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Genome Subtyping of Autochthonous *Bacillus* Species Isolated from *Iru*, a Fermented *Parkia biglobosa* Seed

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Phylogenetic relationship and strains sub-typing of *Bacillus* species isolated from *iru*, a traditional fermented condiment in Africa were studied using polyphasic genomic approaches and the profiles compared with bacilli isolated from similar Asian condiments. The 16S rRNA gene sequencing identified the strains as *Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus cereus, Bacillus licheniformis, Bacillus pumilus,* and *Brevibacillus formosus*. The phylogenetic analysis conducted showed five distinct clusters with genetic relatedness among *B. subtilis* and *B. amyloliquefaciens* strains from Africa and Asia. Amplified ribosomal DNA restriction analysis (ARDRA) successfully differentiated species of *B. subtilis* phylogeny from *B. cereus*. Combined analyses of ARDRA, internal transcribed spacer-polymerase chain reaction (ITS-PCR), ITS-PCR-restriction fragment length polymorphism (ITS-PCR-RFLP) and randomly amplified polymorphic DNA (RAPD-PCR) further confirmed *B. subtilis* and *B. amyloliquefaciens* as the dominant *Bacillus* species associated with fermentation of *iru*, and revealed high strains genetic diversity, while multilocus sequence analysis (MLSA) data distinguished *B. cereus* from *B. thuringiensis*. This information is essential for selection

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of starter cultures with desirable functional attributes to guarantee product consistency and safety quality of traditional fermented foods.

Key Words: phylogenetic relationship; *Bacillus* strains; diversity; fermented condiments; starter cultures

INTRODUCTION

Fermented vegetable proteins used as condiments are widely consumed in many African countries. These include *soumbala* (Burkina Faso), *iru* or *daddawa* (Nigeria and Ghana), *afitin* and *sonru* (Benin Republic), *nététou* (Senegal), and *kinda* (Sierra Leone), all produced by fermentation of African locust bean [*Parkia biglobosa* (Jacq. Benth)] (N'dir et al., 1994; Ouoba et al., 2008; Ouoba et al., 2010; Oguntoyinbo et al., 2010). Roselle seeds (*Hibiscus sabdariffa*) are fermented to produce *bikalga* (Burkina Faso); *dawadawa botso* (Niger Republic); *datou* (Mali); *mbuja* (Cameroon) and *furundu* (Sudan) (Ouoba et al., 2008). Also, Baobab seeds [*Adansonia digitata* (L.)] are fermented to produce *maari* (Burkina Faso) (Parkouda et al., 2010). Similarly, in southeast Asia, various fermented alkaline food condiments reportedly produced mainly from soybeans [*Glycine max* (L.)] include *kinema*, consumed by the people of eastern Himalayan regions of the Darjeeling hills and Sikkim in India, Nepal and Bhutan (Tamang and Nikkuni, 1996; Sarkar et al., 1997; Dahal et al., 2005); *hawaijar*, popular among the Manipuris of northeast India (Jeyaram et al., 2008); *thua nao* from northern Thailand (Leejeerajumnean et al., 2001); Chinese *douchi* (Peng et al., 2004) and Korean *cheonggukjang* (Kim et al., 1996).

Iru is consumed by more than 150 million people in West African subregion and is particularly popular in Nigeria. It is used as both flavoring and thickening agents in soups and stews (Oyeyiola, 1988) and serves as a low cost source of plant protein and seasoning agent (Antai and Ibrahim, 1986). Traditionally, *iru* is prepared by boiling *P. biglobosa* seeds for 24 h followed by dehulling. The cotyledons obtained are again boiled for 4 h with optional addition of *iku iru*, a softening agent made from ground seeds of sunflower (*Hibiscus sabdariffa*). They are then drained using raffia sieve, spread into wide calabash trays while still hot, covered with jute bags and left to ferment for 3–5 d. Salt may be added at the end of the fermentation process as a preservative.

The dominance of *B. subtilis* during fermentation of locust beans for *iru* production has been consistently reported (Antai and Ibrahim, 1986; Odunfa and Oyewole, 1986). Earlier studies on the identification of the microorganisms associated with *iru* were based on phenotypic characterization using biochemical tests that are poorly discriminatory, nonreproducible and often laborious (Odunfa, 1981; Ikenebomeh, 1989). Genotypic typing techniques to study diversity of *Bacillus* species during legume fermentation have been reported in Africa and Asia. Sarkar et al. (2002) used randomly amplified polymorphic

DNA polymerase chain reaction (RAPD-PCR) to study the diversity within the *B. subtilis* isolated from *kinema* and *soumbala*. Jeyaram et al. (2008) characterized dominant *Bacillus* species associated with *hawaijar* using polyphasic genotypic techniques. In Africa, *Bacillus* species responsible for the fermentation of Baobab seeds and *Prosopis africana* (Guill., Perrott and Rich.) Taub. for *maari* and *okpehe* production respectively were recently reported (Oguntoyinbo et al., 2010; Parkouda et al., 2010). These studies did not determine strains clonal relationship, variation, and phylogenetic of *Bacillus* species across different geographical regions; also horizontal gene transfer and recombination that may occur among food-borne microbial strains require typing of diverse strains. Information such as this is essential for identification of strains with functional or virulence characteristics during industrial production of fermented food condiments. In this study, different genomic characterization techniques were used to subtype *Bacillus* species isolated from fermented *P. biglobosa* during *iru* production in W. Africa and also determine the phylogenetic relationship with strains obtained from similar fermented condiments in Asia.

