SINGLE AND MIXED INFECTIONS OF PLANT RNA AND DNA VIRUSES ARE PREVALENT IN COMMERCIAL SWEET POTATO IN HONDURAS AND GUATEMALA

by

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DEDICATION

I dedicate my thesis to my family, for their unconditional support even from afar.

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ABSTRACT

Sweet potato is one of the 15 most important food crops worldwide. At least 30 different virus species, belonging to different taxonomic groups affect sweet potato. Little is known about the viruses present in sweet potato crops in Central America, which is the primary origin of sweet potato. The objective of this study was to design and implement primers for use in polymerase chain reaction (PCR) and Reverse transcription-PCR (RT-PCR) to identify and survey the diversity of plant viruses infecting sweet potato in Honduras and Guatemala. Primers were designed and used to amplify, clone, and sequence a taxonomically informative fragment of the coat protein (CP) gene for whitefly-transmitted geminiviruses (herein, *sweepoviruses*) and potyviruses, and of the heat shock protein 70 (HSP70) for the crinivirus, *Sweet potato chlorotic stunt virus* (SPCSV). The partial genome sequences were used for identification based on phylogenetic relationships with reference sequences available in the GenBank database. All three of the plant virus groups identified in this study were found to occur either in single or in multiple infections. Results of the sequence analyses indicated that the genomic regions amplified in this study were capable of discriminating among potyvirus species, and strains of SPCSV. With respect to potyvirus, all isolates were identified as *Sweet potato feathery mottle virus* (SPFMV) species, except for two, which grouped phylogenetically with *Sweet potato virus G* (SPVG) and *Sweet potato virus C* (SPVC). All sweepoviruses detected in sweet potato plants belonged to a single phylogenetically, well-supported group that contains all other previously described geminiviruses (sweepoviruses) associated with sweet potato or closely related host species. These results demonstrate that the primers designed for amplification of plant virus species commonly recognized to infect sweet potato, effectively detected the viruses singly and in mixtures from symptomatic plants, and that the resultant fragment, when subjected to cloning and DNA

sequenced, was phylogenetically informative at the species and/or strain levels, depending on the virus group.

INTRODUCTION

1.1 Literature review

1.1.1 Importance of sweet potato

Sweet potato *Ipomoea batatas* (L.) is a tropical vine from the *Convolvulaceae*, and it is considered one of the 15 most important food crops worldwide (FAOSTAT 2013). Nearly 97% of the sweet potatoes are cultivated in developing countries, produced as a low cost subsistence or export crop both for human consumption and animal feed (Loebenstein et al. 2009). China is the main producer of sweet potato worldwide, accounting for more than 70% of the production (FAOSTAT 2013). Among other regions where sweet potato is grown commercially for local and export use are South East Asia, Africa, United States and Latin America (Loebenstein et al. 2009)

Key morphological characters of sweet potato plants and wild *Ipomoea* species, together with evidence provided by amplified fragment length polymorphism (AFLP), have been used to predict that the primary origin and center of diversity of sweet potato resides in the region between the Yucatán Peninsula and Orinoco River in Venezuela (Austin, 1998, Zhang et al. 2000). This area includes Guatemala and Honduras, the two locales that are the focus of this study.

Even though Guatemala is located within the center of origin of the sweet potato, commercial production there has been limited. Sweet potato is grown in small areas as a subsistence crop by local farmers. Interest in increasing sweet potato production has been reported, for which the

collaboration of the government supported “Instituto de Ciencia y Teconología” (ICTA) and the International Potato Center (CIP), as well as other research institutions has permitted the improvement of the crop in field trials. Given the high nutritional value of sweet potatoes and the nutritional deficiency that affects people in part of the country, initiatives that promote the increased production of this crop for Guatemala have been considered (Scott et al. 1992).

In Honduras, sweet potato has been exported since year 2000, with the collaboration of external agencies such as United States Agency for International Development (USAID), providing assistance in crop management and marketing. As a result 520 thousand pounds of sweet potatoes have been exported from Honduras to Europe, boosting the economic income for sweet potato producers, and expanding the interest in its cultivation by additional farmers (USAID, 2005).

In many tropical countries, planting material is obtained from stem cuttings taken from the material saved from the currently planted or the most recently harvested crop. In some temperatate regions, the storage roots produced for use as vegetative plant parts is used to plant the next season’s crop. In contrast, in the U.S., virus-free meristem tissue culture is usually the source of planting material, which is multiplied at least once in the fields (Gibson et al. 2015).

