 7200 x g after 5 minutes of ice incubation. The aqueous segment was poured into a fresh tube, isopropanol (1: 0.6) was added and DNA was precipitated at –20 ^{o}C for 16 hours. The DNA was obtained through centrifugation at 7200 x g for 10 minutes, followed by a 500 µl ethanol wash, 30 minutes of incubator drying at 37 ^{o}C, and a final dissolution

in 50 µl sterile distilled water to serve as stock DNA. The 1.5% Agarose gel was used to test the stock DNA, and it ran for about 40 minutes at 120 V. After that, it was examined under a UV lamp to authenticate that bacterial DNA was present.

A sterile pestle was used to macerate 100 mg of fungal mycelia in 1 ml of DNA Extraction Buffer (DEB) containing proteinase K (0.05 mg/ml). The extract was poured into a 1.5 ml Eppendorf tube. After adding 50µl of 20% Sodium Dodecyl Sulphate (SDS), the mixture was placed in a water bath for 30 minutes at 65 ^{o}C. The tubes were cooled to 25±2 ^{o}C. After adding 100µl of 7.5M potassium acetate and quickly mixing, the mixture was centrifuged for ten minutes at 13,000 rpm.

The supernatant was poured into new sterilized autoclaved tubes, and 2/3 volumes of cold Isopropanol/Isopropyl alcohol was dispensed to the supernatant, following three gentle inversions, the tubes were incubated at -20°C for one hour. It was centrifuged at 13000rpm for 10 minutes and the supernatant was discarded. A 500µl of 70% ethanol was added again and centrifuged for 5 minutes at 13000rpm. The supernatant was discarded carefully. After removing any remaining ethanol, the DNA pellets were dried for 10 to 15 minutes at 37 °C. Tris-EDTA (TE) buffer (50µl) was used to re-suspend the DNA pellets. DNA was kept at -20 ^{o}C for further laboratory analysis.

The stock DNA was tested on a 2% Agarose gel run on a voltage of 120V for about 40min. This was later viewed under UV light to authenticate and confirm the presence of the fungal DNA (Amer *et al.,* 2011).

PCR reaction cocktail consisted of 10 µl of 5x GoTaq colourless reaction; 3 µl of 25Mm MgCl2; 1 µl of 10 mM of dNTPs mix; 1 µl of 10 pmol each forward primer and reverse primer, for fungi : (ITS 1)- 5' TCC GTA GGT GAA CCT GCG G 3' and (ITS 4)- 5' TCC TCC GCT TAT TGA TAT GC 3', for bacteria 27F 5'- AGA GTT TGA TCM TGG CTC AG-3' and - 1525R, 5′-AAGGAGGTGATCCAGCC-3′; 0.24µl of 0.3units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water and 8µl working DNA template (Garuba *et al.,* 2022). Amplification was done using a PCR system thermal cycler (Applied Biosystem Inc., USA) with a profile of an initial denaturation (94°C for 5 minutes), 35 cycles of denaturation (94°C for 30 seconds), annealing (55 °C for the 30 seconds), extension (72°C for 1 minute 30 seconds) and a final extension (72°C for 10 minutes). The reaction is summarized in Table 1.

The amplified DNA fragments of target sequences in PCR were ethanol purified to remove the PCR reagents. Briefly 7.6 µl of Na acetate 3 M and 125 µl of 95 % ethanol were added to about 50 µl PCR amplified products in a fresh sterile 1.5 µl Eppendorf tube, mixed by simple inversion and kept at -20 °C for 30minutes. The tubes were centrifuged for 10 minutes at 13000 g followed by the removal of supernatant after which the pellet was washed by adding 500 µl of 70 % ethanol, mixed and then centrifuged for 15 minutes at 7500 g and 4 °C. Repeat pellets were washed with 70 % ethanol and centrifuged. The tubes were re-suspended with 25 µl of sterile distilled water and kept at -20 °C after inverting them on paper tissues to allow them to dry for a full 25 minutes at 37 °C.

The presence or absence of the expected band size of the amplified region sequence was checked in gel electrophoresis. The integrity of the PCR amplicons was viewed on a 1% agarose gel (CSL-AG500, Cleaver Scientific Ltd) stained with EZ-vision® Bluelight DNA Dye.

