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Genetic Diversity and Phylogenetic Relationships of Three Viruses Infecting Yam (*Dioscorea* Spp.) in Guinea Savanna Zone of Nigeria

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Abstract

Yam mosaic virus (YMV), *Yam mild mottle virus* (YMMV) and *Dioscorea alata bacilliform virus* (DaBV) are important viruses infecting different species of yam in Nigeria. Only YMV has been well characterised in sub-Saharan Africa. The aim of this study was to characterise the three viruses in Guinea Savanna zone of Nigeria and compare them with counterpart viruses of yam in other parts of the world. Purified nucleic acid products prepared in the Virology and Molecular Diagnostic Laboratory of the International Institute of Tropical Agriculture, Ibadan, Nigeria were shipped to the Molecular Laboratory, Iowa State University, United States of America for sequencing. Twenty-two (22) sequence data of virus isolates from the two locations in Nigeria were evaluated. Sequences of counterpart viruses were obtained from GenBank. YMMV in isolates from *Dioscorea alata* from the two locations were 100% and 99% related to isolates (*D. rotundata*) from Ghana and Guadeloupe, respectively. YMVs in isolates from Gbakumo and Dan-anachia (*Dioscorea rotundata*) from the two locations were 81% and 65% related to counterpart isolates from Cameroun and Cote d'Ivoire, respectively. The presence of strong geographical clustering and relationship between viruses from isolates in the study and other counterparts suggests regional and widespread distribution of these viruses.

Key words: Characterisation, diversification, viruses, yam, Nigeria

1. Introduction

Yam (*Dioscorea* spp.) is an important staple food for much of the sub-Saharan Africa and in Nigeria where it constitutes the principal root crop in terms of land under cultivation, volume, and value of production (Coursey, 1967; Bamire and Amujoyegbe, 2005; Asala et al., 2013). Yield losses due to pests such as insects, diseases and weeds have constituted a problem to yam cultivation (Akinlosotu,1985; Okoroafor, 2009; Asala et al., 2014). Most edible yam species produce true seeds which are infertile, thus most propagation and multiplication of yam is through vegetative planting of cuttings (seed yam) or pieces of tuber (setts). This vegetative propagation has increased the perpetuation and accumulation of diseases, particularly those that are caused by viruses. The use of small tubers from previous harvest has allowed the selection of infected lines (Asala et al., 2013).

Several viral diseases have been reported to infect yams in Africa, Asia, and Central America (Coursey, 1967). Viruses of the general *Potyvirus, Potexvirus, Badnavirus Cucumovirus* and *Caviavirus* infect yam of different species worldwide. These viruses cause a range of symptoms including mosaic, mottle, vein clearing, chlorosis, stunting and distortion (Kenyon et al., 2001; Atiri et al., 2003; Asala et al., 2012). These have led to chronic, and sometimes, severe disease conditions in all yam growing areas. In sub-Saharan Africa where more than 90% of the world's yam is produced (FAOSTAT, 2009), *Yam mosaic virus* (YMV) Genus *Potyvirus* is particularly important and is the only well characterised virus infecting yam in sub-Saharan Africa (Goudou-Urbinoet al., 1996; Njukeng et al., 2002; Osmond, 2006). Other viruses infecting yam in Africa are: *Dioscorea alata bacilliform virus* (DaBV) Genus *Badnavirus*, *Cucumber mosaic virus* (CMV) Genus *Cucumovirus*, *Dioscorea alata virus* (DAV) Genus *Potyvirus, Dioscorea dumetorum virus* (DdV) Genus *Potyvirus* (Hughes et al., 1997; Odu et al., 2003; Asala et al., 2012). Yam domestication started about 5000 years ago (Dumont and Vernier, 2000) and the process is continuing, providing new sources of diversity (Bousalem & Dallot, 2000). The evolution, dispersion and epidemiological properties of virus are believed to be determined by uncontrolled introduction of infected yam germplasm which have contributed to widespread distribution of viruses in the crop (*Bousalem et al.*, 2003; Odu *et al.*, 2001; Atiri et al., 2003).