Viral diseases in sweet potato

A major constraint for sweet potato production worldwide is infection by plant viruses (Valverde et al. 2007). This is because vegetative propagation by vine cuttings or the storage root promotes

the accumulation of viruses in subsequent generations of planting materials (Zhang et al. 2011). Sweet potato is known to be a host of at least 30 viruses, belonging to nine families (Clark et al. 2012).

Circular, single-stranded DNA viruses: current classification: genus, *Begomovirus*; family *Geminiviridae*: common name: Sweepovirus

Members of the family *Geminiviridae* are known to infect cultivated sweet potato and other wild host species in the genus, *Ipomoea* (*Convolvulaceae*) such as *I. nil, I. setosa* Ker Gawl, *I. purpurea* or tall morning glory (Trenado et al. 2011), and hosts in the genus, *Merremia* (*Convolvulaceae*) including *Merremia quinquefolia* (L.) Hallier f. and *M. aegyptia* (L.) Urb. (Idris et al. submitted). Analysis of the full-length genome sequence for a number of isolates from sweet potato indicates that sweet potato-infecting begomoviruses are monopartite, and phylogenetically divergent from both monopartite and bipartite whitefly-transmitted viruses placed in the genus, *Begomovirus.* Analysis of additional isolates suggests they may constitute a new genus, however, for the time being, the common name "*sweepovirus*" is used to distinguish this group from other geminiviruses (Fauquet et al. 2003, Lozano et al. 2009). Evidence from studies involving all the different continents where sweet potatoes are grown suggests that this group of viruses is distributed worldwide (Gibson et al. 2015).

Sweepoviruses consist of circular, single stranded DNA of about 2.8 kilobases (kb), encapsidated in icosahedral, twinned particles, which are characteristic of the family *Geminiviridae* (Brown et al. 2012). According to the most recent ICTV guidelines, a pairwise identity threshold of 91% or

greater is used to demarcate begomoviral species, whereas, strains are defined as having 94% or greater pairwise nt identity, at least eight species have been identified (Brown et al. 2015).

In addition, sweepoviruses are transmitted by several biotypes of the whitefly vector *Bemisia tabaci* (Genn.) (Valverde et al. 2004, Simmons et al. 2009, Trenado et al. 2011). Among the documented SPLCV hosts, 38 of the 45 different species belong to the genus, *Ipomoea* have been shown to be whitefly transmissible. Additional plant species classified in 30 different families have been tested as possible SPLCV hosts, but thus far SPLCV has not been detected in any non-*Ipomoea* hosts (Ling et al. 2011). Wild perennial *Ipomoea* hosts included in the latter study were previously reported as plant hosts of the whitefly vector *B. tabaci* (Simmons et al. 2009). This suggests that even if the sweet potato crops are not grown throughout the year, alternate wild hosts might act as virus reservoir, which it is an important factor to consider for disease management strategies (Ling et al. 2011). In another study involving naturally-infected wild host species from Puerto Rico *Sweet potato leaf curl virus* (SPLCV) was identified in mixed infection with the New World, bipartite *Begomovirus*, *Merremia leaf curl virus* (MeLCV) in *Merremia* species (Brown and Idris, 2007; Idris et al. submitted). Recently, Kim et al., (2015) reported the transmission of SPLCV through true seed of sweet potato plants, with 15% of the seedlings derived from seed being infected. The SPLCV was present in more than 70% of seeds harvested from infected plants, and virus was detected in dissected endosperm and embryos.

A partial fragment, or the ‘core coat protein’ gene (575 bp) has been shown to be useful for provisional identification and classification of members of the genus, *Begomovirus* (Brown et al. 2001). The functions of the CP of begomoviruses include encapsidation, vector transmission

(Höhnle et al. 2001), and enables systemic infection of monopartite begomoviruses (Melgarejo et al. 2013). The sweepovirus CP gene has been used as a basis for PCR primer design (Clark et al. 2012).

SPLCV infection results in leaf curling and vein yellowing on some hosts, but the virus also can be present in asymptomatic plants, the latter of which is a limitation for selecting virus-free material for propagation when virus detection methods are unavailable (Albuquerque et al. 2012, Gibson et al. 2015). Even when the characteristic foliar symptoms are not observed, SPLCV isolates have been found to result in 10 to 80% yield loss in field trials for different sweet potato cultivars (Ling et al. 2010, Gibson et al. 2015).