MATERIALS AND METHODS

Sampling

Twenty samples of fermented *P. biglobosa* seeds were obtained from local producers and retail markets in four towns in Nigeria (Oyo, Abeokuta, Kaduna, and Ado-Ekiti). The sampling plan followed a sampling technique according to Smith (2001). The fermented condiments were transported immediately with the aid of ice pack into the laboratory, stored in the refrigerator at 4°C, and analyzed microbiologically within few days of collection.

Isolation of Microorganisms and Phenotyping

Homogenate of each of the condiments was made following the method of Guo et al. (2006) with slight modifications. Six-fold serial dilutions were carried out; 100 μL of appropriate decimal dilutions were plated in triplicate on nutrient agar (Scharlau Chemie S.A., Barcelona, Spain) using spread plate technique (Harrigan and McCance, 1976) and incubated at 37°C for 18–24 h. The predominant representative colonies were selected based on colonial morphology and purified by repeated streaking to obtain pure cultures. A total of 280 bacterial cultures obtained were phenotyped using colony characteristics, Gram's reaction, catalase test, and endospore staining using phase contrast microscope (BX61, Olympus, Japan) and subsequently stored at −20°C in 50% v/v glycerol (Calibiochem, San Diego, California, USA). Reference strains of *Bacillus* were also obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India.

Genotypic Characterization

Genomic DNA Extraction

Bacterial genomic DNA was extracted using modified lysozyme-heat lysis method as previously described (Zhang et al., 2002). DNA quantity and purity were determined at absorbance reading of 260 nm (NanoDrop™ 1000 Spectrophotometer, Thermo Scientific, Waltham, Massachusetts, USA).

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

The 16S rRNA gene ca. 1500 bp of the isolates were amplified with the universal primers pair fD1 and rD1 (Table 1), digested with *Hae*III, *Cfo*I, *Hinf*I, *Dde*I, *Taq*I and *Rsa*I (Promega, USA) and analysed on 2% agarose (Promega, USA) containing ethidium bromide (0.5 μg/ml) (E1510, Sigma Aldrich), using 0.5X TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0). A 1 kb DNA ladder (Promega, USA) or 100 bp DNA ladder Cfol (Promega, USA) was used as a size standard marker in each run, and the size of the DNA fragments were measured using Quantity One software 4.6 (BIO-RAD, Berkeley, California, USA).

16S-23S rRNA Gene Internal Transcribed Spacer (ITS) PCR Amplification and Restriction Analysis (ITS-PCR-RFLP)

The amplification of the 16S-23S rRNA gene internal transcribed spacer (ITS) was carried out in a 25 μL reaction mixture containing 50 ng DNA, 1X PCR reaction buffer containing 1.5 mM $MgCl_2$ (P2192, Sigma-Aldrich), 1.0 mM $MgCl_2$ (M8787, Sigma-Aldrich), 0.5 μM each of forward and reverse primers (Sigma-Aldrich) (Table 1), 200 μM each of dNTP (Sigma-Aldrich) and 1.25 U of *Taq* DNA polymerase (D6677, Sigma-Aldrich). Amplification was performed in a master cycler (Eppendorf 5333, USA) with an initial denaturation of 94°C for 5 min followed by 30 cycles of final denaturation at 94°C for 30 sec, annealing at 60 °C for 30 sec and extension at 72°C for 1 min with a final extension at 72°C for 7 min. The ITS PCR products were analyzed on 1.5% agarose and digested with *Cfo*I as previously described.

Randomly Amplified Polymorphic DNA PCR (RAPD-PCR)

Six random primers were used for the RAPD-PCR analysis (Table 1) and analyzed in a 15 μL reaction volume, which contained 25 ng DNA, 1X *Taq* buffer with 1.5 mM $MgCl_2$ (Genei, Banglore), 0.8 pmol/μL each of the primers, 200 μM each of dNTP, and 1.2 U of *Taq* DNA polymerase (Genei, Banglore). Amplification was performed in a thermal cycler (BIO-RAD iCycler, USA) and the first step of the PCR cycling was the initial denaturation at 94°C for 5 min followed by 35 cycles of final denaturation at 94°C for 1 min, annealing at 35°C for 1 min and extension at 72°C for 2 min. The PCR was completed with a final

Table 1: List of PCR primers used in this study.