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Limited research on characterisation of virus in Nigeria has been done in southern humid forest zone (Njukeng et al., 2002; Osmond, 2006) but not in the Guinea Savanna. The aim of this study was, therefore, to characterize the important viruses of yam in the Guinea Savanna zone of Nigeria and assess the degree of genetic diversity and phylogenetic relationships of the viruses within the Guinea Savanna zone of Nigeria and between Nigeria and some other parts of the world.

2. Materials and Methods

2.1 Detection of virus from yam leaf samples

Deoxyribonucleic acid (DNA) was used as the reaction template for Immunocapture Polymerase Chain Reaction (IC-PCR) and Ribonucleic acid (RNA) for Immunocapture Reserve Transcription Polymerase Chain Reaction (IC-RT-PCR).

2.1.1 Deoxyribonucleic acid (DNA) template

The procedure for detection of Badnavirus (Seal & Muller, 2007) was used. The technique involved using a specific primer pair for the Badnavirus (Mumford & Seal, 1997; Seal and Muller, 2007). A 12.5 µl reaction mix was prepared in PCR tubes. The mix contained 2.5 µl green buffer, 0.25 µl dNTPs, 0.75 µl $MgCl_2$, 0.5 µl each of Badna primers forward and reverse, 0.06 µl Taq DNA polymerase (Promega Corporation, USA), 5.94 µl sterile distilled water and 2.0 µl DNA template. Extract from known virus-free leaf (cowpea leaf) was used for negative control, while extract from known virus-infected plant (yam leaf) was used as positive control for each reaction set up. The PCR tubes were placed in the thermal cycler for amplification. The amplification profile for the DNA was as follows: 94^{o}C for 4 min, 94^{o}C for 30 sec for denaturation; one cycle, primer annealing at 50^{o}C for 30 sec, 72^{o}C for 30 sec for primer extension for 40 cycles, and final extension was carried out at 72^{o}C for 5 min for one cycle. The primer sequences were:

Badna FPS 5`-ATG CCITTY GGI ITI AAR AAY GCI CC-3`, Badna RP 5`-CCA YTT RCA IAC ISC CCA ICC-3` (Seal and Muller, 2007). YMMV Forward 5`-GGC ACA CAT GCA AAT GAA RGC-3`, Reverse 5`-CAC CAG TAG AGT GAA CAT AG-3` (Mumford and Seal, 1997). YMV Forward 5`-ATC CGG GAT GTG GAC AAT GA-3` and YMV Reverse 5`-TGG TCC TCCGCC ACA TCA AA-3` respectively.

2.1.2 Ribonucleic acid (RNA) template

A total of 10 RNA extracts out of 15 were amplified in RT-PCR using multiplex primers (source) for YMV, YMMV and CMV, while 12 DNA extracts also out of 15 were amplified using Badna primers from two locations (FCT and Kwara). The amplified products were analyzed on 1.5 % agarose gel I TAE buffer at 120 Volts for one hour. For RT-PCR, the RNA extract was used as the template for the multiplex YMV and YMMV PCR. The primers for YMV and YMMV, forward and reverse, were used for the detection of *Yam mosaic virus* and *Yam mild mottle virus* for the two years. The reaction mixture of 12.5 µl was prepared and loaded into PCR tube. Each of the reactions contained 2.5 µl green buffer, 0.25 µl dNTPs, 0.75 µl $MgCl_2$, 0.25 µl each of forward and reverse of primers YMV and YMMV, 0.06 µl Taq, 0.06 µl RT, 5.88 µl sterile distilled water and 2.0 µl RNA template. Extract from known virus-free leaf was used for negative control, while extract from virus infected plant was used as positive control for each reaction set up in the PCR. Amplification was done in the thermal cycler as described above. The amplification profile for RNA consisted of one cycle denaturation at 44^{o}C for 30 min, 95^{o}C for 5 min, for one cycle; 95^{o}C for 30 sec, 55^{o}C for 1 min for primer annealing, 72^{o}C for 1 min for 35 cycles and finally, one cycle primer extension at 72^{o}C for 10 min. The amplified products were resolved on 1.5 % agarose gel stained with ethidium bromide and observed under UV light.

