Molecular characterisation of ampeloviruses associated with mealybug wilt of pineapple disease in Ghana

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Research

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Abstract

Background: Mealybug wilt of pineapple (MWP) is the most destructive viral disease of pineapple worldwide. The disease is caused by pineapple mealybug wilt-associated virus (PMWaV), a member of the family *Closteroviridae* and the genus *Ampelovirus*, and transmitted by mealybugs.

Methods: In order to understand the association between closteroviruses and MWP in Ghana, 24 pineapple plant samples showing typical symptoms of MWP were collected during a survey of the Central Region in 2019. Three quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assays with PMWaV species specific primers were performed to amplify the heatshock protein 70 gene (HSP70) of PMWaV-1,-2 and -3 in the pineapple samples. Purified qRT-PCR products of thirteen isolates which consist of PMWaV-1 (7 isolates), PMWaV-2 (4 isolates) and PMWaV-3 (2 isolates) were sequenced in both directions. Sequence and phylogenetic analyses were then conducted.

Results: Three different species of *Ampelovirus* namely PMWaV-1, PMWaV-2, and PMWaV-3, were detected from the plant samples, with abundance of mixed infections. Sixteen out of the 24 samples (66.7%) were infected with at least one of the three species of *Ampelovirus* identified. PMWaV-2 had the highest infection rate of 62.5% across the districts; this was followed by PMWaV-1 and PMWaV-3 with infection rates of 33.3% and 8.3% respectively. Purified qRT-PCR products of thirteen isolates which consist of PMWaV-1 (7 isolates), PMWaV-2 (4 isolates) and PMWaV-3 (2 isolates) were sequenced in both directions. Sequence comparison using BlastN showed that all the seven sequences of the Ghanaian PMWaV-1 isolates (GenBank Accession Nos. MN427634 - MN427639 and MN399973) shared 95.2% to 99.7% nucleotide identity with each other and 95.2-100% with sequences of isolates previously published in GenBank. The four sequences of the Ghanaian PMWaV-2 isolates (GenBank Accession Nos. MN427642 - MN427645) shared nucleotide identity of 98.9–100% with each other and 98.2-100% nucleotide identity with sequences of isolates previously published in GenBank. Also, the two sequences of the Ghanaian PMWaV-3 isolates (GenBank Accession Nos. MN427640 and MN427641) shared 98.3% nucleotide identity to each other and 97.5- 99.3% nucleotide identity with sequences of isolates previously published in GenBank. Phylogenetic analyses of the nucleotide sequences of HSP70 gene of the 13 Ghanaian isolates and 24 sequences previously published in GenBank, clustered the PMWaV-1, PMWaV-2 and PMWaV-3 isolates into three distinct genetic groups with > 95% bootstrap support.

Conclusion: The present study shows for the first time the occurrence of PMWaV-1, PMWaV-2 and PMWaV-3 in Ghana pineapple fields as well as in Africa.

Background

Pineapple (*Ananas comosus* L. Merrill), a *Bromeliaceae,* is the third most important fresh fruit crop after citrus and banana worldwide [1]. Brazil, Philippines, and Thailand are the leading producers worldwide whilst in Africa, Côte d'Ivoire, Nigeria, Ghana and Kenya are the main pineapple producing countries [2].

In Ghana, the pineapple sector is the most developed horticultural sector [3,4] cultivated mainly in the areas of Central, Greater Accra, Eastern and Volta regions of Ghana, in small and medium scale. Pineapple production is a source of income for thousands of people ranging from farmers to market women and small-holder farmers. The crop provides raw material to feed industries, leading to establishment of cottage industries. Pineapple is a non-traditional export crop in Ghana and hence a source of foreign exchange. It contributed more than USD 283,000,000 in foreign exchange to the economy of Ghana between 1990 and 2013 [5].

Mealybug wilt of pineapple (MWP) is a destructive viral disease that affects pineapple production in many growing regions worldwide including Ghana [6,7]. In Ghana, fruit yield loss due to MWP attack has been estimated at about US$ 248.00 per hectare [7]. Elsewhere in Hawaii, MWP has been reported to cause reduction in fruit yield by 30-55%, depending on the age of the plant at the onset of the disease [8].

Mealybug wilt of pineapple is caused by pineapple mealybug wilt-associated virus (PMWaV), a member of the genus *Ampelovirus* and family *Closteroviridae.* Pineapple mealybug wilt-associated virus-1 (PMWaV-1), PMWaV-2, PMWaV-3, PMWaV-4, and PMWaV-5 are the five distinct species identified in Hawaii, Australia and Cuba from diseased pineapple fields [9,10,11]. These viruses are transmitted by two species of mealybugs namely the gray pineapple mealybug (*Dysmicoccus neobrevipes*, (Beardsley), and the pink pineapple mealybug (*Dysmicoccus brevipes* (Cockerell) [12], and also by man through inadvertently planting of infected planting materials (suckers, slips or crowns). These mealybugs have a symbiotic association with the ants. The ants help the mealybugs in the foundation of mealybug settlements and consume the honeydew created by the mealybugs and can suppressively affect the mealybugs' natural enemies [13-16]. Mealybug wilt of pineapple disease symptoms are displayed by serious tip dieback, downward curling, reddening, and wilting of the leaves which can prompt a complete breakdown of the plant [17,18] (Figure 1).

Even though PMWaVs have been associated with MWP disease worldwide, there is no record of such an association in Ghana. Knowledge of ampelovirus species associated with MWP in Ghana is very important in devising strategies to manage the disease. Dey et al. [6] reported that PMWaV-2 species alone without the others could make the wilt symptom of pineapple to develop. Also, In Hawaii, MWP symptoms are strongly associated with infection by PMWaV-2 [8]. On the other hand, in Australia, the disease is strongly associated with infections by PMWaV- 3 alone or co-infection by PMWaV-1 and -3 [11].

The aim of this study was to identify and characterize ampeloviruses associated with MWP disease that affects pineapple production in Ghana.

Materials And Methods

Collection of pineapple leaf samples

Twenty-four pineapple leaf samples showing symptoms of MWP (tip dieback, descending curling, reddening, and wilting of the leaves which can prompt a complete breakdown of the plant) were collected from three districts in the Central Region of Ghana, during field survey in 2019. The districts were Abura-Asebu-Kwamankese (AAK), Ekumfi, and Komenda-Edina-Eguafo-Abirem (KEEA), leading pineapple growing centres in Ghana. The location, climate and vegetation types of the three districts are presented in Table 1.