Primer names	Primer sequences	Target regions	Annealing temp. (°C)	References
fD1	5′-AGAGTTTGATCCTGGCTCAG-3′	16S rRNA	65	Weisburg et al.(1991)
rD1	5′-AAGGAGGTGATCCAGCCGCA-3′′	16S-23S rRNA	60	Lechner et al. (1998)
16Sf-R2	5′-CGCGGGATCCTTGTACACACCGCCCGTC-3′			
23Sr-R10	5′-GGCCGTCGACCCTTTCCCTCACGGTACTG-3′	ra	35	This study
OPA 17	5′-GACCGCTTGT-3′			
OPA 18	5′-AGGTGACCGT-3′	ra	35	Pulido et al. (2005)
OPA 20	5′-GTTGCGTCC-3′	ra	35	This study
OPL 14	5′-GTGACAGGCT-3′	ra	35	This study
OPI 04	5′-CCGCCTAGTC-3′	ra	35	This study
M13	5′-GAGGGTGGCGGTTCT-3′	ra	35	This study
gyrB-BC-F	5′-TGAATTGACACGCCGCAAGAGC-3′	DNA gyrase subunit B	55	This study
gyrB-BC-R	5′-AACGCACTTCTGGGTCCATTGTCG-3′	glycerol uptake facilitator protein	55	This study
glpF-BC-F	5′-GCGTTTGTGCTGGTGTAAGT-3′			
glpF-BC-R	5′-CTGCAATCGGAAGGAAGAAG-3′	guanylate kinase putative	55	This study
gmk-BC-F	5′-ATTTAAGTGAGGAAGGGTAGG-3′			
gmk-BC-R	5′-GCAATGTTCACCAACCACAA-3′			

[ra] random amplification.

extension temperature of 70°C for 7 min. The thermocycle program used for the PCR amplifications of M13 consisted of one cycle of 95°C for 5 min followed by 35 cycles of 95°C for 30 sec, 36°C for 1 min and 72°C for 1 min 30 sec, and then one cycle of 72°C for 5 min. The RAPD-PCR products were analyzed on 1.5% agarose. The banding patterns of ARDRA, ITS-PCR, ITS-PCR-RFLP and RAPD-PCR polymorphisms were scored manually and then grouped using NTSYSpc. 2.20e for the generation of clusters in a dendrogram based on the Jaccard similarity coefficient (S_J) and the unweighted pair group method using arithmetic averages (UPGMA).

16S rRNA Gene Sequencing and Phylogenetic Analysis

The 16S rRNA genes of representative strains within the formed clusters were sequenced; closest known relative of the sequences were obtained by comparison with those deposited in GenBank. These sequences were eventually submitted to GenBank NCBI and received the accession no. JN255703 to JN255730. Pairwise and multiple alignments of these sequences including strains of related species were carried out using *CLUSTAL W* 2.0.12 (Thompson et al., 1994), according to the Kimura two-parameter model (Kimura, 1980). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987), and evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* 5 (Tamura et al., 2011). The statistical reliability of the tree was evaluated by bootstrap analysis of 1000 replicates (Felsenstein, 1985).

Multilocus Sequence Analysis (MLSA)

PCR amplification of *gyrB, glpF,* and *gmk* housekeeping genes were used for differentiation of *B. cereus* phylogeny. The reaction mixture consisted of 50 ng template DNA, 1X PCR buffer with 1.5 mM $MgCl_2$, 0.25 μM each of forward and reverse primers (Table 1), 200 μM each of dNTP and 1.25 U of *Taq* DNA polymerase. PCR conditions were fixed at 94°C for 5 min, 36 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 30 sec, with a final extension of 72°C for 7 min. The PCR products obtained were analyzed on 1.5% agarose, sequenced as described previously (Jeyaram et al., 2010), and searches were performed using GenBank with the BLAST program.

RESULTS

Phylogenetic Analysis

The nucleotide sequences of almost complete 16S rRNA genes of the bacterial strains compared with those of NCBI first identified them as species

closely related to *B. subtilis, B. amyloliquefaciens, B. cereus, B. licheniformis, B. pumilus,* and *Brevibacillus formosus* in decreasing order of occurrence. Second, it revealed *Bacillus* species as the major group of bacteria associated with fermentation of *P. biglobosa* during *iru* production. Third, the 16S rRNA gene sequences aided construction of a phylogenetic tree of *Bacillus* strains obtained from African fermented condiments (*iru* or *daddawa*); Asian fermented condiments (*kinema, hawaijar, thua nao, douche,* and *cheonggukjang*) and other typed and referenced bacilli strains deposited in different culture collections. The results of the phylogenetic relationship are as shown in Figure 1, with five major bacilli groups identified. Group I consists of *B. subtilis* and *B. amyloliquefaciens* strains from different condiments. The cluster analysis also showed the strains to be closest relative of *B. subtilis* subspecies subtilis DSM 10^T. Although the analysis could not differentiate *B. subtilis* from *B. amyloliquefaciens*, these strains are clonally related and must have originated from common ancestors before dispersal and domestication in vegetal protein foods. Group II is a subcluster of Group I that consists of species identified as *B. licheniformis*; the two strains clustered with *B. licheniformis* ATCC 14580. This bacterium is common with African condiments but poorly reported in Asia; it appeared as close relative of *B. subtilis*, which must have diverged over time. Group III consists of species closely related to *B. pumilus* ATCC 7061. They completely diverged from *B. subtilis* and *B. licheniformis* groups, phenotypically this species does not hydrolyse starch (data not shown), and it might have lost amylase production due to genetic mutation as a result of adaptation. Group IV comprises *B. cereus* that clustered with *B. cereus* ATCC 14579; many studied strains demonstrated diarrhoeal and emetic toxin production. Group V could be referred to as an outcast; it comprises *Brevibacillus formosus* strain that clustered with the type strain *Brevibacillus formosus* DSM 9885^T.