2.2 Sequencing of purified PCR and RT-PCR products

Twenty-two DNA and RNA extracts amplified through PCR and RT-PCR were used for sequence analysis. The nucleotide sequences from each location were processed and sent to GenBank for accession numbers. The amplified Polymerase chain reaction (PCR) products that were positive were purified using ethanol purification method as described by Kumar (2009). The PCR products for sequencing (42 µl) were transferred into 1.5 ml Eppendorf tubes and labelled. Ninety-five (95) percent ethanol was added to the PCR product in the ratio 2:1. The tubes were inverted gently thrice and incubated at -70^{o}C for 10 mins. The contents of the tubes were later centrifuged at 13,000 rpm for 10 mins. Following removal of each tube from the centrifuge, the supernatant was discarded, while 500 µl of 70 % ethanol was added and spun at 13,000 rpm for 5 mins. The ethanol was discarded, and the nucleic acid pellets were dried at 37^{o}C for 15 mins. The pellets were dissolved in 30 µl of sterile distilled water and stored at -20^{o}C for use in sequence analysis. The preparation of purified nucleic acid products was carried out in the Virology and Molecular Diagnostic Laboratory at IITA, Ibadan, Nigeria before they were sent to a commercial company (Molecular Laboratory, Iowa State University) in the United States of America for sequencing.

The products were sequenced using an ABI Prism 310 in both directions in an automated sequencer and BigDye Terminator Kit (Applied Biosystem, CA USA) by priming the sequenced reactions with the same oligonucleotides.

A total of 22 sequenced data from isolates of the two locations (Abuja FCT and Kwara State) for the specific viruses were evaluated. The sequenced data of virus isolates were received, analysed and again sent to GenBank, National Center for Biotechnology Information (NCBI) for processing. The data were received in the FASTA format from the company's site. Data were manually edited and consensus sequence for each isolate was made along with their electropherograms using the BioEdit software tool. Sequence confirmation was done with similar searches from GenBank using the BLAST program in NCBI. Multiple nucleotide sequence alignments were performed using the CLUSTAL W program (Thompson et al., 1994) with default parameters and manually adjusted. The counterpart sequences were gathered from GenBank with their accession numbers indicated on the bootstraps. YMV/Dr Gha4, YMV/Dr CD11, YMV/Dr Nig8 and YMV/Dr Cmn151 were counterpart isolates from Ghana, Côte d'Ivoire, Nigeria, and Cameroun, respectively. For YMMV, YMMV/Dr Guad2, YMMV/Dr Mart3, YMMV/Da CD137 and YMMV/Da SLK2 were used as counterpart isolates. Badnavirus counterpart isolates were DBV/Dr GH2f, DBV/Dr Gn694, DBV/Dr GH2f and DBV/Dr Gn1633. Phylogenetic relationships between isolates were determined by trees generated in molecular evolutionary genetics analysis (MEGA 5.1) software (Tamura et al., 2007) using the neighbour-joining tree method developed by Saitou & Nei (1987).

3 Results and Discussion

Extracts that showed expected band sizes of 586 bp and 249 bp on the gel after RT-PCR were considered positive for YMV and YMMV, while those that had expected band size of 579 bp on the gel after PCR were considered positive for Badnavirus. The control wells had no bands for both negative and buffer. The extracts used for positive control wells produced fragments of similar sizes with tested sample. In the results from BLAST analysis (Fig. 1), the database is shown to produce counterpart isolates from other countries which were like the ones obtained in the present study. The database also produced out-group isolates which were different from viruses in the present study. The nucleotide sequence clustering of isolates within each virus group and their counterparts when compared with out-groups, showed clear differences between them. Most of the counterpart isolates from other locations were 97-99% identical with the isolates in this study, while out-groups were 100% different from the study isolates (Fig. 1).

The nucleotide sequence clustering of isolates within each virus group and their counterparts when compared with out-groups, showed clear differences between them.

