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Towards understanding the diversity of banana bunchy top virus in the Great Lakes region of Africa

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The genetic variability of banana bunchy top virus (BBTV) isolates from the Great Lakes region of Africa (GLRA) spanning Burundi, the Democratic Republic of the Congo and Rwanda was assessed to better understand BBTV diversity and its epidemiology for improved disease management. DNA-R and DNA-S fragments of the virus genome were amplified and sequenced in this study. These two BBTV fragments were previously used to classify isolates into the South Pacific and the Asian groups. Phylogenetic analyses based on nucleotide sequences involving GLRA isolates and those obtained from the GenBank database were carried out. Sequence similarity for both DNA-R and DNA-S fragments ranged between 99.1 to 100.0% among the GLRA isolates, 96.2 to 100.0% and 89.7 to 94.3% between the GLRA isolates and those previously clustering in the South Pacific and the Asian groups, respectively. These results showed that GLRA isolates belong to the South Pacific group and are phylogenetically close to the reference Indian isolate. The similar banana cultivars and BBTV isolates across the GLRA implied that the disease may have mainly spread through exchange of planting material (suckers) between farmers. Thus, farmers' awareness and quarantine measures should be implemented to reduce BBTV spread in the GLRA.

Key words: Banana bunchy top disease (BBTD), *Musa* spp., *Pentalonia nigronervosa,* virus genome.

INTRODUCTION

Musa spp. (banana and plantain) is a staple food crop for approximately 400 million people worldwide and nourishes over 70 million people in sub-Saharan Africa (AATF, 2003). This crop is ranked the first in terms of contribution to the total annual agricultural production in Burundi and Rwanda while it is the second after cassava in the Democratic Republic of the Congo (DR Congo) (FAOSTAT, 2009). The perennial nature of banana, compared with other staples, allows households to access food all-year round, providing significant amounts of micronutrients (Kumar et al., 2011). Among banana cultivars grown in Africa, plantain types (AAB genome)

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are mainly found in the humid lowlands of West and Central Africa, while the highland cooking and beer banana (AAA-EA) which contribute to approximately 30% of world banana production are common in the Eastern African highlands (Tenkouano et al., 2003). Eastern Africa, including the Great Lakes zone, is considered as a secondary centre of diversity for the highland banana (Karamura et al., 1998; Tenkouano et al., 2003) where smallholder farmers grow a mixture of 5 to 10 different cultivars around their homesteads (AATF, 2003). Banana plantations are subjected to various natural calamities, in particular viral diseases, limiting their production. Among the viral infections, banana bunchy top disease (BBTD) is reported as the most destructive disease (Dale, 1987; Islam et al., 2010; Stainton et al., 2012).

BBTD was first reported from the Fiji Islands in 1889, but its causal agent was only identified 100 years later in 1990s (Magee, 1927; Wardlaw, 1961; Kumar et al., 2011), and was given the name, banana bunchy top virus (BBTV) (Karan et al., 1994). Currently, BBTV has spread to 33 countries worldwide (excluding the Americas) including 13 African countries (IITA, 2010).

BBTV spreads from one location to another by exchange of infected planting material and from plant to plant through the banana aphid, *Pentalonia nigronervosa* Coquerel (Hemiptera, Aphididae), but is not transmitted mechanically (Thomas and Dietzgen, 1991; Foottit et al., 2010). The banana aphid transmits the virus with high host specificity to *Musa* spp. in a circulative and persistent manner (Hafner et al., 1995; Hogenhout et al., 2008; Foottit et al., 2010). In the plant, replication of these circulative viruses is frequently restricted to the phloem providing a route for uptake and inoculation of viruses between plants via stylet-feeding aphids (Hogenhout et al., 2008). The virus is also transmitted over long distances through the movement of BBTV-infected planting materials (Kumar and Hanna, 2008; Vishnoi et al., 2009; Kumar et al., 2011).

BBTD is easily recognizable from other banana diseases by its characteristic symptoms consisting of dark green streaks on leaves and petioles, marginal leaf chlorosis, dwarfing of the plant and leaves that stand more erect and bunched at the top of the pseudostem, forming a rosette with a ‘bunchy top’ appearance (Magee, 1927; Su et al., 2003).