RNA extraction

Viral RNAs were extracted from leaf tissues of each sample using Quick-RNA^{TM} Plant Miniprep Kit (ZymoResearch Corp.) according to the manufacturer's instructions.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Luna Universal One-Step qRT-PCR Kit (BioLabs Inc.) was used for the qRT-PCR amplification of headstock protein 70 (HSP70) gene of PMWaVs, according to the manufacturer's instructions. Briefly, an initial reaction volume of 12.6 µL containing 10 µL of 2× Luna Universal One-Step Reaction Mix, 1 µL of 20x Luna WarmStart RT Enzyme Mix, 0.8 µL of 10 µM reverse primer, 0.8 µL of 10 µM forward primer, was prepared and placed in qPCR tubes. Total RNA template (< 1 µg) was added to the mixture in the qPCR tubes and nuclease-free water was added to make up a final reaction volume of 20 µL. The qPCR tubes were then spun in a centrifuge for 1 min at 2, 500 rpm to remove the bubbles. The tubes were then incubated in a pre-warmed thermocycler (Applied Biosystems StepOnePlus) according to the programme reaction conditions indicated in Table 1, and SYBR scan mode setting on the real-time instrument (thermocycler). The primer sequences are shown in Table 2.

Gel electrophoresis

The amplification products were assessed by electrophoresis in 1.5% agarose gel in TBE buffer (89 mM Tris-borate and 2 mM EDTA, pH 8.3) and stained with ethidium bromide using a 2 kb ladder. The gel was then visualized in UV light in a gel documentation system and the gel photograph was then taken for further analysis.

Cleaning and sequencing of PMWaV-1, PMWaV-2 and PMWaV-3

Purified qRT-PCR products of thirteen isolates which consist of PMWaV-1, PMWaV-2 and PMWaV-3 were sequenced according to Sether *et al.* [20] and Gambley *et al.* [11] in order to assess variation within a virus isolate and to ensure consistent and reliable sequence data. The DNA bands were purified and

sequenced in both directions using the BrilliantDye™ Terminator Cycle Sequencing Kit V3.1 (NimaGen BV, The Netherlands).

Sequence data were edited and assembled using BioEdit version 7.0.5 [21]. The quality of each nucleotide in the sequence was examined in order to detect and evaluate changes in nucleotides and for each amplicon construct consensus sequences. Both primer and non-coding sequences from the alignments were also removed. Additional published sequences obtained from GenBank were verified and added to the data sets (Table 3). After editing, the final sequences analysed were the partial HSP70 gene of 420 nt of PMWaV-1, 591 nt of PMWaV-2 and 486 nt of PMWaV-3. The deduced amino acid sequences analysed included 140 aa for PMWaV-1, 197 aa for PMWaV-2 and 162 aa for PMWaV-3. The sequences obtained in this work were analysed together with those retrieved from the GenBank (Table 3).

Multiple sequence alignments were made using the ClustalW programme implemented in MEGA version 7.0 [22]. Alignments were also manually altered to guarantee right reading frames. The analyses included a total of thirteen isolates of PMWaV-1, PMWaV-2 and PMWaV-5 nucleotide sequences of HSP70 genes from pineapple fields in Ghana, and 24 corresponding sequences of isolates previously published in GenBank.

Sequence comparisons and phylogenetic analyses

For HSP70 homologous genes of PMWaV-1, PMWaV-2 and PMWaV-3, the nucleotide and the deduced amino acid sequence identities were determined using BioEdit v7.0.5 [21] and BlastN. For HSP 70 homologous genes between PMWaV-1, PMWaV-2 and PMWaV-3 sequence alignments, MODELTEST [23] implemented in MEGA version 7 program [22] was conducted to select the most suitable nucleotide substitution model using the Akaike Information Criterion, the Bayesian Information Criterion [24] and the hierarchical probability ratio test. The best fit nucleotide substitution model was then used for phylogenetic analyses using the maximum likelihood method with 1000 replicates of bootstrapping using the MEGA 7 software [22]. The neighbour-joining method also implemented in MEGA 7 was used for comparison.

Genetic diversity

The following genetic diversity indices for all samples of the HSP70 homologous gene each of the PMWaV-1, PMWaV-2 and PMWaV-3 were determined using the DnaSP V.5.0 programme [25]: haplotype diversity (h), nucleotide diversity (π), number of segregating sites (S) and total number of mutations (Eta).

Determination of genetic distance and selection pressure

For each of PMWaV-1, PMWaV-2 and PMWaV-3 sequence dataset, the overall genetic distance (the number of base substitutions per site from averaging across all sequence pairs in a population) within

HSP70 homologous nucleotide sequence data sets were estimated using the Maximum likelihood model [26]. Bootstrap method (1000 replicates) was used to obtain standard error estimates. The analyses were conducted in MEGA 7.

The HyPhy package Maximum Likelihood analysis of the natural codon-by-codon selection technique [27] implemented in MEGA 7 [22] was used to predict the number of synonymous substitutions inferred per synonymous site (dS) and the number of non-synonymous substitutions per non-synonymous site (dN). These estimates were produced using the joint Maximum Likelihood reconstructions of ancestral states under the defaults Muse-Gaut model [28] and General Time Reversible model [29]. The dN-dS test statistic was used to detect codons that were under positive selection.

An overabundance of non-synonymous substitutions shows a positive value for the test statistics. In this case, Kosakovsky and Frost [30] and Suzuki and Gojobori [31] methods were used to calculate the probability of rejecting the null hypothesis of neutral evolution (P-value). Values of P less than 0.05 are considered significant at a 5% level. The overall ratio dN/dS was also calculated from the mean values of dN and dS to compare the selection pressures acting on the HSP 70 gene of each PMWaV-1, PMWaV-2 and PMWaV-3 species. The gene is under positive (or diversifying) selection when the dN/dS ratio is > 1, negative (or purifying) selection when the dN/dS ratio < 1, and neutral selection when dN/dS ratio = 1.

Neutrality test

Tajima D and Fu and Li's D and F statistics were used to test the hypothesis that PMWaV diversity trends are consistent with the neutral molecular evolution theory [32-34]. The neutral theory of molecular evolution says that the great majority of molecular-level evolutionary modifications are caused by selectively neutral mutants shifting randomly [32]. The importance of each test statistics was estimated by 10,000 permutations.