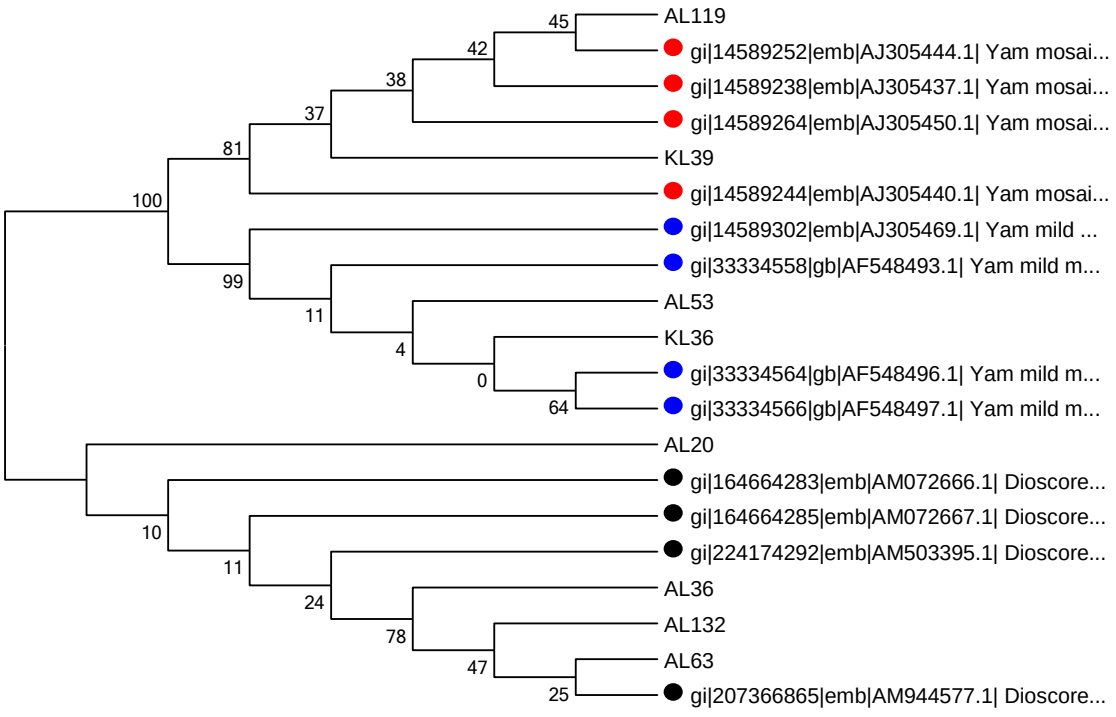

Figure 1. Phylogenetic tree of Isolates of YMV, YMMV and DaBV

The phylogenetic tree shows 2 isolates of YMV, YMMV and 4 isolates of Badnavirus from Kwara State, Abuja FCT and isolates from other countries infecting yam, based on their nucleotide sequences using the neighbour-joining methods. Red colours indicate counterpart isolates; YMV from Nigeria, Cameroun, Côte d'Ivoire and Ghana, blues colours are YMMV from Guadeloupe, Martinique, Côte d'Ivoire, and Sri Lanka, while black colours are isolates BDV from GH and Gn694/1633.

Figure 2a shows the phylogenetic tree generated from clustering of nucleotide sequence of YMV KL39 (Local variety Gbakumo), AL 119 (Local variety Dan-anachia), isolates from Kwara and FCT Abuja, isolates from counterparts (Gha4, CD11, Nig8 and Cmn151) and out-groups (PG102 and India). The genetic distance of isolates of study area and that of counterparts ranged from 0.027 to 0.049 while the distance to that of out-groups ranged from 1.926 to 2.087. The tree suggested the existence of two major clusters based on coefficient of genetic distance and similarities. The first main cluster showed the isolates from this study and counterpart, while the second cluster showed isolates from out-groups. The sub-cluster consisting of isolates from Cameroon, Nigeria and Côte d'Ivoire were 100% related while the isolates from study and Ghana were 41-48% related. The analysis of nucleotide alignment substitution showed some differences in nucleotide displayed, which could have been due to changes in amino acids and phenotypic variations. In some groups, few nucleotide substitutions were observed, and this might have been due to few amino acid changes and diversity within the virus populations in this study. Variations were also observed within counterpart isolates which generally showed that yam viruses are genetically unstable. Bousalem *et al.* (2003) noted that potyviruses have the most variable coat protein nucleotide and genetic diversity potential, because of their high mutation and recombination rates or bio-geographical environment contribution.