The Sanger Sequencing of the purified DNA fragments was carried out. The fragments were sequenced using the Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000 according to the manufacturer's instructions (https://www.nimagen.com/). Cycle Sequencing Mastermix contained 88 µL BrilliantDye™ Terminator Kit V3.1 sequencing mix, 132 µL 5x BrilliantDye sequencing buffer, 88 µL 21M13 (Forward) or M13Rev (Reverse) primer (5 pMol/ µL) and 484 µL Molecular Biology grade water. Nine microliters of the 792 µL Mastermix were distributed among the 80 wells arranged in columns 1 through 10 on a fresh 96-well plate. By multichannel pipetting, 1 µL of the cleaned PCR products was transferred to the corresponding wells of the prepared sequencing

plate. The plate was sealed tightly for cycle sequencing.

SeqTrace version 0.9.0 was used to generate consensus DNA sequences from both forward and reverse. The Basic Local Alignment Search Tool (BLAST) was carried out at the National Center for Biotechnology Information (NCBI) website to obtain local similarity. DNA sequencing alignments were obtained using AliView version 1.17-beta1 software. Phylogenetic analysis was carried out using Molecular Evolutionary Genetics Analysis (MEGA version 11) software.

Results and Discussion

One fungus (isolate 1) and two bacteria (isolates 2 and 3) were isolated from the samples. The mycelia of the fungus appeared dense, white to pale yellow. The reverse of the plate was uncoloured and turned yellow. The microscopic view revealed the conidial head radiates, splitting into columns. The vesicles elongated to globose, biseriate, and metule densely packed, covering most of the vesicular surface with phialides. The conidia were globose to broadly ellipsoidal (Figure 1A and 1B). The pure cultures of the bacterial isolates are shown in Figure 2. The selective biochemical tests for the identity of the two dominant bacteria are summarized in Table 2. There were DNA bands of the target regions of the genome of the fungus and bacteria in gel electrophoresis (Figure 3). The molecular studies of the ITS region of encoded rDNA of the fungal isolate (represented as *Aspergillus wentii UIL* in phylogeny tree with accession number OR840584 generated after deposition in the GenBank)) showed 737 query length and 100% identity with *Aspergillus wentii* (Figure 4). *A. wentii* is closely related to *A. europaeus*, and *A. dimorphicus* (Hubka *et al*., 2016), belonging to Section Cremei (Peterson, 1995). *Aspergillus* species is one of the fungal genera that reduce the postharvest quality of freshly harvested and processed kola nuts (Aduama-Larbi *et al*., 2022). *A. parasiticus, A. niger and A. flavus* have been previously reported to be responsible for kola nut rot (Idris *et al*., 2017; Oduwaye *et al*., 2018). *A. wemtii* is another species reported in this work. It is an asexual filamentous fungi usually found on a variety of organic substrates including decaying plant materials and moist grains (Nadumane, *et al*., 2016). It has also been previously isolated from white field corn (Schroeder and Verrett, 1969), marine alga (Li *et al*., 2014) and deep-sea sediment (Li *et al*., 2016). *Aspergillus wentii* as a soil-borne fungus is found on decayed leaves and other organic substrates. *A. wentii* has also been reported to produce aflatoxin and emodin (Well *et al*., 1975; Hamasaki and Kimura, 1983). Other secondary metabolites reported to be produced by this *Aspergillus* species include kojic acid, β-nitropropionic acid, 1-amino2-nitrocyc1opentane-l-carboxylic acid, wentilactone A and B, and hexadecylcitraconic acid (Dorner *et al*., 1980; Hamasaki and Kimura, 1983). *A. wentii* produces enzymes that are industrially important and synthesize compounds that contribute towards anticancer research (Nadumane, *et al*., 2016). For instance, wentilactone B isolated from *A. wentii* and its derivatives were examined for their anticancer potentials against many cell lines and it was observed that wentilactone B was the most effective with an IC_{50} value of 17µM and similar success was also recorded *in vivo* tumour growth (Zhang *et al*., 2013; Nadumane, *et al*., 2016).