Results

Detection of the viral species responsible for MWP disease

Three different species of *Ampelovirus* namely PMWaV-1, PMWaV-2, and PMWaV-3 were detected by qRT-PCR from the plant samples during the study (Table 4). Sixteen out of the 24 samples (66.7%) were infected with at least one of the three species of *Ampelovirus* identified. PMWaV-2 had the highest infection rate of 62.5% across the districts, indicating that it is the most prevalent virus species in the region; this was followed by PMWaV-1 and PMWaV-3 with infection rates of 33.3% and 8.3% respectively (Table 4; Figure 1). All the three viral species were detected from pineapple samples from AAK district, whilst only two (PMWaV-1 and PMWaV-2), were detected from Ekumfi and KEEA districts, implying that PMWaV-3 was found only in AAK district.

Performance of the PMWaVs primers on samples across the growing area

Fig. 2 shows qRT-PCR amplification of the ampeloviruses with PMWaVs primers of cDNA fragment size that varies from 495 – 610 bp from all the 24 diseased leaf samples (lanes 1-12 and 13- 24) but no band for negative control (NTC).

The amplicon of PMWaV-1 was obtained from pineapple samples using 225 / 226 primer pair of band size 590 bp. The primer pair detected the virus from two out of eight samples from KEEA, and six out of eight samples from AAK, whilst none of the eight samples from Ekumfi was infected by PMWaV-1.

With PMWaV-2 amplicon obtained from pineapple samples using 223 / 224 primer pair of band size 610 bp, the primers detected the virus from two out of eight samples from Ekumfi, four out of eight samples from KEEA, whilst all the eight samples from AAK were infected by PMWaV-2.

PMWaV-3 was detected from only two out of eight samples from AAK using 263/264 primer pair with band size 495 bp. The virus, however, was not detected from any of the samples from KEEA and Ekumfi districts.

Mixed viral infections of pineapple samples by pineapple mealybug wilt associated viruses (PMWaVs)

Mixed viral infections by two or all three viruses identified (PMWaV-1**,** PMWaV-2, PMWaV-3), were detected in the pineapple samples from two out of the three districts (Table 5). Mixed infections were detected in 6 out of 8 pineapple samples in AAK where they were co-infected by PMWaV-1 and either PMWaV-2 or PMWaV-3 in double infections (4 samples) or co-infected by all three virus species, i.e triple infections (2 samples). Mixed infection was detected on only one sample from KEEA where it was co-infected by PMWaV-1 and PMWaV-2. Mixed infection however was not detected from any of the samples from Ekumfi. District.

Sequence analysis

Heat shock protein 70 (HSP70) gene was sequenced to confirm identities and estimate the genetic variability among PMWaV-1, PMWaV-2 and PMWaV-3 isolates. Sequence comparison showed that all the seven sequences of the Ghanaian PMWaV-1 isolates (GenBank accession numbers MN427634 - MN427639 and MN399973) shared 95.2 to 99.7% nucleotide identity with each other and 95.2-100% with sequences of isolates previously published in GenBank (Accession numbers KT322152, KT322148, EF620774, HE583225 JX645771, HQ129930, MH704740, AF414119, KJ872494, EU79113, KC800714 and HG940514). The two sequences of the Ghanaian PMWaV-3 isolates (GenBank accession numbers MN427640 and MN427641) shared 98.3% nucleotide identity to each other and 97.5- 99.3% nucleotide identity with sequences of isolates previously published in GenBank (Accession numbers NC043406,

MH704742, GU563497, JX508638 and FJ209047). Also, the four Ghanaian PMWaV-2 isolates (GenBank Accession numbers MN427642 - MN427645) shared 98.9 to 100% nucleotide identity with each other and 98.2 – 100% nucleotide identity with sequences of isolates previously published in GenBank (Accession numbers EU769115, KT322167, FN825676, MH704741, HE583226 ,NC043105, EU016675 and JX645772).

The deduced amino acid sequences of the Ghanaian isolates also ranged from 86.5 to 99.2% for the PMWaV-1, 97.1 to 100% for PMWaV-2 and 95.3% for the PMWaV-3 (Table 6), indicating narrow variability (close identities) within each viral species. Deduced amino acid sequences of the Ghanaian isolates shared identities of 86.5 - 100% for PMWaV-1, 95.5 - 100% for PMWaV-2, and 93.3 - 98.0% for PMWaV-3 with isolates previously published in (Table 6).

3.5 Phylogenetic analyses

The maximum likelihood tree for the partial HSP70 gene nucleotide sequence dataset revealed that the 37 sequences of PMWaV-1, PMWaV-2 and PMWaV-3 isolates from symptomatic pineapple samples formed three main genetic groups corresponding to three clades supported by bootstrap values of greater than 95%. Ther seven Ghanaian PMWAV-1 isolates (Accession numbers: MN427634 - MN427639 and MN399973) clustered on clade 1 together with twelve isolates previously published in GenBank with 99% bootstrap support. The two PMWaV-3 isolates from Ghana (Accession numbers MN427640 and MN427641) clustered on clade 2 with four corresponding PMWaV-3 isolates previously published in GenBank with 96% bootstrap support. With 99% bootstrap support the four Ghanaian PMWaV-2 isolates (Accession numbers: MN427642 - MN427645) clustered on clade 3 with the corresponding PMWaV-2 isolates previously published in GenBank.

The maximum likelihood phylogenetic tree for the HSP70 ORF amino acid sequences had similar topology as that of nucleotide sequence (three genetic groups or clades) with bootstrap support of greater than 88% (Figure 4). Group 1 containing seven PMWAV-1 isolates from Ghana (Accession numbers MN427634 - MN427639 and MN399973) clustered with PMWAV-1 isolates previously published in GenBank with 89% bootstrap support. Two Ghanaian PMWaV-3 isolates (Accession numbers MN427640 and MN427641) clustered on clade 2 with four corresponding PMWaV-3 isolates previously published in GenBank with bootstrap support of 91%. Group 3 consists of four isolates of PMWaV-2 obtained from pineapple fields in Ghana (Accession numbers MN427642 - MN427645) clustering on clade 3 with PMWaV-2 isolates from GenBank (Figure 4).

From both HSP70 nucleotide (Figure 3) and deduced amino acid (Figure 4) phylogenetic trees, it is clear that PMWaV-2 isolates have long evolutionary relationship with both PMWaV-1 and PMWaV-3. It is also evident from Figures 3 and 4 that PMWaV-1 and PMWaV-3 are more closely related i.e. they have close evolutionary relationship.

Genetic diversity within HSP70 gene of PMWaV-1, PMWaV-2 and PMWaV-3 isolates

Analysis of genetic diversity within the HSP70 genes of PMWaV-1, PMWaV-2 and PMWaV-3 isolates showed that the genes were variable with high number of mutations, high number of polymorphic sites and very high haplotype diversity but low nucleotide diversity (Table 7).