The BBTV is a member of family *Nanoviridae,* genus *Babuvirus* belonging to a group of circular single-stranded DNA (cssDNA) viruses (Allen, 1987; Amin et al., 2008; Karan, 1995). It is an isometric virus with a genome consisting of at least 6 fragments (Harding et al., 1993; Horser et al., 2001; Hu et al., 2007) and two components were considered in this study. The DNA-R encodes the ‘master’ Rep (M-Rep) that directs self replication in addition to replication of other BBTV genome fragments (Harding et al., 1993; Karan et al., 1994; Theresa, 2008). On the other hand, the coat protein (CP) is encoded by DNA-S for the integral BBTV fragment (Horser et al., 2001). Based on sequence analysis of DNA-R and DNA-S (CP) fragments, respectively, Karan et al. (1994), Wanitchakorn et al. (2000) and Kumar et al. (2011) demonstrated that BBTV isolates can be clustered into two distinct groups. The ‘South Pacific’ group comprising isolates from Australia, the South pacific region, South Asia (that is, India, Pakistan) and Africa; while the ‘Asian’ group comprises isolates from China, Indonesia, Japan, the Philippines, Taiwan and Vietnam (Horser et al., 2001). Although BBTD has long been recognised (Magee, 1927), molecular characterisation of BBTV began in the early 1990s (Harding et al., 1993). In Africa, a handful of BBTV isolates from sub-Saharan Africa (SSA) have been characterized (Wanitchakorn et al., 2000; Kumar et al., 2011) which includes only a single isolate originating from Burundi (accession AF148943). To date, significant molecular characterization using a substantial number of samples from the African Great Lakes region is lacking. To better understand BBTV diversity and its epidemiology for accurate BBTD management, knowledge of the molecular nature of BBTV in Africa is required. In this study, the DNA-R fragment and the coat protein (CP) (Wanitchakorn et al., 2000; Horser et al., 2001; Furuya et al., 2005; Kumar et al., 2011) were used to characterize BBTV isolates from the African Great Lakes region. BBTV isolates from the GLRA were compared with isolates already available in existing GenBank databases to assess their relationship with the Asian and South Pacific groups. In addition, the likely sampling site at different altitudes and influence of banana cultivar on sequence mutations within the GLRA were considered.

MATERIALS AND METHODS

Sampling

Banana leaf samples were collected in regions affected by BBTD in three countries namely Burundi, DR Congo and Rwanda from April to May 2010. Duplicate pieces of banana leaves of approximately 4 cm^2 each were taken from the youngest leaf of a banana plant displaying advanced BBTD symptoms. Leaf pieces were placed in individual Petri dishes lined with silica gel for the duration of the transport and transferred to the laboratory, where they were extracted and stored at -20°C pending use (Chase and Hills, 1991). In all, 37 samples were collected from five Provinces of Burundi (Bubanza, Bujumbura Rural, Bururi, Cibitoke and Makamba), 22 from three districts in the Eastern South Kivu DR Congo (Kabare, Nyangezi and Kamanyola) and 20 from the Rusizi district of the Western Province of Rwanda, giving a total of 79 samples. These samples were collected from diverse local banana genotypes namely AAA-EA, ABB, AAB and AABB types cultivated at different altitudes across the three countries. Diagnostic tests confirming the viral status of samples were performed using previously described PCR analysis (Harding et al., 1993; Thomson and Dietzgen, 1995).

PCR analyses and sequencing based on DNA-R and DNA-S virus genome

Leaf pieces were placed in mesh plastic bags (Agdia Biofords,

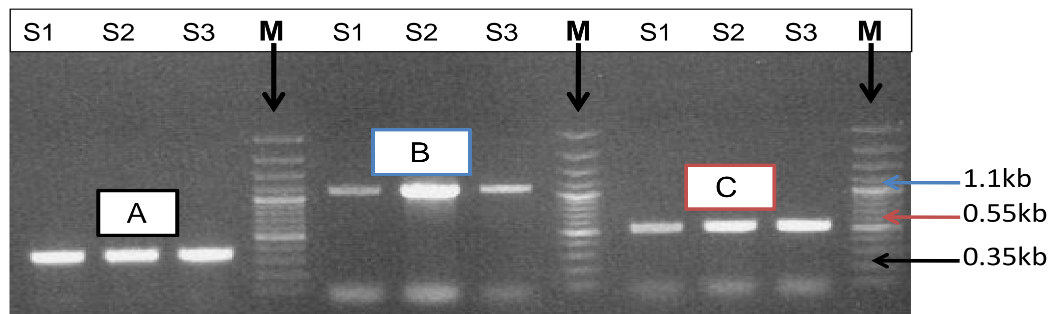

Figure 1. Molecular detection profile of banana bunchy top virus putative replicate gene (A=349 bp), DNA-R (B=1111bp) and coat protein (C=550 bp) components. Lane M: 100-bp ladder (Fermentas, France). S1, S2, S3 are examples of amplified samples from the African Great Lakes region.