However, it is also used in industrial enzyme production. *A. wentii* produced lipase at 30^{o}C at pH 6.0 in 3 days and also produced galactose, fructose, mannitol, sucrose, lactose and maltose along with lipid degrading enzymes in a growth medium supplemented with glucose. In addition, this species of fungus has also been used in the production of glucoamylase and the manufacture of biodiesel from lipids (Lago *et al*., 2021).

The sequence of Isolate 2 (represented as *Bacillus cereus* UIL in the phylogeny tree with accession number OR838808) with a query length of 1003 and query cover of 99% had 95% identity with *Bacillus cereus* and that of isolate 3 (represented as *Bacillus subtilis CnB UIL* in phylogeny tree with accession number OR838807*)* had 99% identity with *B. subtilis* The phylogenetic relationships of the consensus sequences of the two bacterial isolates with those extracted from National Centre for Biotechnology Information (NCBI) after BLAST are shown in Figures 5 and 6. *Bacillus cereus* is an endemic, soil-dwelling, rod-shaped and Gram-positive bacterium. The bacterium is close to *B. anthracis, B. thuringiensis, B. weihenstephanensis, B. mycoides* and *B. pseudomycoides* (Elsharkawy *et al*., 2022).

It is cosmopolitan and diverse, and found on plants, in soil and enteric tracts of mammals and insects (Marrollo, 2016). The bacterium is also one of the major food-related pathogenic bacteria subsisting in food production environments due to its adhesive endospores that spread to foods and their product. It causes enteric diseases that are mild and of short duration manifesting in the form of diarrheal infection or an emetic syndrome (Marrollo, 2016). *B. cereus* causes food poisoning when its concentration exceeds 10^4 spores or vegetative cells per gram (Yu *et al*., 2019). *B. cereus* outbreaks are linked to mortality as well as morbidity in people who consume contaminated food, and they happen all over the world (Juneja *et al*., 2017). Schmid *et al* (2016) opined that most of the diseases caused by *B. cereus* are self-limited and have a short duration.

B. subtilis is as ubiquitous as *B. cereus* living in water, air, soil and plant residues. The bacterium produces endospores making it to withstand extreme heat and desiccation. In a numerical classification of rod-shaped bacteria, *B. cereus, B. mycoides* and *B. thuringiensis* clustered at 89 - 92% relatedness while *B. subtilis* group joined the *B. cereus group* at 72% similarity (Priest *et al*., 1981). *B. licheniformis, B. pumilus* and *B. subtilis* are closely related and clustered (78%) in the *subtilis* group but are delimited by the use of pyrolysis-gas chromatography (O'Donnell *et al*., 1980). Unlike *B. cereus, B. subtilis* is considered a non-threatening bacterium because it is not toxic to human, plants and animals but produces many enzymes such as proteases that help in nutrient cycles. Gill (1982) reported that *B. subtilis* produce an extracellular toxin called subtilis in with low toxigenic properties. It causes allergic reactions in individuals who are continuously exposed to it (Edberg, 1991). *B. subtilis* has also been found as an endophyte citrus without causing any apparent disease (Munir *et al*., 2020). In this study, the bacterium was isolated from kola nut which might exist as an endophyte because it was not established if it caused any postharvest disease to the sample.

Conclusion

Kola nut is an important fruit that is often contaminated with *Aspergillus wentii, Bacillus cereus* and *B. subtilis*. The isolation of the mould and bacteria may serve as a foundation to improve postharvest handling of kola nuts and pave the way for further research on the ability of the isolates to instigate disease in the fruits.

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Table 1: Polymerase Chain Reaction Condition

Activity	Temperature	Duration
initial denaturation	**96°C**	**60 sec**
	96°C	**10 sec.**
28 cycles	**50°C**	**5 sec.**
	72°C	**1 min. 15 sec.**
hold	**4°C**	∞