Bacillus Characterization and Strains Subtyping

The genotypic diversity among the *Bacillus* strains was further determined using polyphasic genomic approaches. Analysis of *Hinf*I digestion distinctly differentiated the *Bacillus* strains into two phylotypes of *B. subtilis* and *B. cereus* (Fig. 2). The restriction digested products of *Rsa*I clearly differentiated *B. subtilis* from *B. amyloliquefaciens; B. subtilis* from *B. pumilus; B. amyloliquefaciens* from *B. licheniformis; B. pumilus* from *B. licheniformis* (Fig. 3). However, *Rsa*I failed to distinguish *B. amyloliquefaciens* from *B. pumilus* and also *B. subtilis* from *B. licheniformis*, as they were observed to possess similar polymorphisms. The difficulty in differentiating *B. subtilis* from *B. licheniformis* was overcome with *Cfo*I digestion. These results clearly indicated that ARDRA can be successfully used for differentiation of *Bacillus* species, particularly *B. subtilis* phylogeny, when appropriate restriction endonucleases are employed. PCR amplification of the 16S-23S rRNA gene internal transcribed

Figure 1: Dendrogram showing multiple sequence alignment of 16S rRNA gene sequences of *Bacillus* species isolated in Africa from *iru* or *daddawa; hawaijar, cheonggukjang, kinema, douchi, thua nao* in Asia; referenced and typed strains. Pairwise phylogenetic distances were calculated based on 1400nt of 16S rRNA gene.

spacer (ITS-PCR) could not bring about any strain differentiation; rather it maintained the interspecies differentiation generated by ARDRA, except for *B. licheniformis*. However, ITS-PCR and restriction analysis with *Cfo*I showed intraspecies variation among the *B. subtilis* strains aside from differentiating *B. pumilus* from *B. amyloliquefaciens*. Dendrogram constructed based on combined analysis of the gel fingerprints obtained from ARDRA, ITS-PCR and ITS-PCR-RFLP is shown in Figure 4. Two major clusters were identified, cluster 1 identified as *B. subtilis* phylogeny consisting of 17 strains clustered together at 64% and cluster 2 identified as *B. cereus* phylogeny consisting

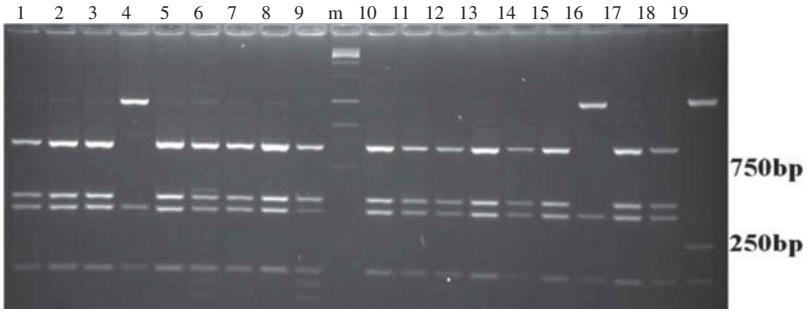

Figure 2: ARDRA gel profile based on *Hinf*I showing differentiation of *B. subtilis* and *B. cereus*. Lanes 1: *B. subtilis* MTCC 2451; 2: *B. amyloliquefaciens* MTCC 1270; 3: *B. licheniformis* MTCC 429; 4: *B. cereus* MTCC 430; 5: *B. circulans* MTCC 490; Lanes 6, 9, 10, 12, 13 & 15: *B. subtilis* strains; Lanes 7 & 8: *B. amyloliquefaciens* strains; Lanes 11 & 14: *B. pumilus* strains; Lane 16: *B. cereus* U175; Lanes 17 & 18: *B. licheniformis* strains; Lane 19: *Brevibacillus formosus* U185B; m 1kb DNA ladder.

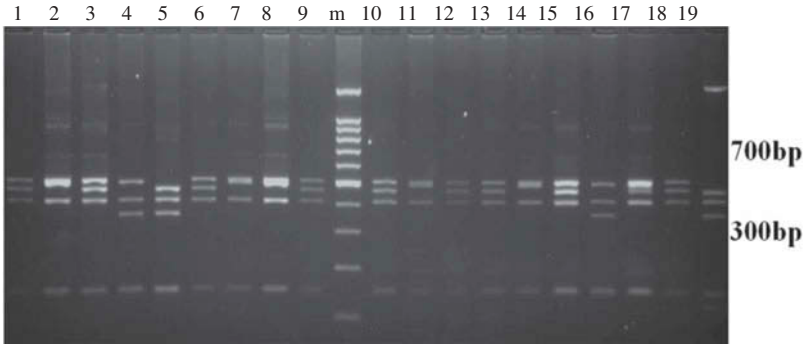

Figure 3: ARDRA gel profile based on *Rsa*I showing differentiation among *B. subtilis* phylogeny. Lanes 1: *B. subtilis* MTCC 2451; 2: *B. amyloliquefaciens* MTCC 1270; 3: *B. licheniformis* MTCC 429; 4: *B. cereus* MTCC 430; 5: *B. circulans* MTCC 490; Lanes 6, 9, 10, 12, 13 & 15: *B. subtilis* strains; Lanes 7 & 8: *B. amyloliquefaciens* strains; Lanes 11 & 14: *B. pumilus* strains; Lane 16: *B. cereus* U175; Lanes 17 & 18: *B. licheniformis* strains; Lane 19: *Brevibacillus formosus* U185B; m 1kb DNA ladder.