France) and 2 ml of extraction buffer at 4°C was added (Busogoro et al., 2009). The crude extract was obtained for each sample by thoroughly crushing banana leaves before being dispensed in three 200 µl aliquots and stored at -20°C pending PCR analyses. PCR amplifications were carried out using diluted extracts (1:100 in distilled water). The PCR amplification was performed using specific primer pairs previously described for BBTV CP and DNA-R fragments (Harding et al., 1993; Thomson and Dietzgen, 1995; Amin et al., 2008). All 79 samples were first subjected to the PCR amplification of a 349 bp fragment of the putative BBTV replicase gene using primer pairs BBT1 forward (5'-CTCGTCATGTGCAAGGTTATGTCG-3') and BBT2 reverse (5'-GAAGTTCTCCAGCTATTCATCGCC-3') for detection of the virus in different samples (Harding et al., 1993; Thomson and Dietzgen, 1995). The selected representative positives samples were then amplified using primer pairs, MREPF forward (5'-GAATTCAAGAATGGAATAATTC-3') and MREPR reverse (5'-GAATTCCTCTAATAACCC-3') described by Amin et al. (2008) targeting amplification of DNA-R fragment, whereas primer pairs CPXI.PRI forward (5'-GCTAGGTATCCGAAGAAATCC-3') coupled with BBTV3C.EXP reverse (5'-ATAAAGCTTTCAAACATGATATGT-3') described by Wanitchakorn et al. (2000) were used to amplify the BBTV DNA-S coat protein fragment.

The PCR reactions were set up in a final volume of 50 µl comprising 5 µl of crude extract (diluted 1:100 in distilled water), 5 µl of 10x PCR buffer (Roche), 6 µl of $MgCl_2$ (25mM), 1.2 µl (200 µM/each) of dNTPs mix, 1 µl of each primer (0.5 µM), 0.25 µl (1.25 u/50 µl) of *Taq* DNA polymerase obtained from Fermentas-France and sterile distilled water (30.55 µl) was added to make the final volume (Amin et al., 2008; Burns et al., 1995). The PCR procedure was performed using MyCycler from Bio-Rad, Belgium. The thermo-cycling scheme consisted of denaturation at 94°C for 4 min; 40 cycles of 30 s to 1 min at 94°C, 1 min at 52°C and 2 min at 72°C followed by a final elongation step at 72°C for 10 min. The amplified products were visualized by electrophoresis in a 1% (w/v) agarose gel using ethidium bromide staining along with 100 bp ladder from Fermentas, France. Gels were then photographed on a digital gel documentation system. PCR products were quantified in ng/µl using NanoDrop ND-1000 spectrophotometer machinery with a limit of 1.80 values at A260/280 absorbance ratio. Amplified specific products to each of the DNA-R and CP fragments were then shipped for subsequent sequencing, using the same primer pairs, at Macrogen in South Korea.

Phylogenetic analyses (sequence alignment and phylogenetic tree) of BBTV nucleotide sequences

The nucleotide sequences of BBTV DNA-R and CP fragments of the GLRA isolates were compared in a pairwise matrix with existing BBTV and *Abaca bunchy top viruses (ABTV)* sequences obtained from the GenBank database using the Basic Local Alignment Search Tool (BLAST) available on the National Centre for Biotechnology Information (NCBI) (Theresa, 2008; Vishnoi et al., 2009). Multiple alignments for sequence comparison were performed using CLUSTALO (Thomson and Dietzgen, 1995; Amin et al., 2008). The genetic diversity of BBTV isolates was determined between GLRA isolates and those representing reference isolates from the previously described Asian and South Pacific groups including a previously sequenced Burundian isolate (AF148943) and other isolates from sub-Saharan Africa (Kumar et al., 2011). The consensus trees were generated using neighbour-joining algorithms with 100 bootstrap replications with Sea View Version 4.2.9 (Gouy et al., 2010).