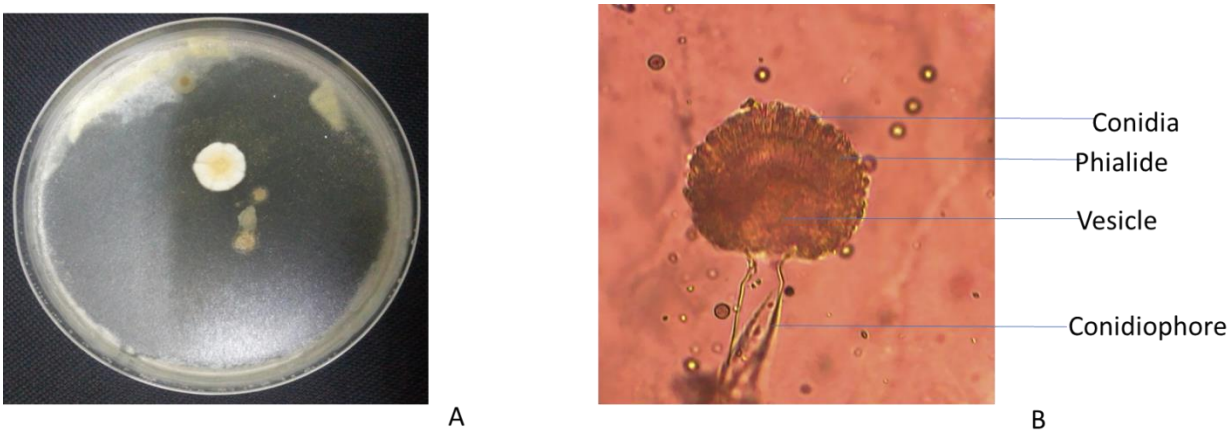


Figure 1: The macromorphological (A) and micromorphological (B) structures of *Aspergillus wentii*

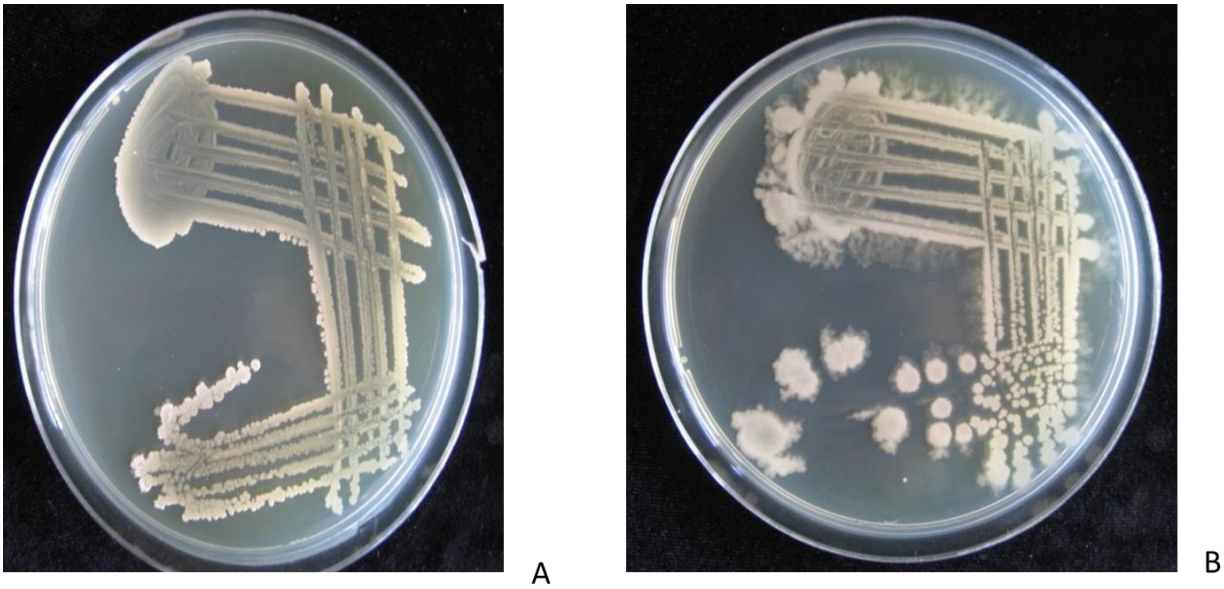
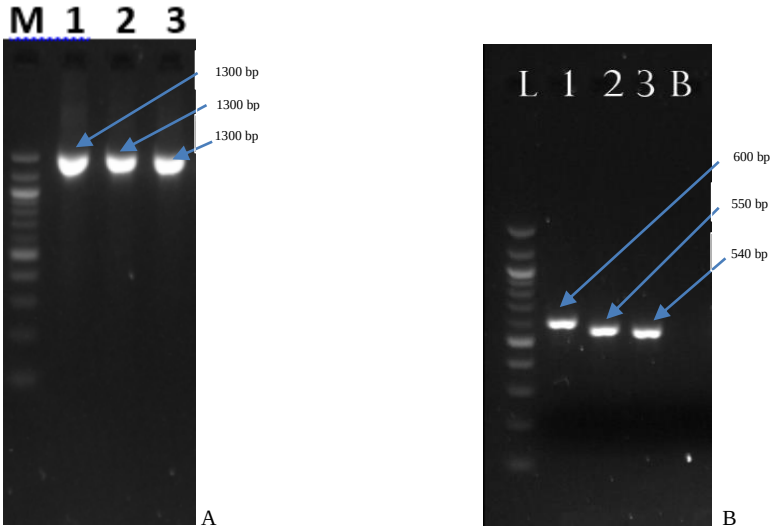