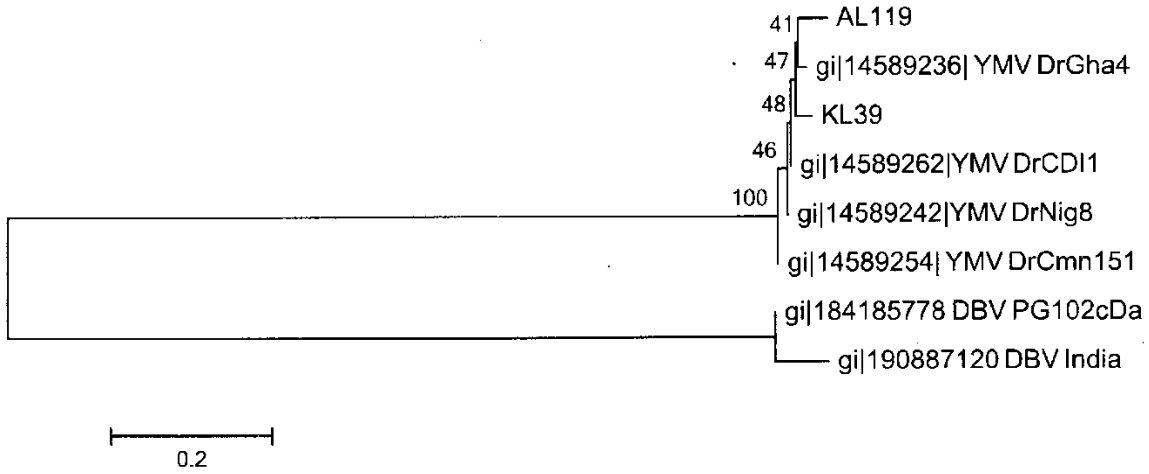

Figure 2a. Phylogenetic tree of isolates of YMV

This result also shows genetic distances of 2 isolates of YMV infecting yam species from Kwara State, FCT Abuja, 4 isolates of YMV infecting yams from other countries and 2 isolates of DBV (out-groups) infecting yam in other locations based on their nucleotide sequences using the neighbour-joining methods. Virus isolates: AL119 = Dan-anachia, KL39 = Gbakumo, DrGha = Ghana, DrCDI = Cote d'Ivoire, DrNig = Nigeria, DrCmn = Cameroon, PG = Papua New Guinea and India.

The out-group isolates which form the second main cluster from Papua New Guinea and India form a separate sub-cluster that was 100% related and were not genetically distanced from each other as shown in the bootstrap tree (Fig. 2b).

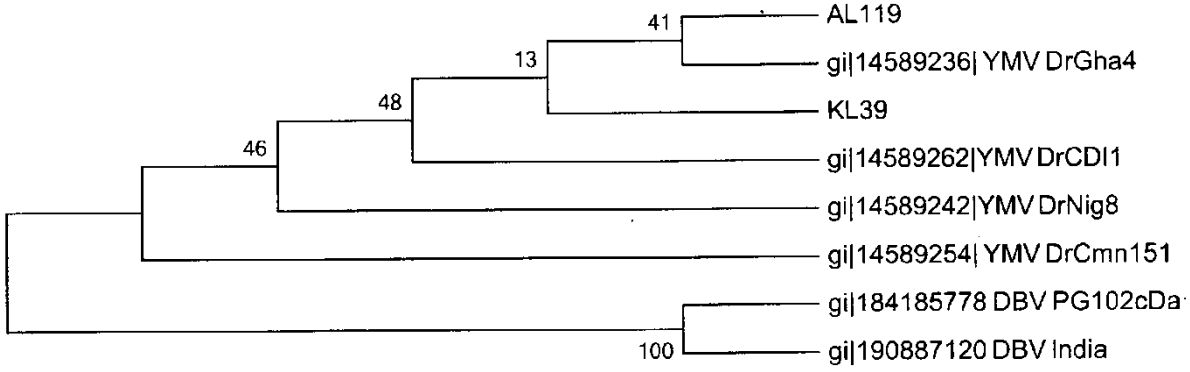

Figure 2b. Bootstrap of isolates of YMV

This shows the bootstrap of genetic distances of 2 isolates of YMV infecting yam species from Kwara State, FCT Abuja, 4 isolates of YMV infecting yams from other countries and 2 isolates of DBV (out-groups) infecting yam in other locations based on their nucleotide sequences using the neighbour-joining methods. Virus isolates: AL119 = Dan-anachia, KL39 = Gbakumo, DrGha = Ghana, DrCDI = Côte d'Ivoire, DrNig = Nigeria, DrCmn = Cameroon, PG = Papua New Guinea and India.