RNA viruses infecting sweet potato crops

Genus*, Potyvirus; family, Potyviridae*

The genus *Potyvirus*, which is classified in the family *Potyviridae*, has a genome composed of a single-stranded, positive sense RNA that is encapsidated in a flexible, filamentous particle that has helical symmetry. The ssRNA genomes of the sweet potato-infecting potyviruses are about 10.8kb in size. They encode a single polyprotein that is then self-cleaved into smaller functional proteins (Tugume et al. 2010, Adams et al. 2012).

A number of plant species have been reported as hosts of the potyviral species, SPFMV, including 22 species of *Ipomoea, Hewittia sublobata* (L. f.) Kuntze, and *Lepistemon owariensis* (Beauv.) Hall. f., which are all members of the *Convolvulaceae* (Tugume et al. 2008).

Sweet potato potyviruses are transmitted in a non-persistent, non-circulative manner by the aphids *Myzus persicae* (Sulzer) and *Aphis gossypii* (Glover), as are many members of the genus, *Potyvirus* (Byamukama et al. 2004, Adams et al. 2012; Atreya et al. 1995). The CP in potyviruses, besides encapsidating the virus, has functions related to the transmission of potyviruses by aphids (Atreya et al. 1995), cell-to-cell movement, and virus replication (Andrejeva et al. 1999).

Given the relatively large size of the potyviruses genome, the full-length CP gene coding region is used to assess the taxonomic status and phylogenetic relationships among potyviruses (Tugume et al. 2010). In addition, PCR primers have been also designed to target the three prime untranslated region (3'UTR) (Clark et al. 2012, Tugume et al. 2010, Tairo et al. 2005), which is highly conserved within potyvrus species, and therefore is useful for taonomic purposes (Adams et al. 2005). The most recent report of the ICTV indicates that the species demarcation criteria of potyviruses is established at a threshold of 80%, based on the amino acid sequence similarity of the CP, and 76% for the percentage nucleotide (nt) identity for either the CP or the complete viral genome (Adams et al. 2012).

Among the sweet potato potyviruses, SPFMV has been considered to be the most widespread, and it has a worldwide distribution (Karyeija et al. 1998, Tugume et al. 2010). Infection frequency of the virus varies, but in some fields, infection has been documented to occur in 100% of the sweet potato plants sampled (Bryan et al. 2003). Economic losses associated with single infection of SPFMV is not considered significant, and in many cases no symptoms are

observed (Gibson et al. 1997, Karyeija et al. 1998). Conflicting results have been reported in Kenya and Uganda, where yield losses in field experiments can average 50% (Gibson et al. 2005). When symptoms are present, they have been found to vary according to the virus strain and sweet potato variety, and can involve leaf decoloration and damage to the root that manifests as russett cracking and internal corking (Bryan et al. 2003).

Other species within the *Potyvirus* genus that have been found less frequently in sweet potato, compared to SPFMV, are: SPVG, *Sweet potato virus 2* (SPV2) formerly referred as *Sweet potato virus Y, Ipomoea vein mosaic virus*, and *Sweet potato vein mosaic virus* (Li et al. 2012); SPVC, previously recognized as a SPFMV strain C (Untiveiros et al. 2010); *Sweet potato latent virus,* and *Sweet potato mild speckilng virus.* SPFMV, SPVC, SPVG, and SPV2 are phylogenetically related and represent an *Ipomoea*-specific lineage (Untiveiros et al. 2008).

Sweet potato chlorotic stunt virus* (SPCSV), Genus *Crinivirus*; family *Closteroviridae

The plant virus that is considered to cause the most damage to sweet potato crops is SPCSV. Members of this genus are helical flexous filaments, with a bipartite genome that consists on linear positive sence single stranded RNA, with a total size of 17.6 kb. (Martelli et al. 2012, Cuellar et al. 2011) The virus is transmited in a semi-persistent manner by *B. tabaci* (Genn.) (Cohen et al.1992).

Species classification criteria include particle size, genome structure and size, vector annd host specificity and a 75% similarity threshold for amino acid sequece in polymerase, CP and HSP70 (Martelli et al. 2012).