RESULTS

PCR detection and sequencing of BBTV

The BBTV was confirmed in all 79 samples collected from symptomatic banana plants using a primer pair targeting the putative replicase gene (349 bp) of the BBTV genome. Among these positive samples, BBTV DNA-R and CP fragments of 27 representative samples covering the different localities and banana varieties were amplified using corresponding primer pairs of each fragment. Among those samples, 14 same isolates were successfully amplified for DNA-R and CP in addition to 8 and 5 different isolates for DNA-R and CP, making a total of 22 and 19 isolates, respectively. The PCR products of DNA-R (1111bp) and CP (550bp) fragments (Figure 1) were sequenced and used in comparisons.

Sequence analysis of BBTV based on coat protein fragment

The phylogenetic analysis was carried out using the sequences of a 475 bp product representing the BBTV-CP fragment. The sequence comparisons showed a nucleotide sequence identity between the BBTV isolates from the GLRA (sequenced in this study) greater than

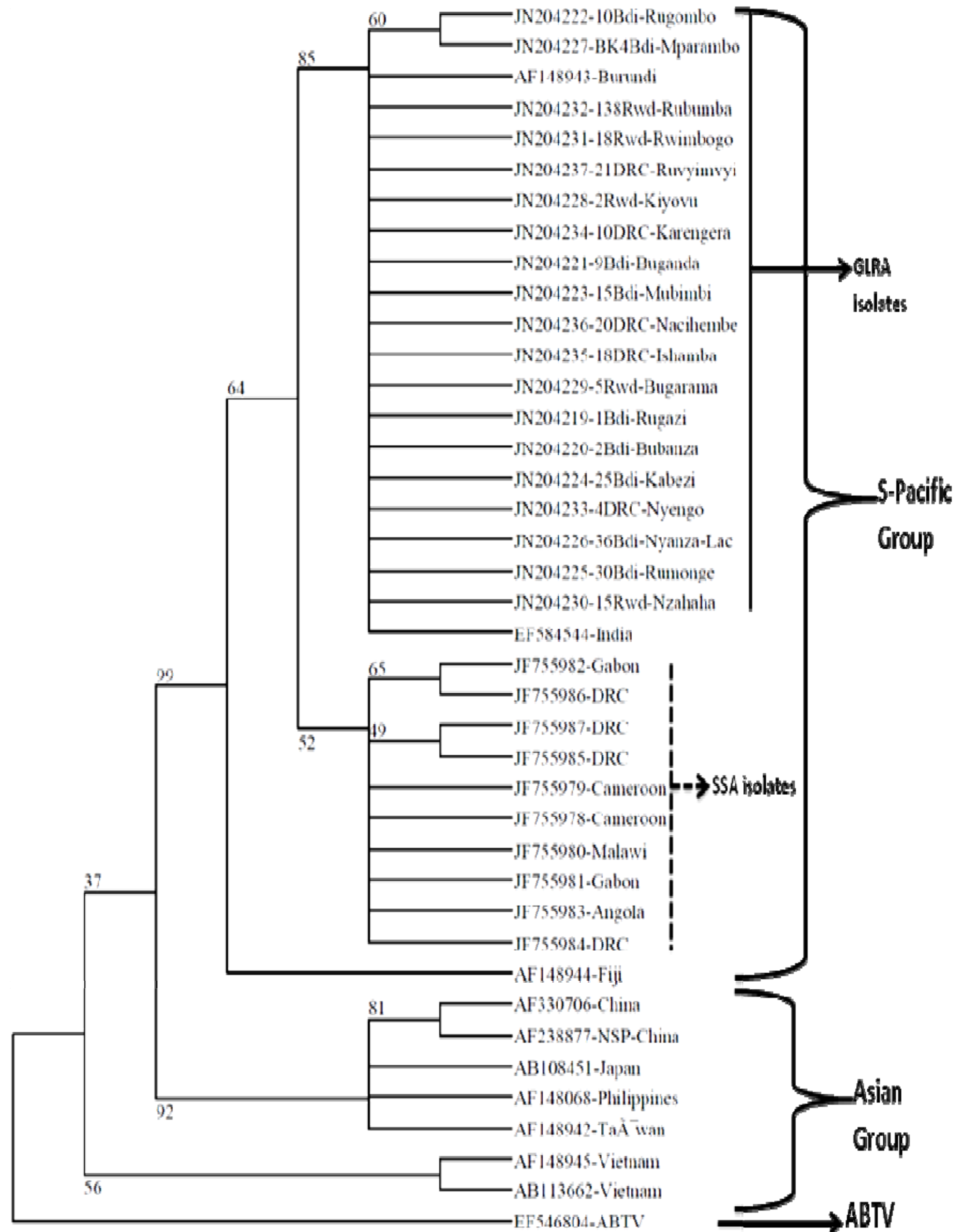

Figure 2. Neighbour-joining tree showing relationships based on BBTV CP nucleotide sequences of 19 isolates collected in the African Great Lakes Region (GLRA) compared with representative BBTV and ABTV isolates both obtained from the GenBank database.

99%. The GLRA isolates showed nucleotide sequence identity ranging from 97.2 to 99.7% with the South Pacific group which include isolates from sub-Saharan Africa (SSA), whilst they share only between 89.8 and 94.3% identity with CP nucleotide sequences of Asian isolates. On the other hand, pairwise comparisons between CP nucleotide sequences from the Asian and South Pacific groups showed higher sequence variability among isolates of the Asian group (99.3 to 92.0%) than among isolates from the South Pacific (100.0 to 95.2%). Phylogenetic analysis based on the BBTV CP nucleotide sequences confirmed the clustering of BBTV isolates into two major groups, the Asian and the South Pacific groups. The South Pacific group consists of all GLRA isolates including Burundian isolate (AF148943) that was deposited earlier in GenBank and Indian isolate (EF584544) followed by isolates from sub-Saharan Africa and Fiji (Figure 2). Within the South Pacific group, three