Analyses of genetic distance and the natural selection within HSP70 gene of the PMWaV-1, PMWaV-2 and PMWaV-3 isolates

The overall mean genetic distances within the nucleotide sequence datasets for HSP70 gene for PMWaV-1, PMWaV-2 and PMWaV-3 were determined using Maximum Likelihood model [32]. The mean genetic distance within the isolates of PMWaV-1, PMWaV-2 and PMWaV-3 were 0.018±0.002, 0.007±0.002 0.020±0.004 respectively (Table 8).

Using the Maximum Likelihood method via the HyPhy package [35] 17 detected codon positions in the HSP70 gene of PMWaV-1, 7 for PMWaV-2 and 4 codon positions in the HSP70 gene for PMWaV-3, were under significant positive selection ($P < 0.05$) (Table 8). This provided evidence of heterogenous selection pressures among codon sites in HSP70 genes for PMWaV-1, PMWaV-2 and PMWaV-3 datasets. There was also comparison for the overall selection intensity in the HSP70 genes. The results showed that the selection intensity (mean pairwise d_N / d_S) for this gene was 0.2587 for PMWaV-1, 0.2696 for PMWaV-2 and 0.1545 for PMWaV-3) (Table 8). Thus, overall, the values of the d_N/d_S were low, i.e. $d_N/d_S < 1$, implying that the HSP70 gene of PMWaV-1, PMWaV-2 and PMWaV-3 was under negative selection.

Neutrality tests

The results for the various neutrality tests are summarised in Table 9. Tajima's D test and Fu and Li's F* test for PMWav-1 were significant in terms of neutrality deviation ($P < 0.05$), but the rest of the tests (Fu and Li's D* and Fu and Li's F* tests) did not detect significant neutrality deviation ($P > 0.05$) for the PMWaV-1, PMWaV-2, and PMWaV-3 populations.

Discussion

Over the years, symptoms alone have not been effective in the detection of the plant viral disease [36]. The detection of PMWaVs by molecular means has however been shown to be reliable and efficient [11, 20]. To identify ampeloviruses associated with MWP disease, viral RNA was detected by qRT-PCR using species specific primers developed by Sether et al. [20] and Melzer et al. [37] and subsequently confirmed by phylogenetic analyses of nucleotide and deduced amino acid sequences of HSP70 gene of the PMWaV isolates. The qRT-PCR assays successfully detected three ampeloviruses namely PMWaV-1-PMWaV-2 and PMWaV-3 from MWP-symptomatic pineapple samples collected from the three districts,

which are leading pineapple producing centres in Ghana. This finding is consistent with report of Dey et al. [6] which states that PMWaV is a currently recognized as a complex of viruses belonging to three recognized species, designated as Pineapple mealybug-wilt associated virus 1 (PMWaV-1), PMWaV-2, PMWaV-3, and the putative PMWaV-4 and PMWaV-5. This is the first time these three ampelovirus species have been identified in one country in Africa. Similarly, all the three virus species have been identified from Hawaii [6], Australia [11], Taiwan [38], Mexico, China, Cuba etc. These countries are major pineapple countries in the world, suggesting that these viruses are prevalent in all major pineapple growing countries worldwide as reported by Sether et al. [20] and Dey et al. [6] The presence of these three ampelovirus species in Ghana, has serious consequence in the epidemiology of MWP disease in the country, hence threatening the pineapple industry. It has been reported by Dey et al. [6] that PMWaV-2 species alone without the others could make the wilt symptom of pineapple to develop. Also, whilst in Hawaii, MWP symptoms are strongly associated with infection by PMWaV-2 alone [17] in Australia, the disease is strongly associated with infections by PMWaV-3 alone or co-infection by PMWaV-1 and PMWaV-3 [11].

The study also detected multiple viral infections of pineapple samples from AAK and KEEA districts by these three ampeloviruses (PMWaV-1, PMWaV-2 and PMWaV-3). This has serious consequences in the epidemiology and management of MWP in these districts especially at AAK where double and triple infections were detected from six out of eight pineapple samples tested. Mixed infections can result in recombination of viral species leading to the generation of variants showing novel genetic features, which may cause severe damage to crops [39].

It was observed that AAK district had higher levels of PMWaV infection than KEEA and Ekumfi districts. This finding could be attributed to the presence of large numbers of viruliferous mealybugs feeding on the pineapple plants in the AAK districts as reported by Dey et al. [6]. The least PMWaVs infection of pineapple samples from Ekumfi could also be as a result of the small number of samples tested or exposure to non-viruliferous mealybugs under field conditions or farmers practising proper agronomic practices such as good sanitation and proper pest management which reduce the abundance of the viruliferous mealybugs on their farm as reported by Sether and Hu [17].

Genetic variability of PMWaV-1, PMWaV-2 and PMWaV-3 populations infecting pineapple crops in the Central Region of Ghana was analysed using the sequences encoding HSP70 homologous genes of the viral genome. The results revealed that the PMWaV-1, PMWaV-2 and PMWaV-3 isolates clustered into three different genetic groups (evolutionary divergent lineages) corresponding to three clades supported by bootstrap values more than 98%, irrespective of the geographical origin. Thus all the Ghanaian PMWaV-1, PMWaV-3 and PMWaV-2 isolated clustered together with This affirms the reports of Dey et al. [6] and Gambley et al. [11] which describe PMWaV-1, PMWaV-2 and PMWaV-3 isolates as three distinct closterovirus species. It was however interesting to note that the HSP70 ORF amino acid phygenetic tree revealed that PMWaV-1 isolates which clustered at clade 1 had two significant sub-clusters supported by 92% bootstrap support with our field isolate with accession number MN427634 clustring with isolate from Genbank with accession number HG940514 (Figure 4). On the other hand, PPMWaV-2 and PMWaV-

3 isolates did not form sub-clusters. This suggests that HSP70 gene of PMWaV-1 is more diverse than that of PPMWaV-2 and PMWaV-3. This is supported by the relatively higher nucleotide and haplotype diversities recorded for PMWaV-1 than that of PMWaV-2 and PMWaV-3 (Table 7). This could be due to the greater number of mutations and recombination in the gene of and PMWaV-1 compared to that PMWaV-2 and PMWaV-3, which is consistent with the finding of Melzer et al. [18]. According to report by Roossinck, [40] mutation and recombination are the initial sources of variation in populations. RNA viruses use all known genetic variation processes to guarantee their survival, mutation and recombination are the main cause of errors that occur during replication of RNA viruses resulting in a high degree of variability [41].