of 3 strains clustered at 45%. Subclusters of *B. subtilis* phylogeny showed a high degree of strain diversity. *B. subtilis* U104 clustered with strains isolated from *hawaijar* and *kinema*. Similar strain relatedness was observed among *B. licheniformis* and *B. amyloliquefaciens* isolated from *iru* and reference strains from Asian fermented foods. Analysis of colonial morphology using stereo zoom microscope also showed diversity of colonial types among bacilli strains isolated from different *iru* samples in Nigeria (data not shown).

The two dominant species in *B. subtilis* phylogeny identified as *B. subtilis* and *B. amyloliquefaciens* were further subjected to genomic strain diversity using RAPD-PCR OPA 18 primer. Figure 5 showed 60% similarity level, between two clusters I and II identified as *B. subtilis* and *B. amyloliquefaciens*

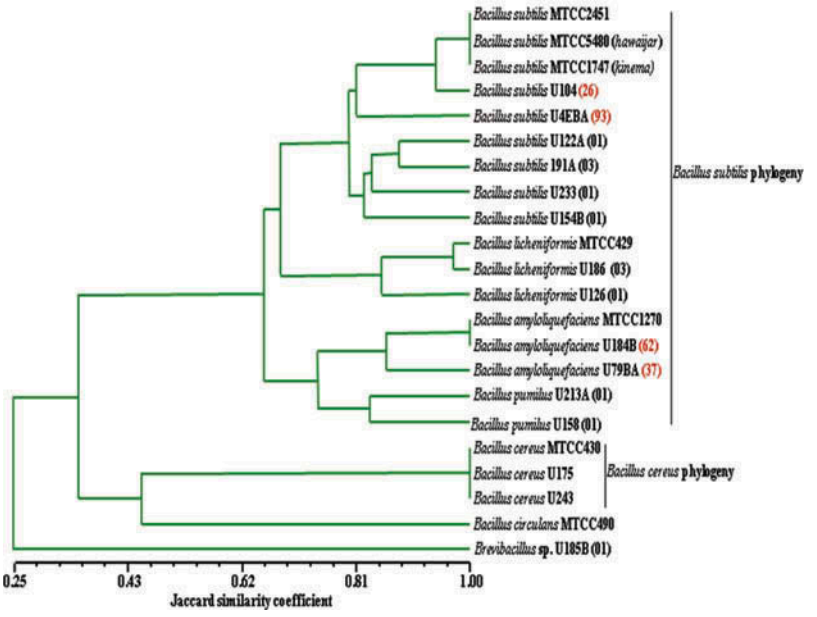

Figure 4: Dendrogram based on UPGMA clustering of Jaccard similarity coefficient (S_j) of normalized combined ARDRA, ITS-PCR and ITS-PCR-RFLP fingerprint patterns of *Bacillus* isolated from *iru* and reference strains.

strains. Also, at about 70% similarity level, two subclusters each of *B. subtilis* and *B. amyloliquefaciens* strains were identified, which were genetically distinct from the reference strains *B. subtilis* MTCC 5480 and MTCC 1747 from *hawaijar* and *kinema,* respectively, and *B. amyloliquefaciens* MTCC 1270. The dominant strains of *B. subtilis* and *B. amyloliquefaciens* were further studied using RAPD-PCR with M13 as it gave better diversity than OPA 18. At 85% similarity level, high strain diversity was found within *B. subtilis* (19 strains), and are genetically distinct from the reference strain *B. subtilis* MTCC 2451 (Fig. 6). Also, at 63% similarity level, high strain diversity was also observed within *B. amyloliquefaciens* (13 strains), which are genetically different from reference strain *B. amyloliquefaciens* MTCC 1270 (Fig. 7). Thus, polyphasic genomic techniques as used in this study were useful in identification, strain differentiation and comprehensive understanding of the diversity of *Bacillus* strains.

MLSA for Subtyping *Bacillus cereus sensu lato*

The *B. cereus* phylogeny comprising *B. cereus, B. mycoides, B. pseudomycoides, B. weihenstephanensis, B. anthracis,* and *B. thuringiensis* showed high similar ARDRA, ITS-PCR, ITS-PCR-RFLP, and RAPD profiles, without any species differentiation. This necessitated PCR amplification of *gyrB, glpF,* and