The HSP70 is postulated to be involved in processes such as virus particles assembly, assembly of complexes required for replication, and subgenomic RNA synthesis and cell-to-cell movement (Martelli et al. 2012). Given the large size of SPCSV genome, sequencing data is mostly restricted to few open reading frames (ORFs). The HSP70 is highly conserved among isolates of the same strain, thus is a useful taxonomic marker for members of *Closteroviridae*. The HSP70 gene of SPCSV has been successfully amplified by RT-PCR using primers for molecular detection. (Alicai et al. 1999, Qin et al. 2013, Clark et al. 2012)

Two distinct strains of SPCSV have been identified and named East Africa and West Africa strain. The isolates differ in nt and amino acid sequence data, and are phylogenetically distant from each other. The West Africa strain is known to have a worldwide distribution, except in East African countries, where the East African strain is prevalent. The East Africa strain was originally thought to be restricted geographically to East African countries such as Uganda, but recently, it has been reported in Peru, Tanzania, and China (Qin et al. 2013, Tugume et al. 2013).

Infection by SPCSV alone may cause significant symptoms and yield reductions as great as 34-44%, for example, in Peru (Gutiérrez et al. 2003). Yield loss has been observed to be higher when the SPCSV-EA strain is prevalent (Gibson et al. 2015).

Synergistic interactions among viruses

Infection of sweet potato by multiple viruses is quite common, owing to their persistence in the tuberous roots that are vegetatively propagated, allowing viruses to be passed on to the offspring. In some instances certain viruses have been found to interact in a synergistic manner, in which on virus aids the others in accumulating to higher levels than possible on their own, often leading to the development of more severe symptoms. In this scenario, the virus that is synergized by the presence of the other, reaches a higher virus titer in the plant, due to increased replication and/or enhanced viral movement. Plants that experience this type of mixed infection usually exhibit increased in the symptoms severity and lower yield (Untiveiros et al. 2007).

Sweet potato virus disease (SPVD), produced by the synergistic interactions between SPCSV and SPFMV in East Africa can cause losses as great as 90% (Karyeija et al. 1998, Clark et al. 2012). Also, in Peru more than 80% of plants with SPVD developed severe foliar symptoms and yield was reduced by 65-72% (Gutiérrez et al. 2003). Such symptoms can be variable, but usually are manifest as severe stunting, leaf distortion, chlorosis, clearing of the major veins, and purpling of leaves (Gibson et al. 1998). An analysis of virus titers in these plants indicates that SPCSV titer is similar to that in a singly infected plant, or even lower. In contrast, the titer of SPFMV can incerease by as much as 600-fold (Karyeija et al. 2000). It has been shown that SPCSV encodes an RNA endoribonuclease III (RNase-3), which functions as a strong silencing suppresor of plant host defenses, thereby mediating the synergistic effect of the mixed infection (Cuellar et al. 2009). Also, a synergistic interaction of SPCSV with sweepoviruses has been reported in which co-infection leads to an increase in sweepovirus titer, albeit, the enhancement

varies among sweepovirus isolates (Cuellar et al. 2014). Synergism between SPCSV and plant viruses of the genera: *Cavemovirus* (Cuellar et al. 2011), *Ipomovirus, Carlavirus,* and several different *Potyvirus* has also been reported (Untiveiros et al. 2007).

Sweet potato viruses reported in Central America

Few studies have been carried out to assess the viruses that infect sweet potato in Central America, except for a few publications of results with limited number of samples. Small RNA deep sequencing has been used to detect and identify the plant viruses in 12 sweet potato samples from Honduras, Guatemala, and the Galapagos Islands. The viruses detected were SPCSV (West Africa strain), SPFMV, SPVC, *Sweet potato leaf curl Georgia virus* (SPLCGV), *Sweet potato pakakuy virus* (*Badnavirus*), and *Sweet potato Symptomless virus* (*Mastrevirus*), being SPCSV the most common isolate (Kashif et al 2012). Two of the sequences obtained by this study for SPCSV were further analyzed by Tugume et al. 2013, in which the identity of SPCSV as West Africa strain was confirmed, and sequences were found to be highly similar (98-100% identity on the RNAse-3 and p7 ORFs). In addition, germplasm from the CIP that originated in Guatemala has been analyzed and sweepoviruses were detected on 4 samples. Partial AC1 and AC2 open reading frames (ORFs) sequence, which encode for the replication-associated protein and transcriptional activator protein respectively, revealed that this isolates were highly similar to the majority of sweepoviruses detected in other Latin American countries (Cuellar et al. 2015, Brown et al. 2012).