In spite of the high number of mutations and the consequent high number of haplotypes recorded for the PMWaV-1, 2 and 3 HSP70 genes, the genetic diversity was low (0.0172 ± 0.0032 for the PMWaV-1, 0.0199 ± 0.0024 for PMWaV-3 and 0.0074 ± 0.0016 for PMWaV-2), suggesting genetic homogeneity. This is in line with Sacristan and García-Arenal [42] and Elena et al. [41] which indicated that populations of plant viruses are not extremely variable despite high genetic variation potential and high mutation rates are not necessarily adaptive as a portion of the mutations are deleterious. It has also been reported that analysed populations of plant viruses are genetically stable, and this is so regardless of the many haplotypes that may occur in the population [41] This assertion is further supported by the finding from our study on the selection pressure where HSP70 gene for all the three ampelovirus species (PMWaV-1, PMWaV-2 and PMWaV-3) were found to be under negative selection (Table 8).

The high rate of mutation in RNA viruses could not be due to an evolutionary strategy but to the need for replication of their chemically unstable RNA genome [40]. However, high mutation rates for RNA viruses have been revealed to represent an evolutionary strategy [42].

An indication of population substructuring was the important neutrality deviation observed from the neutrality trials. All the tests for neutrality showed negative values (see Table 24), indicating that all PMWaV-1, PMWaV-2 and PMWaV-3 populations were in active evolution.

Conclusions

Three different ampeloviruses namely PMWaV-1, PMWaV-2 and PMWaV-3, were detected from the plant samples following qRT-PCR assays with PMWaVs species specific primers and sequence and confirmed phylogenetic analyses of nucleotide and amino acid sequences of HSP70 gene. The HSP70 gene of the PMWaV-1, PMWaV-2 and PMWaV-3 had low nucleotide diversity and was under negative selection. Mixed viral infections by 2 or all three viral species were detected in the pineapple samples from two out of the three districts in the Central region. This is the first report of PMWaV-1, PMWaV-2 and PMWaV-3 in Ghanaian pineapple and in Africa.

Declarations

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Authors' contributions

EAB conceived and designed the manuscript; JN performed the experiment and wrote the draft; GVP and ATA performed analyses. All authors read and approved the manuscript

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Availability of data and materials

The HSP70 nucleotide sequences of the Ghanaian PMWaV-1, -2, and -3 isolates used in the study have been deposited in GenBank under the accession numbers as follows: MN427634-MN427639 and MN399973 (PMWaV-3 isolates); MN427642-MN427645 (PMWAV-2 isolates) and MN427640and MN427641 (PMWaV-3 isolates)

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests

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Abbreviations

HSP70 heatshock protein 70 gene; PMWaV pineapple mealybug wilt associated wilt**;** qRT-PCR quantitative reverse transcription-polymerase chain reaction; MWP mealybug wilt of pineapple

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Tables

Table 1 Location, Climate and Vegetation types of the three districts collected from Ghana meteorological service during the study

District	Altitude	Longitude/ Latitude	Relative humidity (%)	Temperature (^{o}C)	Rainfall (mm)	*Vegetation type
KEEA	31.1	01^{O} 15’ W 05^{O} 06’ N	86	26-35	9201	Coastal savannah
AAK	31.1	01^{O} 20’ W 05^{O} 05’ N	86	22-30	1940.2	Deciduous forest
Ekumfi	15.2	00^{O} 37’ W 05^{O} 22’ N	81	24-28	631.2	Coastal savannah

(Source: * MoFA, [19]; Ghana Meteorological Service, 2018)

Table 1: Reaction conditions used for qRT-PCR amplification of heatshock protein 70 gene of PMWaVs

Cycle step	Temperature (°C)	Time	Cycles
Reverse transcription	55	10 minutes	1
Initial denaturation	95	1 minutes	1
Denaturation	95	10 seconds	40-45
Extension	60	30 seconds	
Melt curve	60-95	Various	1

Table 2 Primers used for qRT-PCR detection of *Ampelovirus* species

Primer name	Primer sequence (5’ – 3’)	Viral species	Reference
225	ACA GGA AGG ACA ACA CTC AC	PMWaV-1	Sether et al., 2001
226	CGC ACA AAC TTC AAG CAA TC		
224	CAT ACG AAC TAG ACT CAT ACG	PMWaV-2	Sether and Hu, 2002b
223	CCA TCC ACC AAT TTT ACT AC		
264	ATT GAT GGA TGT GTA TCG	PMWaV-3	Sether et al., 2001
263	AGT TCA CTG TAG ATT TCG GA		

Table 3 Pineapple mealybugs wilt associated viruses isolates from GenBank

Name of isolates	GenBank accession Numbe r	Country	Genomic region	Authors
PMWaV-1				
KT322148	KT322148.1	Thailand	Partial genome	Srikumphung and Chiemsombat (2015)
HQ129930	HQ129930.1	Cuba	Partial genome	Hernandez and Ramos (2012)
KT322152	KT322152.1	Thailand	Partial genome	Srikumphung and Chiemsombat (2015)
EU769113	EU769113.1	Taiwan	Partial genome	Shen *et l.* (2009)
HG940514	HG940514.1	Thailand	Partial genome	Koohapitagtam (2014)
MH704740	MH704740.1	USA	whole genome	Green *et al.* (2018)
HE583225	HE583225.1	Thailand	Partial genome	Koohapitagtam and Hongprayoon (2011)
EF620774	EF620774.1	Thailand	Partial genome	Chiemsombat and Maneechote (2007)
KC800714	KC800714.1	Mexico	Partial genome	Ochoa-Martinez *et al.* (2013)
KJ872494	KJ872494.1	China	whole genome)	Yu *et al.* (2014)
AF414119	AF414119.3	USA	whole genome)	Melzer *et al.* (2009)
JX645771	JX645771.1	Cuba	Partial genome	Hernandez-Rodriguez (2012)
PMWaV-2				
KT322167	KT322167.1	Thailand	Partial genome	Srikumphung and Chiemsombat, (2015)
FN825676	FN825676.1	Cuba	Partial genome	Hernandez et al. (2010)
EU769115	EU769115.1	Taiwan	Partial genome	Shen *et l.* (2009)