Figure 2: Pure cultures of *Bacillus cereus* (A) and *B. subtilis* (B)

Table 2: Biochemical tests of Bacterial isolates

Selective Biochemical reactions	*Bacillus cereus*	*Bacillus Subtilis*
Gram reaction	+	+
Anaerobic	-	+
Aerobic	+	-
Motility	Non-Motile	Non-Motile
Catalase test	+	+
Oxidase test	-	+
Urease hydrolysis	+	+
Citrase hydrolysi	+	+
Starch hydrolysis	+	+
Nitrate production	+	+
Casein hydrolysis	+	-
H_2S production	+	-
Gelatine hydrolysis	-	-



A B

Figure 3: DNA fragments from fungal (A) and bacterial (B) isolates run on 2% agarose gel electrophoresis. M-100bp DNA ladder; L- 100bp DNA ladder

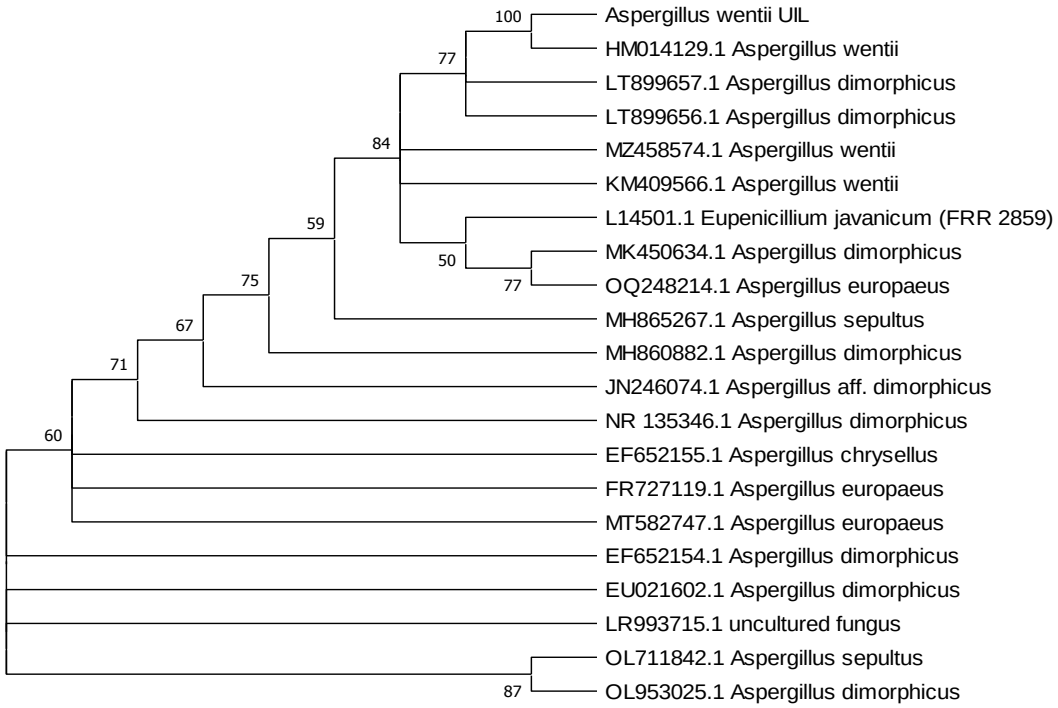


Figure 4: Molecular Phylogenetic analysis of *Aspergillus wentii* by Maximum Likelihood method