Figure3a shows the phylogenetic tree of YMMV rooting from two main groups with genetic distance of 0.05. The first main group was divisible into two subgroups. The first subgroup consisted of isolates from Kwara and Abuja (KL36: TDa 05/00129) and Abuja FCT (AL53: TDa 05/00129) that were 100% related. The same accession was planted in Kwara State and Abuja FCT and suggests that the seed yam was virus infected from source and transferred to the two locations in the study. The other group consisted of isolates from Guadeloupe2, Martinique3, Côte d'Ivoire137 and Sri Lanka2 that were also closely related to isolates in the study (Fig.3a). The other main group consisted of isolates from out-group DBV Papua New Guinea102 and DBV India, which were closely related to each other. The genetic distance of isolates from the present study and those of counterpart areas ranged from 0.035 to 0.054, while that from the present study and out-group ranged from 0.017 to 3.060.

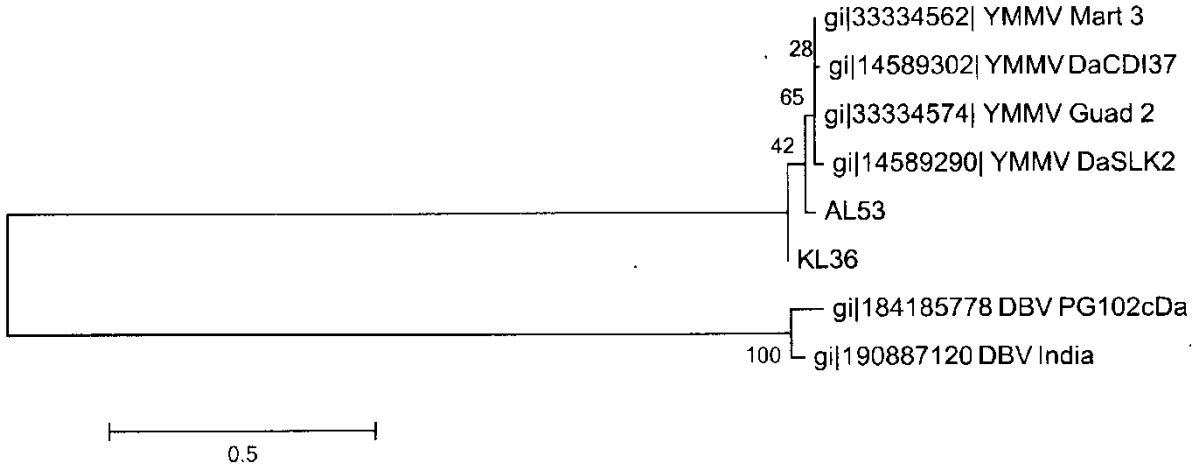

Figure 3a. Phylogenetic tree of isolates of YMMV and YMV

This shows genetic distances of 2 isolates of YMMV infecting yam species from Kwara State, FCT Abuja, 4 isolates of YMV infecting yams from other countries and 2 isolates of DBV (out-groups) infecting yam in other locations based on their nucleotide sequences using the neighbour-joining methods. Virus isolates: AL53 = TDa 05/00129, KL36 = TDa 05/00129, Mart = Martinique, CDI = Cote d'Ivoire, Guad = Guadeloupe, DaSLK = Sri Lanka, PG = Papua New Guinea and India.

The genetic distance of isolates from Kwara and Abuja was 0.017 as shown in the bootstrap tree (Figure3b). Generally, studies have shown the prevalence of YMV, YMMV and Badnavirus on yam in Nigeria, Africa, and other parts of the world where yam is grown (Odu *et al.*, 2006; Bousalem *et al.*, 2003; Eni *et al.*, 2009). These support the strong geographical clustering and relationship among isolates in the study with other counterparts. Inter-group (counterpart) diversity values were larger than in intra-group one (this study).