MH704741	MH704741.1	USA	whole genome	Green *et al.* (2018)
HE583226	HE583226.1	Thailand	Partial genome	Koohapitagtam and Hongprayoon (2011)
NC043105	NC_043105.1	USA	Partial genome	Melzer *et al.* (2000)
EU016675	EU016675.1	Thailand	Partial genome	Chiemsombat *et al.* (2007)
JX645772	JX645772.1	Cuba	Partial genome	Hernandez-Rodriguez (2012)
PMWaV-3				
GU563497	GU563497.1	Cuba	Partial genome	Hernandez *et al.* (2010)
MH704742	MH704742.1	USA	Whole genome	Green *et al.* (2018)
NC_043406	NC_043406.1	USA	Whole genome	Sether *et al.* (2009)
JX508636	JX508636.1	Cuba	Partial genome	Hernandez-Rodriguez (2012)

Table 4 Ampelovirus species detected from 24 diseased pineapple plant samples from three districts in the Central Region

District	Sample number	Virus species		
		PMWaV-1 (Ct)	PMWaV-2 (Ct)	PMWaV-3 (Ct)
AAK	17	+	+	-
AAK	18	+	+	-
AAK	19	+	+	-
AAK	20	+	+	+
AAK	21	+	+	-
AAK	22	+	+	+
AAK	23	-	+	-
AAK	24	-	+	-
Ekumfi	1	-	+	-
Ekumfi	2	+	-	-
Ekumfi	3	-	+	-
Ekumfi	4	-	-	-
Ekumfi	5	-	-	-
Ekumfi	6	-	-	-
Ekumfi	7	-	-	-
Ekumfi	8	-	+	-
KEEA	9	-	-	-
KEEA	10	-	+	-
KEEA	11	-	+	-
KEEA	12	-	-	-
KEEA	13	-	+	-
KEEA	14	-	-	-
KEEA	15	+	+	-
KEEA	16	-	-	-

Present (+) and Absent (-); KEEA: Komenda-Edina-Eguafo-Abirem; AAK: Abura-Asebu-Kwamankese

Table 5 Mixed infections of pineapple samples by pineapple mealybug wilt associated viruses

District	Mixed virus infections (%)		Total (%)
	Double	Triple	
AAK	4	2	6 (75)
Ekumfi	0	0	0 (0)
KEEA	1	0	1 (12.5)

Table 6 Nucleotide (nt) and amino acid (aa) sequence identities of pineapple mealybug wilt associated virus (PMWaV) isolates from Ghana and selected published isolates retrieved from GenBank

Isolates	Sequence identity (%)	
	Nucleotide	Amino acid
PMWaV-1 (HSP70)		
Between sequenced isolates	95.2 - 99.7	86.5 – 99.2
Between sequenced isolates and published isolates	95.2 – 100	86.5 – 100
PMWaV-2 (HSP70)		
Between sequenced isolates	98.9 – 100	97.1 – 100
Between sequenced isolates and published isolates	98.2 – 100	95.5 – 100
PMWaV-3 (HSP70)		
Between sequenced isolates	98.3	95.3
Between sequenced isolates and published isolates	97.5 - 99.3	93.3 – 98.0

Table 7 Genetic variability within HSP70 DNA sequences of PMWaV-1, PMWaV-2 and PMWaV-3 isolates

Dataset	Number of sequences	Number of polymorphic sites (S)	Total number of mutations (Eta)	Nucleotide diversity (π) [a]	Haplotype diversity (h) [a]
PMWaV-1	19	64	69	0.0172 ± 0.0032	1.000 ± 0.0029
PMWaV-2	13	23	23	0.0074 ± 0.0016	0.9870± 0.0035
PMWaV-3	6	27	28	0.0199 ± 0.0024	1.000 ± 0.0076

Table 8 Mean pairwise genetic distance and the selective pressures within HSP70 genes of the PMWaV-1, 2 and 3 isolates

Specie	Mean genetic distance[a]	d_N	d_S	dN/dS	Total number of codons	Codon positions under positive selection[b]
PMWaV-1	0.018±0.002	0.0652	0.2521	0.2587	189	17
PMWaV-2	0.007±0.002	0.0199	0.0738	0.2696	196	7
PMWaV-3	0.020±0.004	0.0214	0.1385	0.1545	160	4

Table 9 Neutrality test for HSP70 gene of PMWaV-1, PMWaV-2 and PMWaV-3

Species	Tajima's D	*P*-value	Fu and Li's D*	*P*-value	Fu and Li's F*	*P*-value
PMWaV-1	-2.07401	< 0.05[a]	-2.26722	> 0.05[b]	-2.57250	< 0.05 [a]
PMWaV-2	-1.79390	<0.05[a]	-2.14661	>0.05 [b]	-2.34611	>0.05 [b]
PMWaV-3	-0.87754	<0.5[a]	-0.70049	>0.05 [b]	-0.81754	>0.05 [b]

[a]Significant at $P < 0.05$ [b] Not significant ($P > 0.05$)

Figures

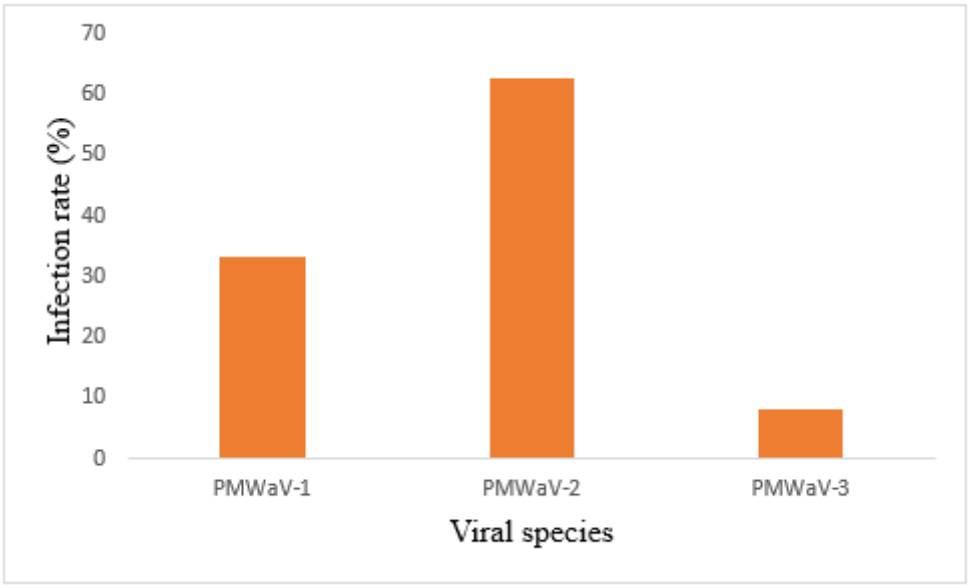

Figure 2

Infection rate of pineapple mealybug wilt associated virus-1 (PMWaV-1), PMWaV-2 and PMWaV-3 on 24 pineapple samples from three districts in the Central Region of Ghana