sister subgroups with high bootstrap support (99%) were distinguished. The first subgroup includes all GLRA isolates (sequenced in this study), the Burundian isolate (AF148943) previously reported and the Indian isolate (EF584544). The second subgroup is represented by all sub-Saharan isolates, while a single isolate from Fiji (AF148944) was classified in the third subgroup. The Asian group was divided into two main subgroups, the subgroup which includes isolates from China, Japan, Philippines and Taiwan and the subgroup of Vietnam's isolates with bootstrap support of 92 and 56%, respectively (Figure 2).

Sequence analysis of BBTV based on DNA-R genome fragment

Sequence analysis was carried out using a 238 bp DNA fragment for each of the 22 isolates, corresponding to the core region of the BBTV DNA-R. The core region was considered for the purpose of sequences comparisons using the same size of the majority of reported BBTV sub-Saharan Africa isolates available in GenBank database. Nucleotide sequence comparisons showed greater than 99% identity among GLRA isolates. BBTV GLRA isolates showed high levels of nucleotide sequence similarity with the South Pacific group (96.2 to 100.0%) compared with the Asian group isolates (89.7 to 93.4%). In addition, the nucleotide sequence variability was rather high within the Asian group (99.3 to 89.3 %) compared with those of the South Pacific group including the GLRA isolates (100.0 to 95.8%).

Phylogenetic analysis based on Rep sequences using the neighbour-joining method has also confirmed the previous reports of the clustering of BBTV isolates into the Asian and the South Pacific groups with high bootstrap support (100%). Four subgroups were distinguished among South Pacific isolates, the first includes all GLRA and SSA isolates followed by the Indian isolate (AF 416470-In); the second subgroup includes isolates from Australia, Fiji, Tonga, Hawaiï and Pakistan, while Egypt is in its own subgroup. The GLRA and other SSA isolates show the closest relationship with a Maharashtra isolate from India.

Among the GLRA isolates, the 2 isolates collected in DR Congo (JN204218-18DRC and JN204217-13DRC) at different altitudes and from different banana cultivars ('Malaya' and 'Yangambi Km5') were grouped together, whereas four isolates from the south of Burundi (JN204206-36Bdi, JN204204-33Bdi, JN204205-35Bdi and JN204203-30Bdi), collected in similar locations but from different banana cultivars ('Yangambi Km5', 'Igisahira', 'Indarama' and 'Kayinja'), formed another subgroup. Interestingly, among SSA isolates, the Cameroun isolate (JF755989-TV13.1) grouped together with the GLRA isolates, while 9 other isolates from SSA belonged to a different subgroup (Figure 3).