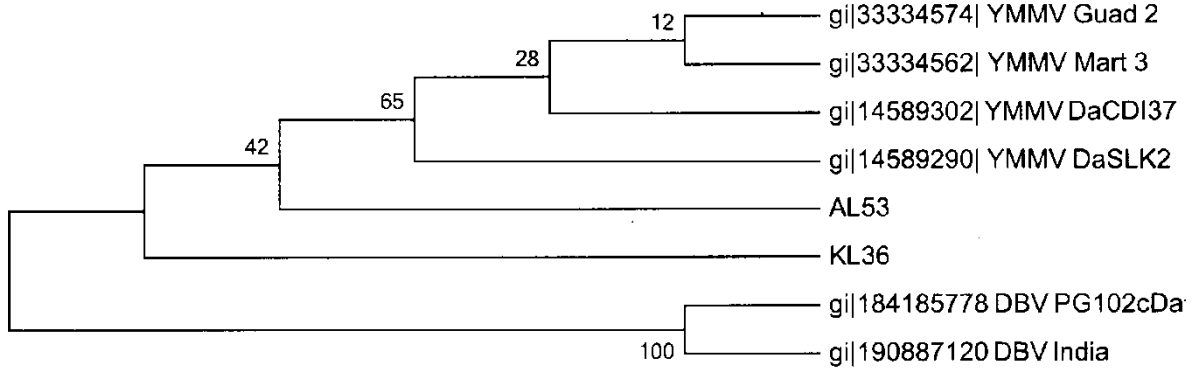

Figure 3b. Bootstraps of isolates of YMMV and YMV

This Figure shows the genetic distances of 2 isolates of YMMV infecting yam species from Kwara State, FCT Abuja, 4 isolates of YMV infecting yams from other countries and 2 isolates of DBV (out-groups) infecting yam in other locations based on their nucleotide sequences using the neighbour-joining methods.

Virus isolates: AL53 = TDa 05/00129, KL36 = TDa 05/00129, Mart = Martinique, CDI = Cote d'Ivoire, Guad = Guadeloupe, DaSLK = Sri Lanka, PG = Papua New Guinea and India.

For Badnavirus, the phylogenetic tree showed three main groups at 1.0 (Figure 4a). The first main group consisted of isolate of out-group YMV NIB CP, the second group consisted of isolate from YMV Nib/CP while the third group were isolates from present study and counterpart (GHL2f, GHL2f, Gn694 and Gn1633). The third main group also comprised of three sub-groups; first sub-group consisted of three isolates from Abuja 99% related to each other, and the second sub-group had isolates from Abuja (AL20) while the last sub-group had isolates of DBV from counterpart.