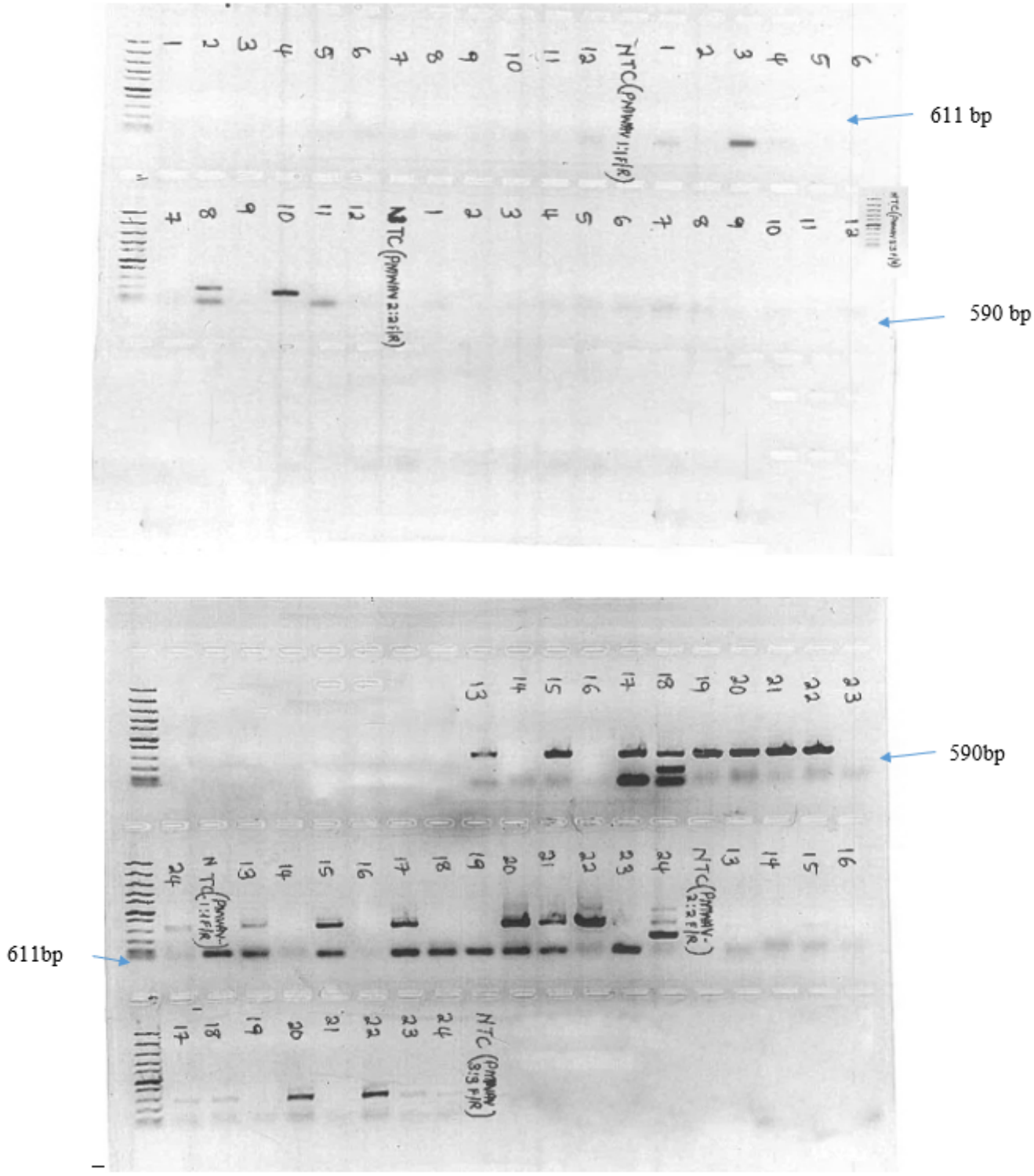

Figure 4

The amplicons of pineapple mealybug wilt associated virus-1 (PMWaV-1), PMWaV-2 and PMWaV-3 obtained from MWP symptomatic-pineapple samples using 225/226, 223/224, 263/264 primer pairs respectively. The amplification was done in two parts; Samples 1 to 12 and samples 13 to 24 with band sizes of 500 – 610 bp; Samples 1-8 were obtained from Ekumfi district; Samples 9-16 were obtained from KEEA district and samples 17-24 were obtained from AAK district.