A publication of viruses detected in sweet potato from Costa Rica, Central America (Valverde et al. 2004) reported the presence of SPFMV, SPCSV and SPLCV (listed in order of abundance) from the analysis of 42 samples. The survey reported also the presence of mixed viral infections, with co-infection of SPFMV and SPCSV being the most common. A low incidence of SPLCV was found with samples analyzed in the study (2 samples were positive). The overall detection frequency of SPFMV, SPCSV and SPLCV in Costa Rica ranged from 30-90% (Valverde et al. 2004).

Detection methods for DNA and RNA sweet potato viruses

Detection of sweet potato viruses presents many challenges, such as low titers of the viruses, the presence of inhibitors in plant tissue, diversity of viral strains, uneven virus distribution among the plant, and mixed viral infections, in which some viruses might mask the presence of others (Valverde et al. 2007).

Grafting onto *Ipomoea setosa* Kerr Gawl, which is considered a universal indicator plant for sweet potato viruses, has been commonly used. A limitation to this approach is that it does not confirm the identity of the viruses, given that some symptoms are common among viruses. In addition, it requires maintenance of plants in greenhouse or growth chambers. Grafting has also being used to enhance the titer of the viruses in a susceptible indicator host, leading to improved detection using serological and nucleic acid based methods (Clark et al. 2012).

Among available serological techniques, the enzyme-linked immunosorbent assay (ELISA) has

been used to detect a number of sweet potato-infecting RNA viruses. Direct blotting of sweet potato sap on nitrocellulose membrane, for immune-dot-blot detection using virus-specific antibodies, has also been performed (Gutierrez et al. 2003, Valverde et al. 2007, Abad 1992). One of the advantages of the latter approach is that is can be performed with limited resources (Abad 1992).

Molecular hybridization has also been for detection of SPLCV, and some of the earliest studies use this approach for virus detection in total DNA isolated from sweet potato plants (Lotrakul et al. 1998). In another example, an RNA probe has been implemented for RNA-cDNA hybridization to detect SPFMV, a method that was shown to provide greater detection sensitivity than the immunoassay approach (Valverde et al. 2007, Abad 1992).

PCR and RT-PCR have been shown to be useful for detecting sweet potato viruses (Li et al. 2004, Li et al. 2012, Qin et al. 2013). In general, PCR amplification detection is highly sensitive, and therefore is useful for detecting viruses that are present in low titer, and/or that are difficult to isolate from vascular tissues, or whose detection is confounded by inhibitors and require dilution prior to PCR amplification. This is the approach selected for use in the present study.

Rolling circle amplification (RCA) has been successfully used to amplify the circular, single-stranded DNA viruses, such as begomoviruses (Nagata et al. 2004, Wyant et al. 2012, Dean et al. 2001, Johne et al. 2009), and herein, sweepoviruses. Among the advantages, is that no previous knowledge of the sequence is required, and full length genomes can be amplified which provides more information about genetic diversity. Within the disadvantages, is that it is limited to the

detection of circular DNA viruses, such as sweepoviruses (Kreuze et al. 2009). Among other limitations, methodological problems have been reported due to the presence of defective DNA, and host plant mitochondrial plasmids, which are amplified non-specifically (Paprotka et al. 2010).

Next generation sequencing (NGS) platforms, such as Illumina, allow high throughput sequencing of small-RNAs (sRNAs), and therefore are important tools for the detection and identification of viruses, including sweet potato viruses (Kreuze et al. 2009, Kashif et al. 2012). In this technique, virus detection relies on the plant RNA interference or silencing mechanism, in which sRNAs are abundantly produced by the plant to target different regions of the virus RNA by sequence homology (Kreuze et al. 2009). NGS was shown to be highly useful when there is no prior knowledge of the viruses that might be infecting the host (Kashif et al. 2012). Among the limitations, is the difficulty in the elimination of host nucleic acids that include sRNA reads produced by the host for endogenous gene regulation, as well as the high cost of the analysis per sample (Kreuze et al. 2009, Pallas et al. 2011).

PRESENT STUDY

The thesis is organized in one appended manuscript that includes the Methods, Results, Discussion, and Literature Cited sections describing the results of this research effort. The research is summarized in the following paragraph.