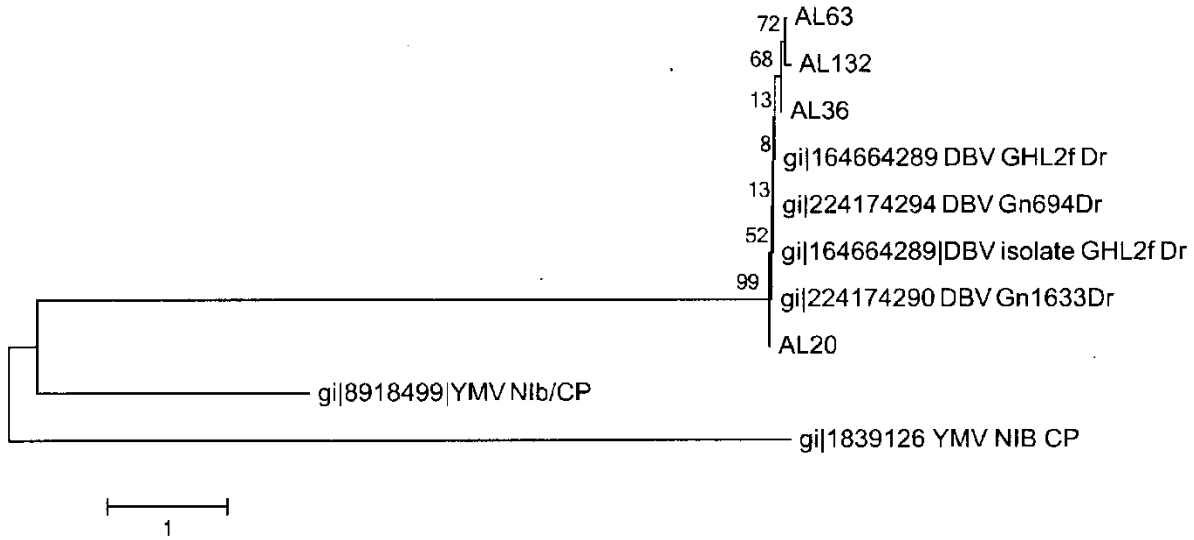

Figure 4a. Phylogenetic tree of isolates of YMV and BaDV

This Figure shows the genetic distances 4 isolates of Badnavirus infecting yam species from FCT Abuja, 4 isolates of YMV infecting yams from other countries and 2 isolates of DBV (out-groups) infecting yam in other locations based on their nucleotide sequences using the neighbour-joining methods. Virus isolates: AL63 = Makakusa, AL132 = Dan-anachia, AL36 = TDa 05/00129, AL20 = TDr 89/02665, GHL = Ghana, Gn = Guyana, Nib and NIB.

The genetic distance between isolates from present study and counterpart was 0.02, while that between it and out-groups was 1.0 as shown in bootstrap tree (Figure 4b). The Africa clades of Badnavirus (Gn1633, Gn694, GHL2F and GHL2f) probably arose by bio-geographical diversification. The regional and world-wide evolutionary and epidemiological distribution of these viruses might have been due to intensive introduction and exchange of yam cultivars locally within location, importation of infected seed yams, climatic changes or emerging and re-emerging of viruses.

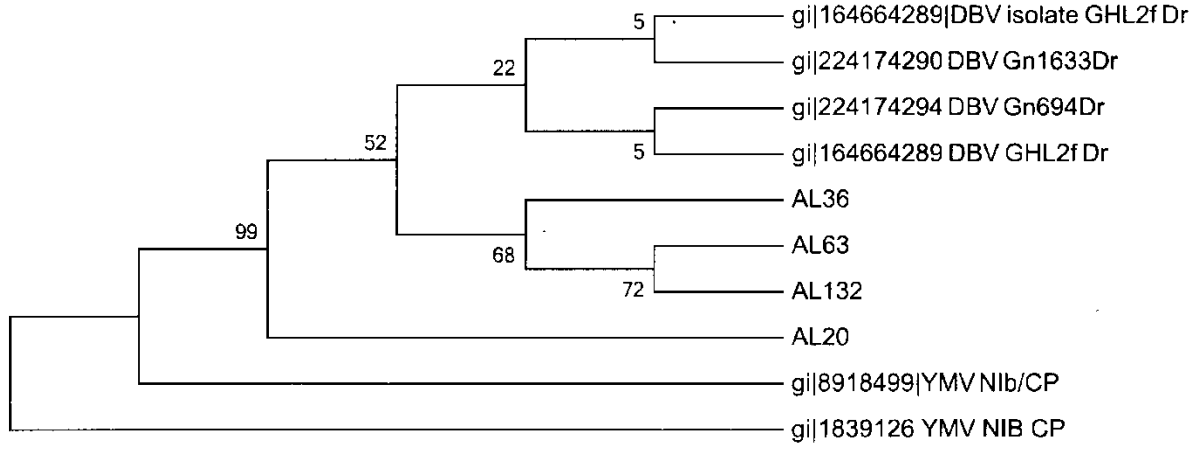

Figure 4b. Bootstraps of isolates of YMV and BaDV

This Figure shows the genetic distances of 4 isolates of Badnavirus infecting yam species from FCT Abuja, 4 isolates of YMV infecting yams from other countries and 2 isolates of DBV (out-groups) infecting yam in other locations based on their nucleotide sequences using the neighbour-joining methods. Virus isolates: AL63 = Makakusa, AL132 = Dan-anachia, AL36 = TDa 05/00129, AL20 = TDr 89/02665, GHL = Ghana, Gn = Guyana, Nib and NIB.

4 Conclusion

Phylogenetic analysis of sequence results revealed that genetic distances among isolates of the present study and those of counterparts were close, suggesting bio-geographical diversification and world-wide distributions of these viruses.

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