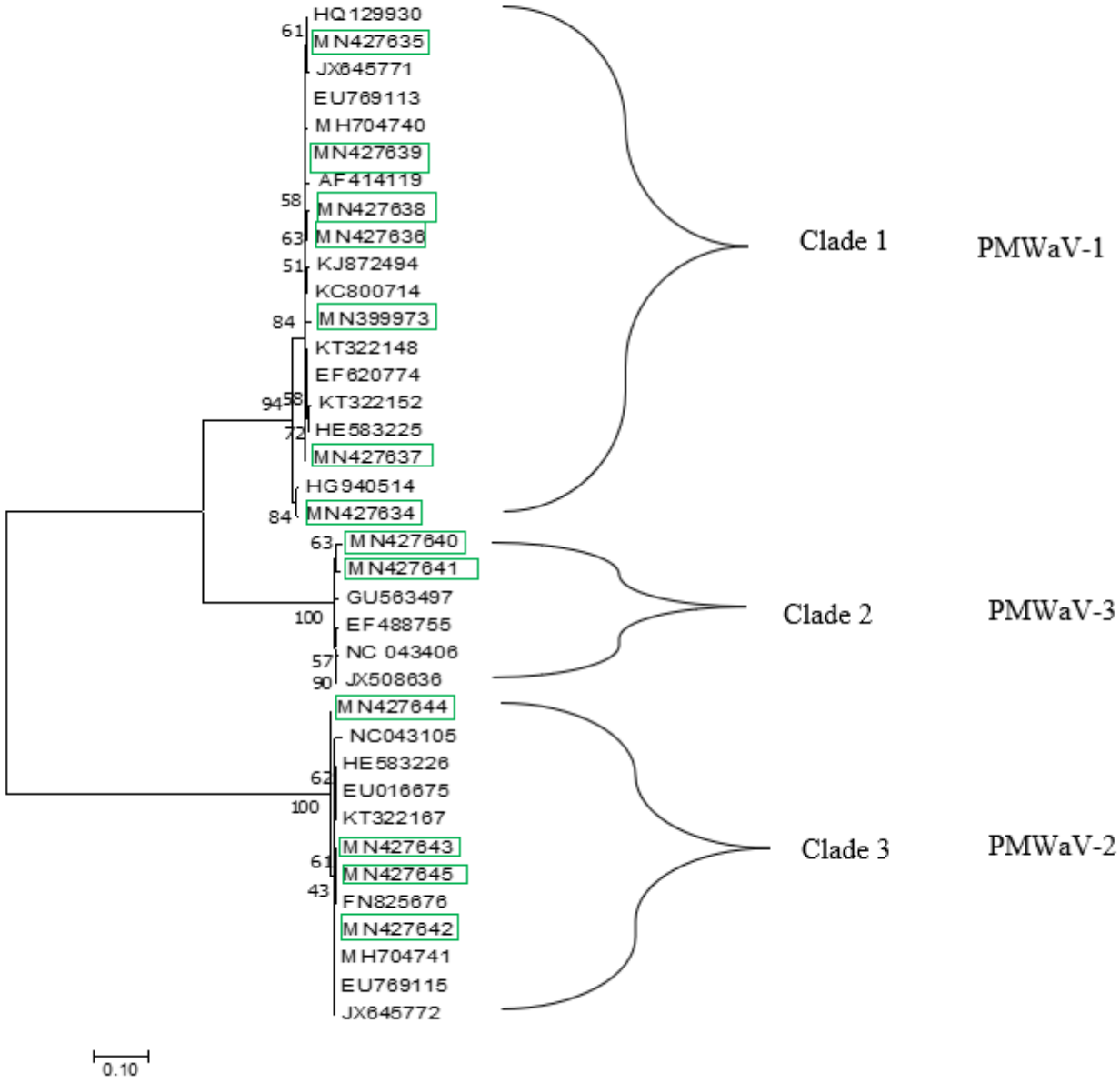

Figure 6

Maximum-likelihood phylogenetic tree of nucleotide sequences of partial HSP70 genes of PMWaV-1, -2, and -3 isolates (n=37) sampled from the Central Region of Ghana and those from GenBank. Accession numbers of Ghanaian isolates are in green boxes whilst the rest are accession numbers of isolates published in GenBank. The scale bar signifies a genetic distance of 0.10 nucleotide substitutions per site.

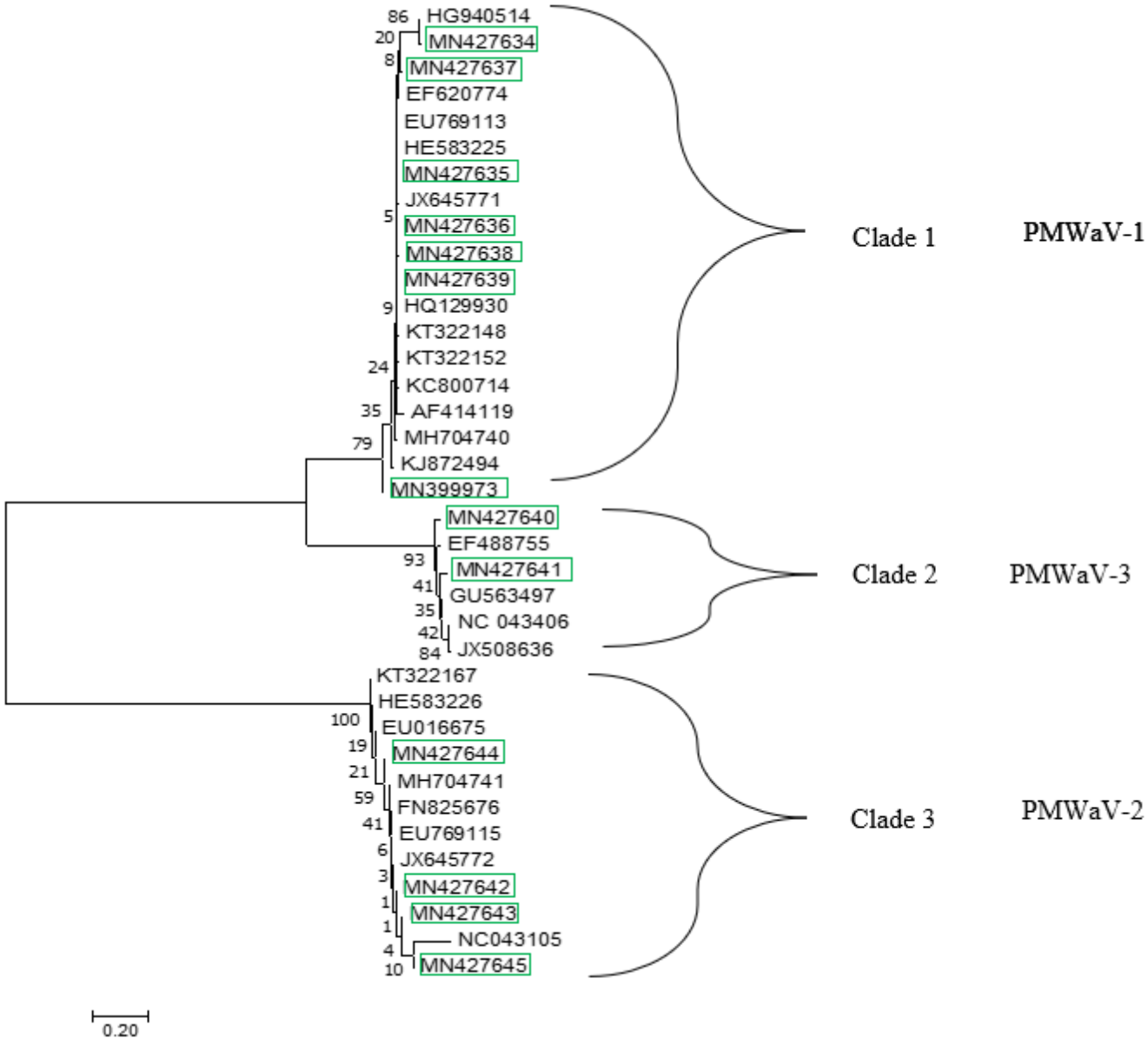

Figure 8

Maximum likelihood phylogenetic tree (abridged) of hsp70 amino acid sequences of PMWaV-1, -2 and -3 isolates (n= 37) sampled in the Central Region of Ghana and corresponding isolates from GenBank. Accession numbers of Ghanaian isolates are in green boxes whilst the rest are accession numbersof isolates from GenBank. The scale bar signifies a genetic distance of 0.20 nucleotide substitutions per site.