2.1 Single and mixed infections of plant RNA and DNA viruses are prevalent in commercial sweet potato in Honduras and Guatemala

Summary

Sweet potato is one of the 15 most important food crops worldwide, and the main biological constraint to growing sweet potato as a food crop is yield loss and storage root quality reduction caused by virus infection. At least 30 different virus species, belonging to different taxonomic groups, affect sweet potato. Little is known about the viruses present in sweet potato crops in Central America, which is the proposed primary origin of sweet potato. The objectives of this research were: 1) to design PCR and RT-PCR primers to detect sweet potato begomoviruses (also known as sweepoviruses), potyviruses, and SPCSV in sweet potato plant samples from Honduras and Guatemala, 2) identify the virus to strain or species using comparative DNA sequence analysis, and 3) to determine the phylogenetic relationship between the virus isolate sequence and all available reference isolates for the strain or species, respectively. Oligonucleotide primers for PCR or RT-PCR amplification were designed and validated for the detection of three of the major groups or species of sweet potato viruses known to occur in the

Americas, sweepoviruses (family, *Geminiviridae*), potyviruses (family, *Potyviridae*), and SPCSV (genus *Crinivirus*; family, *Closteroviridae*). The primers were designed based on the viral sequences for the respective groups or species, available in the GenBank database (www.ncbi.nlm.nih.gov/genbank). The primer design was based on the most conserved regions of the CP gene for the sweepoviruses and potyviruses, and for SPCSV, on the HSP70 sequence. The resultant amplicons were cloned and the DNA sequence was determined, to verify the validity of the primers in relation to the respective positive virus control. The virus isolates used as the positive control were shown to be detectable with the respective primer pair, and the viral sequence identity was confirmed for each. The validated primers were used to screen symptomatic leaf samples collected from commercial sweet potato fields in Honduras and from subsistence farms in Guatemala.

When the primers were used for detection and identification of viruses associated with the field samples from Honduras and Guatemala, one or more viruses was detectable in many but not all of the samples. Pairwise identity and phylogenetic analyses were carried out to determine the identity, sequence diversity, and the taxonomic status of each isolate. The frequency of sweepoviruses was higher in Honduras at 41%, compared to Guatemala (24%). Samples from Honduras shared 88-91% nt identity, whereas, for the isolates from Guatemala the shared nt identity was 91% or greater. The most frequently detected plant virus in samples from Honduras was SPCSV at 61%, for which only the West Africa strain was identified, followed by potyvirus at 53%, of which SPFMV was the most commonly detected species. Most viruses were detected in mixed infection, with three viruses being detected in 23% of samples, including members of the three different viruses, followed by the co-infection of SPCSV and SPFMV (22%). Given

the variability in the incidence of the different viruses detected in the field samples, and that symptoms were not widespread early in the growing season, the results suggest that at least some of the viruses that infect sweet potato are present in the locales where production occurs, and not all of them accompanied the seed pieces upon planting. The results of sequence analyses indicated that the genomic region selected for amplification and sequencing were capable of discriminating among potyviruses species and between strains of SPCSV. For the sweepovirus isolates, all of the CP sequences were found to belong to a single phylogenetically well-supported group. Efforts are needed to better manage sweet potato-infecting viruses to reduce yield losses attributable to plant virus infection arising from infected seed pieces and/or from infections that occur post-planting, the latter being the most likely source of the majority of infections. In addition, studies of the potential occurrence of other groups of plant viruses known to infect sweet potato in the Eastern Hemisphere that have yet to be reported in the Americas to help avoid and/or manage new introductions immediately after they occur. Given the now known mixtures of viruses that are prevalent in sweet potato throughout the Americas, the design of multiplex molecular assays would provide the most cost-effective diagnostics for plant virus detection.

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APPENDIX A

Single and mixed infections of plant RNA and DNA viruses are prevalent in commercial sweet potato in Honduras and Guatemala