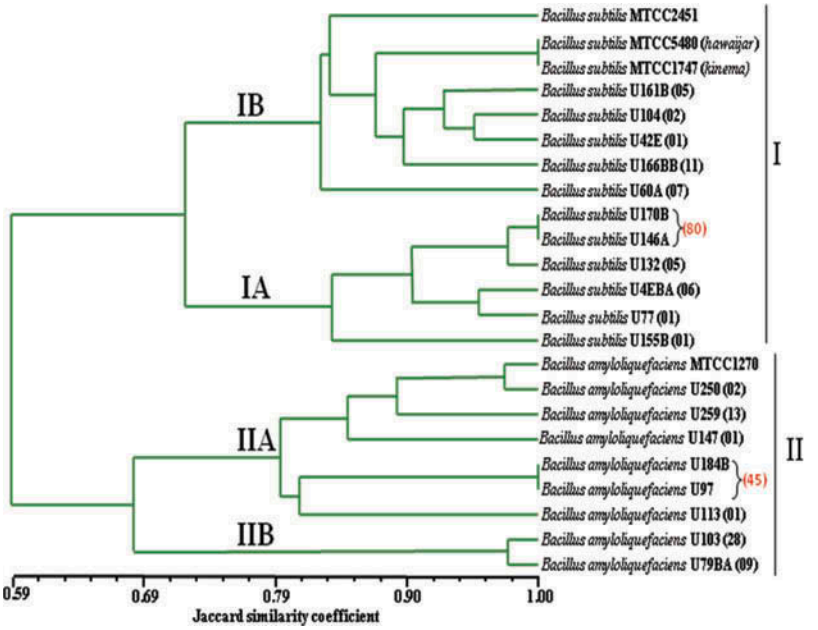

Figure 5: Dendrogram based on UPGMA clustering of Jaccard similarity coefficient (S_j) of normalized OPA 18 RAPD-PCR fingerprints of dominant *B. subtilis* and *B. amyloliquefaciens* strains.

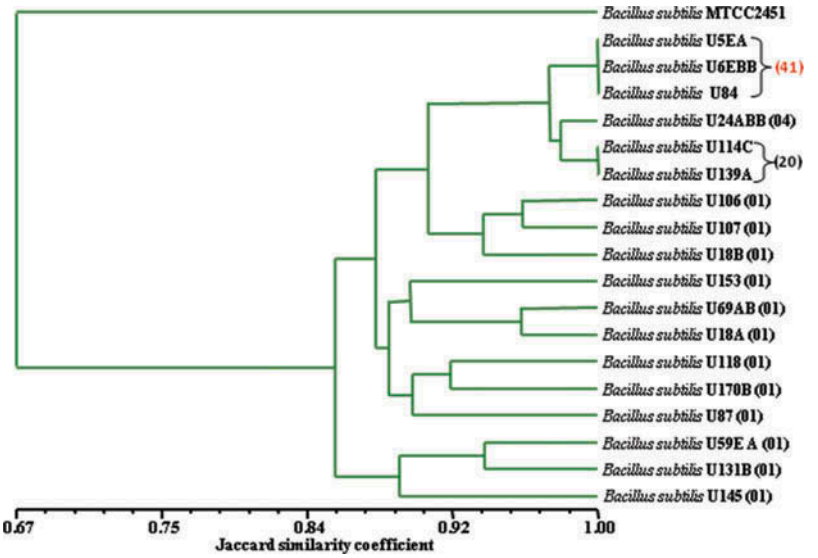

Figure 6: Dendrogram based on UPGMA clustering of Jaccard similarity coefficient (S_j) of normalized M13 RAPD-PCR fingerprints of dominant *B. subtilis* strains.

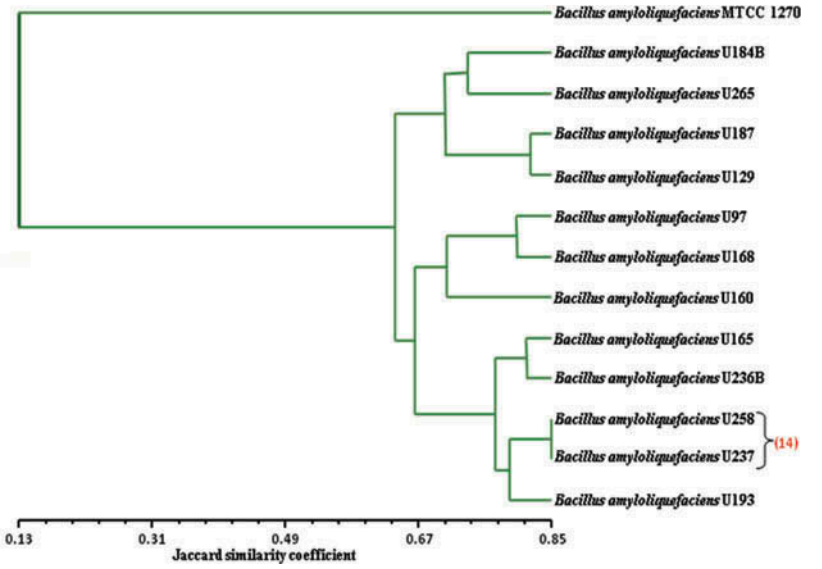

Figure 7: Dendrogram based on UPGMA clustering of Jaccard similarity coefficient (S_j) of normalized M13 RAPD-PCR fingerprints of dominant *B. amyloliquefaciens* strains.

gmk housekeeping genes for possible discrimination. DNA sequences obtained were compared with those deposited in GenBank using the BLAST program; this identified 96% of the *B. cereus* group as *B. cereus* and 4% as *B. thuringiensis*. *gyrB, glpF* and *gmk* gene sequences of *B. cereus* and *B. thuringiensis* strains and other strains deposited in GenBank database were further analyzed by the construction of phylogenetic trees as described earlier. This revealed phylogenetic and clonal relationship of common ancestral origin between *B. cereus* and *B. thuringiensis*; it also established *B. cereus* as the major member of the *B. cereus sensu lato* associated with fermented *iru*. Also, *B. cereus* and *B. thuringiensis* strains from the present study are totally diverged from other strains, information that shows that the strains are geographically domesticated (Figs. 8–10).