DISCUSSION

This study contributed to better knowledge of the GLRA BBTV genome in comparison with other isolates from South Pacific and Asian zones based on two BBTV genome fragments (DNA-R and CP). The BBTV-CP GLRA sequences compared with those of the South Pacific and the Asian groups showed nucleotide differences ranging from 0.7 to 2.8 and 5.7 to 10.2%, respectively. This corroborates previous estimations of 3% variability among the South Pacific group isolates and around 6% across the Asian group isolates (Wanitchakorn et al., 2000). On the other hand, using BBTV DNA-R fragment (Rep) of GLRA isolates, a range of 0.9 to 3.8% and 6.6 to 10.3% of nucleotide differences were comparable to the previous averages of 3.8% among South Pacific group isolates and approximately 10% between the two groups (Karan et al., 1994). Additionally, the phylogenetic analysis using these two genome fragments strongly confirmed that all GLRA isolates belong to the South Pacific group (Figures 2 and 3).

Among the South Pacific isolates, based on the CP nucleotide sequences, the Indian isolate (EF584544) showed a closer relationship with the GLRA isolates than other SSA isolates. Using the core region of the DNA-R fragment, all BBTV isolates from sub-Saharan Africa grouped together followed by the Indian isolate. Additionally, the interrelationships among the SSA isolates showed that Cameroon isolate (JF755989) fell within the group of GLRA isolates. Karan (1995) had suggested that BBTV infections should have two major sources, one in Asia and another in the South Pacific, while Stainton et al. (2012) based on the evidence of re-assortment and recombination events within and between the Asian and the South Pacific BBTV subgroups support the hypothesis of the same geographical origin of both subgroups. Irrespective of the means of the first BBTV introduction, the GLRA isolates fall within the South Pacific group and may spread through either the traditional farmers' practices of intra-and inter-regional exchange of suckers for planting material or introduction of infected plants from research stations (that is, as observed in Rwanda during the survey, banana field of ISAR research station at Bugarama, Rusizi valley, have been reported to have contributed to the spread of BBTV in surrounding areas). Aphids may have extended the spread between plantations at a local level (Kumar et al., 2011). The two gene sequence-based phylogenies (Figures 2 and 3) suggest that the virus isolates from the GLRA could have originated from India rather than from other countries of the South Pacific through exchange of non indexed virus-free banana plantlets before development of diagnostic molecular tools.

In the Great lakes region, previous survey work (Sebasigari and Stover, 1988) suggested in 1987 that BBTD might have been present since the early 1970s in