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ABSTRACT

Sweet potato is one of the 15 most important food crops worldwide. At least 30 different plant virus species, belonging to different taxonomic groups, are known to infect sweet potato. Little is known about the viruses present in sweet potato crops in Central America, which is considered to be a primary origin of sweet potato. The objectives of this research were to design and implement primers for use in polymerase chain reaction (PCR) and reverse transcriptase-PCR (RT-PCR) amplification to identify and survey the diversity of sweet potato begomoviruses (also known as sweepoviruses), potyviruses, and the *Sweet potato chlorotic stunt virus* (SPCSV) infecting sweet potato plants in Honduras and Guatemala. PCR primers were designed for amplification of the most well-known plant viral species that infect sweet potato. All primer pairs detected the target viruses, both singly and in mixtures from symptomatic plants. The amplified the viral partial coat protein (CP) or heat shock protein 70 (HSP70) sequence, respectively, was phylogenetically informative at the species level for the potyviruses, and at the strain level for SPCSV isolates. Based on the partial coat protein sequence, all sweepovirus isolates detected in sweet potato plants belonged to a single phylogenetically well-supported group that contains previously described sweepoviruses infecting sweet potato plants, or closely related host species in the *Convolvulaceae*.

Key Words: *Bemisia tabaci*, crinivirus, coat protein, *Geminiviridae*, heat shock protein, potyvirus, *Sweet potato chlorotic stunt* virus, *Sweet potato feathery mottle virus,* sweepovirus,

INTRODUCTION

Sweet potato *Ipomoea batatas* (L.) is a tropical vine classified in the *Convolvulaceae*, and it is considered one of the 15 most important food crops worldwide (FAOSTAT 2013). Nearly 97% of the sweet potatoes grown worldwide are cultivated in developing countries as low cost subsistence or export crops either for human consumption or animal feed (Loebenstein et al. 2009). China is the main producer of sweet potato worldwide, at 70%, followed by South East Asia, Africa, the United States, and Latin America (Loebenstein et al. 2009, FAOSTAT 2013).

Plant viruses are the greatest constraint to the production of sweet potato as a viable crop (Valverde et al. 2007). This is because vegetative propagation by vine cuttings or storage roots favors virus accumulation (Zhang et al. 2011). Sweet potato affected by at least 30 viruses, belonging to nine families (Clark et al. 2012). Many sweet potato infecting-viruses that have been characterized belong to the genus, *Begomovirus*, and at least eight species have been identified based on percentage nucleotide pairwise sequence identities (Brown et al. 2015). Sweet potato begomoviruses have a monopartite, circular, single stranded DNA genome, and they are transmitted by the whitefly *Bemisia tabaci* (Genn.) (Valverde et al. 2004, Simmons et al. 2009, Trenado et al. 2011). Sequence data show that they are phylogenetically divergent from the other members of the genus, and so the name "*sweepovirus*" has been used provisionally to denote this until taxonomic evidence is further solidified (Fauquet et al. 2003, Lozano et al. 2009). Evidence suggests that this group of viruses is distributed worldwide (Gibson et al. 2015).

The best-studied sweet potato-infecting viruses are represented by several groups of RNA-containing plant viruses. This is primarily due to their high disease incidence (Clark et al. 2006) and to the severity caused by a mixture of two viruses that are responsible for causing the sweet potato virus disease (SPVD). The SPVD is caused by synergistic interactions between the whitefly-transmitted crinivirus, SPCSV, and the aphid-transmitted potyvirus, *Sweet potato feathery mottle virus* (SPFMV). In East Africa, SPVD has been shown to cause as much as 90% loss in sweet potato production (Karyeija et al.1998, Clark et al. 2012), and in Peru, 80% of the plants analyzed were found to be infected with SPVD with yield reduction ranging from 65 to 72% (Gutiérrez et al. 2003).

Thus far, the majority of diseases caused by infection of a single plant virus, have been attributed to sweepoviruses and potyviruses, which cause either mild, transient symptoms in plants, or are asymptomatic. Even so, these seemingly mild infections impose limitations on the selection of materials for propagation that are virus-free (Gibson et al. 2015). Infection by sweepoviruses have been shown to cause considerable yield loss, ranging from 10 to 80%, based on field trials involving different sweet potato cultivars (Ling et al. 2010, Gibson et al. 2015). Infection by SPCSV can cause symptoms and yield loss, for example, a 34-44% reduction in crop yield has been reported in Peru (Gutiérrez et al. 2003).