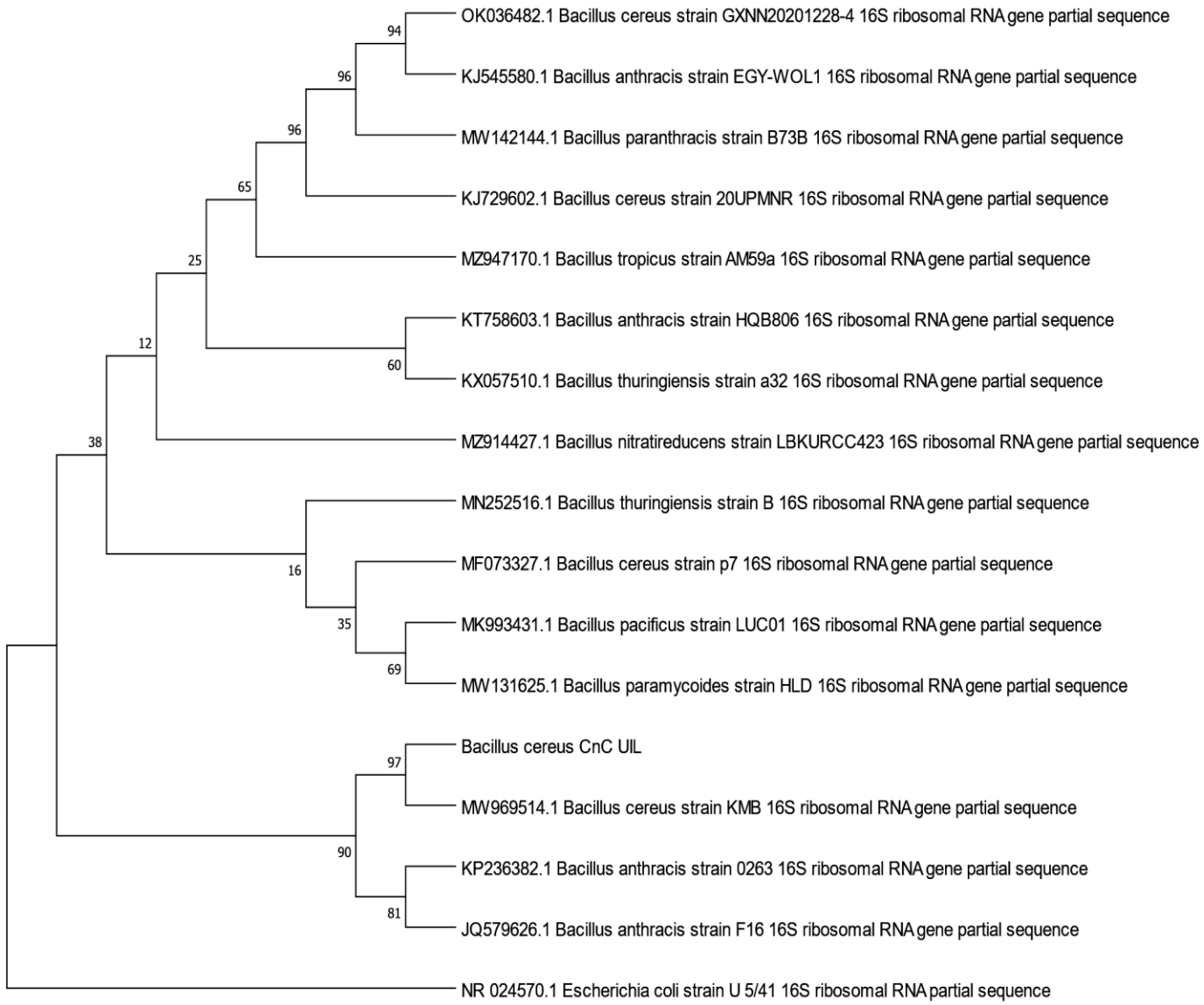


Figure 5: Molecular Phylogenetic analysis of *Bacillus cereus* by Maximum Parsimony method