DISCUSSION

Different genomic typing techniques were used to determine phylogenetic relationship and diversity among autochthonous *Bacillus* species isolated from African and Asian traditional fermented condiments. This provides a foundation for the requirement for screening and selection of starter cultures for industrial production of condiments with desirable functional properties as well as food safety and quality.

The results of the phenotypic characterization based on colonial features, Gram's staining, catalase reaction, and endospore staining identified the

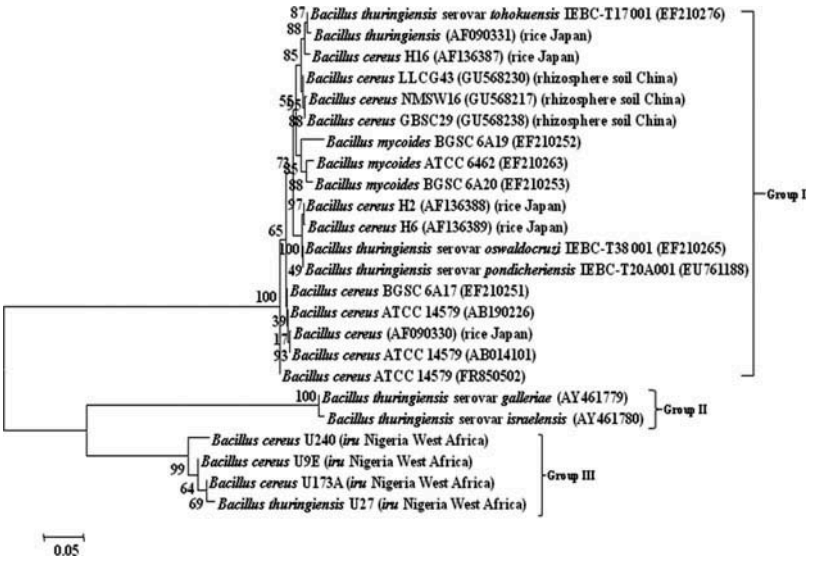

Figure 8: Phylogenetic relationship of *B. cereus* phylotype based on nucleotide sequences of *gryB* gene.

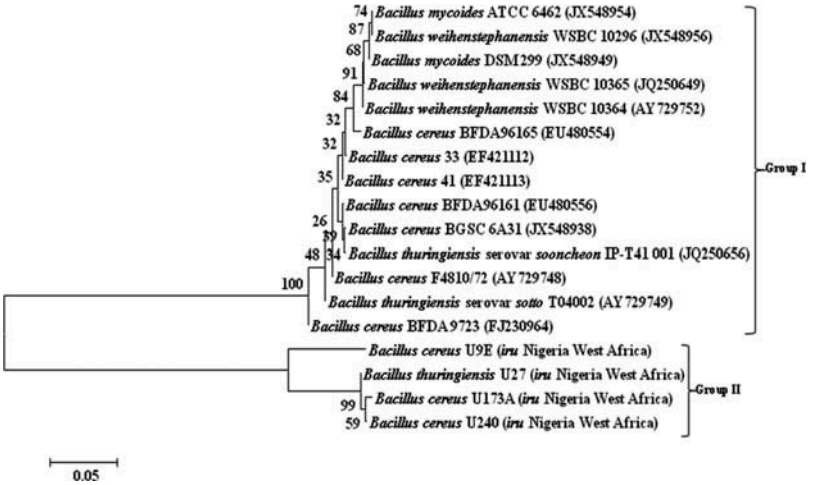

Figure 9: Phylogenetic relationship of *B. cereus* phylotype based on nucleotide sequences of *glpF* gene.

strains as *Bacillus* species and its closest relatives. Previous studies in Africa employed conventional phenotypic characteristics for the identification of *Bacillus* species (Odunfa, 1985; Ikenebomeh, 1989). Although emerging information on the use of genetic characterization for identification of bacilli isolated from *okpehe* and *soumbala* are in agreement with our results (Ouoba et al., 2004; Oguntoyinbo et al., 2010), none of these studies described the

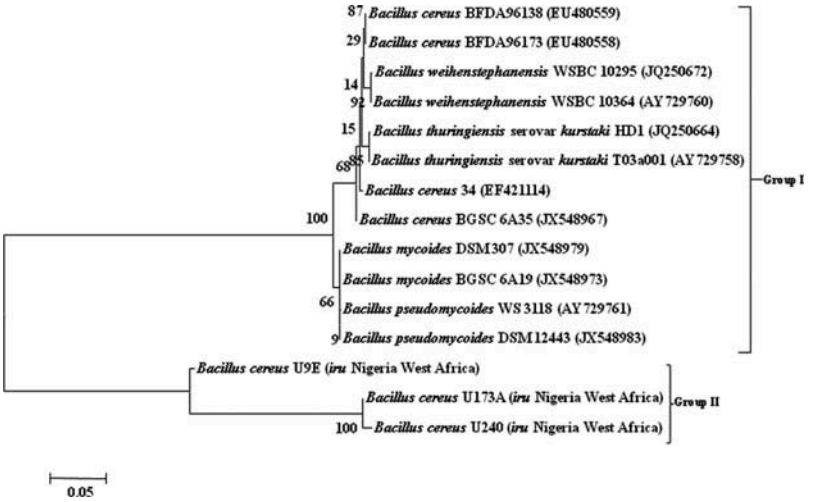

Figure 10: Phylogenetic relationship of *B. cereus* phylotype based on nucleotide sequences of *gmk* gene.