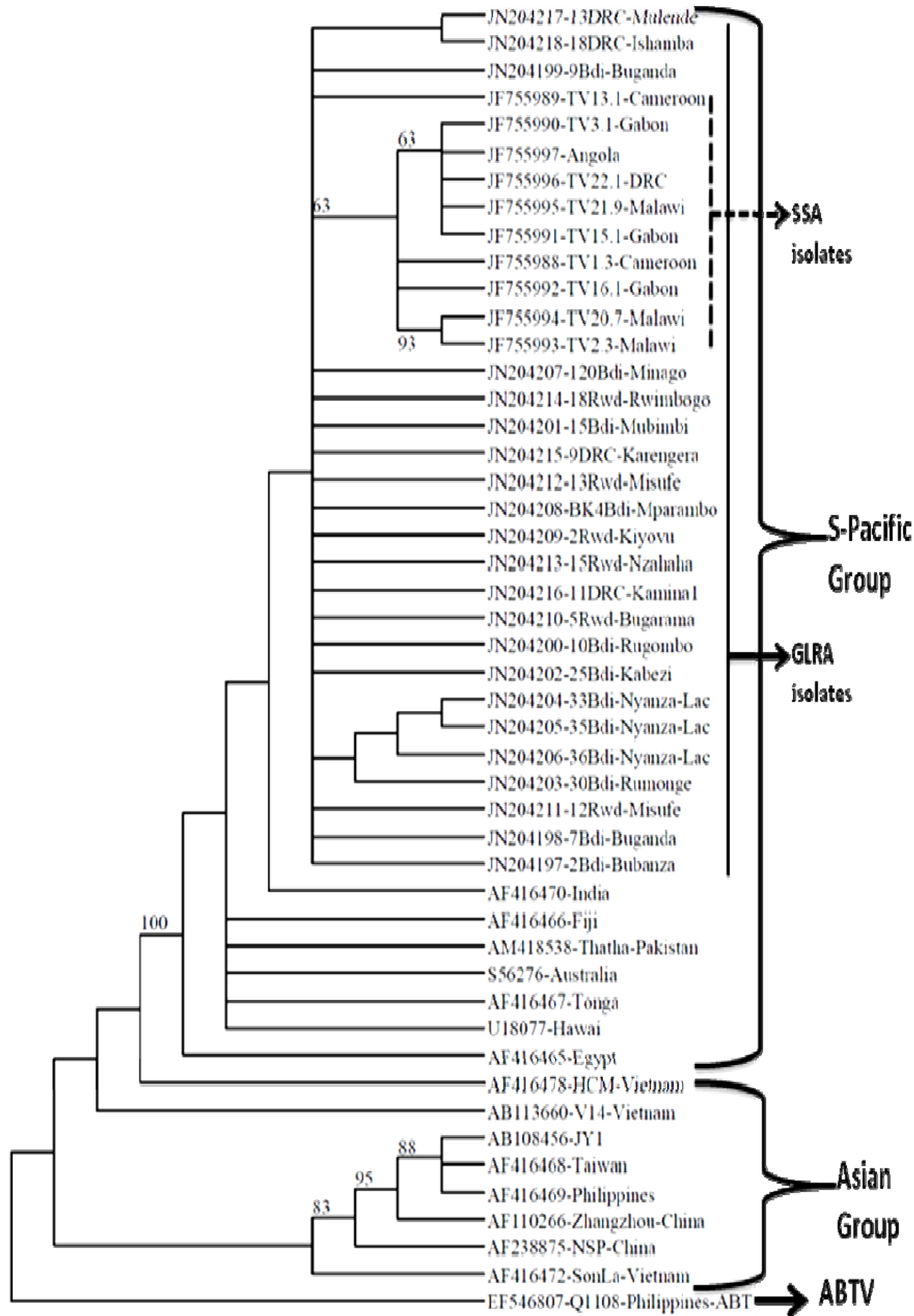

Figure 3. Neighbour-joining tree illustrating the BBTV DNA-R sequence relationships among 22 isolates from the Great Lakes Region of Africa (GLRA) compared with 26 representative isolates from GenBank database.

the Rusizi valley (encompassing parts of Burundi and Rwanda) while the DNA-R sequence variation was around 0.9% among GLRA isolates. In other countries within the south Pacific group such as Pakistan, the DNA-R sequence variations of 0.45% were reported 20 years after disease identification compared with 2% reported over 80 years in Australia (Karan et al., 1994).

The BBTV isolates across the three countries of the GLRA were grouped together. This suggests similarity in origin of GLRA isolates which were most likely distributed

through exchange of planting material. It is likely that the virus was introduced in the Rusizi valley and its surrounding regions of Burundi, Eastern South-Kivu DR Congo and Rwanda by the exchange of ‘Yangambi Km5’-(AAA genome) banana variety commonly grown in those regions. This is a cultivar that originated from INEAC Yangambi Agricultural Research station in the central DR Congo where BBTV was reported as early as the 1950s (Wardlaw, 1961; Kavino et al., 2007).

The BBTV isolates from the same region, but of different altitudes and banana cultivars, were grouped together. This implies the likely virus mutation according to the introduction period regardless the different types of banana cultivars or altitudes, corroborating previous reports which stated that sequence variation of the virus is strongly dependent on the period of time it has spent in a region (Tenkouano et al., 2003, Amin et al., 2008).

Overall, the use of tolerant cultivars should be associated with collective eradication of BBTV-infected mats to reduce virus inocula for long term BBTV management. In fact, the lack of farmers’ awareness on transmission and management practices could be the main factor explaining the continuous spread of BBTD in the GLRA. Therefore, there is a need of stricter regional policy in an attempt to manage BBTD and prevent further spread of the virus in areas not yet affected by the disease. This involves raising farmers’ awareness and implementation of quarantine measures. Further research on the virus using all six BBTV fragments should study the sequence variation and provide a complete view of the evolutionary processes based on BBTV likely recombination and re-assortment within the African Great Lakes region.

Conflict of Interest

The authors have not declared any conflict of interest.

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