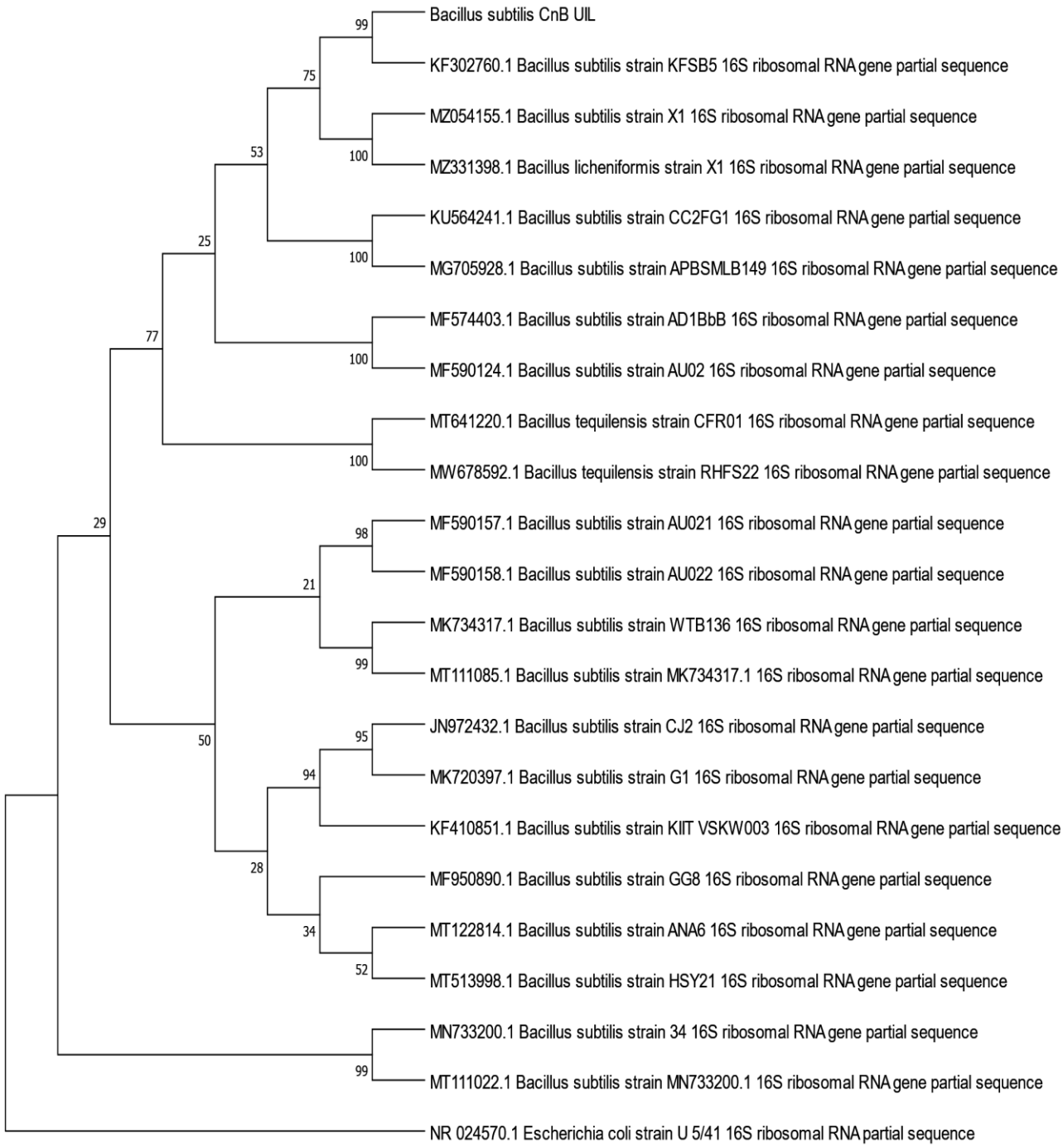


Figure 6. Molecular Phylogenetic analysis of *Bacillus subtilis* by Maximum Parsimony method