phylogenetic relationship among bacilli strains from different regions. The phylogenetic analysis based on 16S rRNA gene showed the genetic relationship among the bacilli strains and established that most of the species must have originated from common ancestor, adapted and domesticated into vegetable protein environment both in Africa and Asia.

The species and strains divergence especially between *B. subtilis* and *B. cereus* group strains may be due to horizontal gene transfer and recombination. Also, reported phenotypic characteristics of these strains showed that *B. subtilis* are highly amylolytic and proteolytic, while *B. pumilus* are nonamylolytic but proteolytic. Whereas, *B. cereus* are weakly proteolytic and amylolytic but must have acquired genes for production of toxins such as emetic and diarrhea over time via recombination and transposable elements (De Palmenaer et al., 2004; Oguntoyinbo and Sanni, 2007; Didelot et al., 2009). Apart from the dominant *Bacillus* species isolated from *iru*, another closely related genus, *Brevibacillus* was detected; this confirmed the earlier reports of the implication of *Brevibacillus bortelensis* in *soumbala* and *bikalga* (Ouoba et al., 2004, 2007). The results of the phylogenetic analysis indicated five different clades, which clustered separately on the basis of species; it also established divergence, evolutionary, and clonal relationship of bacilli from Africa. Although we observed poor 16S rRNA gene sequences discrimination of *B. subtilis* from *B. amyloliquefaciens,* this is also in agreement with previous reports confirming the inability of this gene to differentiate closely related species of *Bacillus* because of their clonal relatedness (Stackebrandt et al., 2002; Santos and Ochman, 2004).

Various molecular fingerprints have been developed to identify clonally related *Bacillus* species. Wu et al. (2006) used group-specific primer combined with ARDRA to distinguish *Bacillus* species and other related genera. Oguntoyinbo et al. (2010) described ARDRA as effective technique to differentiate *B. subtilis* from *B. cereus*. ARDRA used in the present study successfully differentiated the *B. subtilis* group, which hitherto has been a difficult task (Vaerewijck et al., 2001; Parkouda et al., 2010); this makes it a simple, rapid, and reliable molecular technique for distinctly discriminating closely related species of bacilli. The combined results of ITS-PCR and ITS-PCR-RFLP showed high intraspecies variation among the *B. subtilis* strains as observed previously (Jeyaram et al., 2008, 2010). However, in a related study, ITS-PCR-RFLP only allowed genomic typing at species level (Ouoba et al., 2004). The dendrograms obtained from RAPD-PCR analyses of OPA 18 and M13 primers showed high strain level diversity among the dominant *Bacillus* species from *iru*, and they were also phylogenetically different from *hawaijar, kinema,* and other reference strains. Different studies have confirmed the effectiveness of RAPD-PCR for precise strain typing of *B. subtilis* (Sarkar et al., 2002; Matarante et al., 2004; Inatsu et al., 2006; Jeyaram et al., 2008). The *B. cereus* phylogeny showed no species differentiation even with the combined efforts of the various techniques employed (i.e. colony morphology and genomic characteristics). This is perhaps because all the species in this group have high degree of sequence similarity, which makes them closely related (Manzano et al., 2003). Several attempts were made previously using 16S rRNA sequence-based analysis; single-strand conformation polymorphisms of amplified 16S rRNA gene; virulence parameters; pulsed-field gel electrophoresis; ITS-PCR; restriction fragment length polymorphisms (RFLP), including DNA:DNA hybridization for possible discrimination of the *B. cereus* group (Harrell et al., 1995; Keim et al., 1997; Ramisse et al., 1996; Borin et al., 1997; Yamada et al., 1999; Ahaotu et al., 2013) but none of these was able to differentiate *B. cereus* from *B. thuringiensis*. Multilocus sequence analysis of housekeeping genes used in this study prove to be a better and promising technique for differentiation of closely related *B. cereus* species and can have potential application during industrial processing of fermented foods. Other investigators have also used this technique to study the genetic relationship in the *B. cereus* group obtained from different sources (Cardazzo et al., 2008; Didelot et al., 2009).

Our results showed phylogenetic relationship between bacilli isolated from different condiments in Africa and Asia with genomic strains differentiation indicating high level of diversity among strains within species. This study can be of technological interest, since many functional traits are strain-specific rather than being species-specific. Phylogenetic markers developed can be effectively used in differentiating and authenticating *Bacillus* strains from fermented *iru* and other commercially available strains. It is also important to properly identify divergent functional properties associated with different

strains identified in this study. Such data will facilitate development of predictable process through selection of strains with desirable functional quality characteristics as well as improve shelf life and safety quality.

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