Studies have predicted that the primary origin and center of diversity of sweet potato are in the (extant) region located between the Yucatán Peninsula and Orinoco River in Venezuela (Austin, 1998; Zhang et al. 2000), which includes Guatemala and Honduras. Even so, few studies have been carried out to assess the effects of the viruses that infect sweet potato crops in Guatemala

and Honduras, except for three studies with a very limited number of samples. In one of those studies, small RNA deep sequencing has been used to identify the plant viruses associated with 12 sweet potato samples from Honduras, Guatemala, and the Galapagos Islands. The viruses found to be present among the samples were SPCSV, SPFMV, *Sweet potato virus C* (SPVC), *Sweet potato leaf curl Georgia virus* (SPLCGV), *Sweet potato pakakuy virus* (*Badnavirus*), and *Sweet potato symptomless virus* (*Mastrevirus*), being SPCSV being the most common, which was characterized as West Africa strain. (Kashif et al. 2012, Tugume et al. 2013). In addition, sweet potato germplasm from Guatemala were analyzed and analyzed by PCR and DNA sequencing at the International Potato Center (CIP), Lima, Peru were reported to harbor sweepoviruses (Cuellar et al. 2015). Partial AC1 and AC2 open reading frames (ORFs) sequence revealed that this isolates were highly similar to the majority of accessions from Latin American countries (Cuellar et al. 2014).

In general, PCR amplification detection is highly sensitive, and therefore useful for virus detection. And the utility of PCR and RT-PCR have been previously been shown for the detection of sweet potato-infecting viruses (Li et al. 2004, Li et al 2012, Qin 2013). The objectives of this study were to design and implement PCR and RT-PCR primers for virus amplification, cloning, and sequencing of a partial genomic sequence from each of three different sweet potato-infecting viruses, potyviruses, *Sweet potato chlorotic stunt virus* (SPCSV), and sweepoviruses to determine the prevalence of these different viruses in sweet potato samples collected in Honduras and Guatemala from 2010 to 2014.

MATERIALS AND METHODS

Plant samples. Sweet potato leaves showing virus-like disease symptoms were collected from three and four locations each in Guatemala and Honduras, respectively (Figure 1). Samples from Guatemala were landraces collected during 2013, and shipped as total DNA extracts (as described below). Samples from Honduras were commercial plantings collected from 2010 to 2014, and were received in the US as fresh tissue or as fresh tissue, in glycerol. Plant samples infected with sweet-potato viruses whose identities were previously determined, were used as positive controls in the PCR and RT-PCR reactions, and included SPFMV, SPV2, SPCSV East Africa, and West Africa strain, were generously provided by J. Valkonen (University of Helsinki). D. Golino (University of California, Davis) provided a sweet potato plant sample from the UC-Davis germplasm collection known to be positive for SPLCV, which was used as a positive control for sweepovirus detection, and virus-free sweet potato leaves used as negative control.

DNA and RNA isolation. Total DNA and RNA were isolated from sweet potato leaf samples using the RNeasy Plant Mini Kit (QIAgen, Hilden, Germany), according to the manufacturer's instructions, with the following modifications, as suggested by Dr. J. Abad (USDA-ARS, Beltsville, MD) (personal communication), which included the addition of 1% β-mercaptoethanol and 2% polyvinylpyrrolidone (PVP-40) to the RLT lysis buffer (Qiagen cat. number 79216).

Quality evaluation of total nucleotide extract from Guatemala. To obtain a sense of the quality and concentration of nucleic acids isolated from the sweet potato field samples, eight samples, consisting of at least two samples from each collection site in Guatemala, were selected for quantification of DNA and RNA using a NanoDrop 2,000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The total RNA and DNA concentration among the samples ranged from 11.5 to 171.2 ng/uL and 13.9-213.8 ng/uL, respectively. An aliquot (5 ul) of DNA and RNA isolated from each sample was analyzed by agarose (0.8%) gel electrophoresis with 1X final concentration of GelRed (Biotium, Hayward, CA, USA) in 1X Tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer, pH 8.0. Gel bands were visualized and photographed using a Molecular Imager® Gel Doc™ XR System (Bio-Rad, Hercules, CA, USA) ultraviolet light transilluminator, using Image Lab 4.0.1 software (Bio-Rad, Hercules, CA, USA).

Primer design. A conserved region of the CP or HSP70 was identified for each genus/species of virus from sequences that were downloaded from GenBank (NCBI). MUSCLE (Edgar, R.C. 2004), an alignment tool in CLC Sequence Viewer (version 7.0.2, CLC Bio, Aarhus, Denmark), was used to align each group of virus sequences separately. Primer3 software (Untergrasser et al. 2012) was used to select the pair of primers defined as having the best nucleotide sequence match, and optimal criteria (annealing temperature, GC content, absence of primer dimers) for primer design to target the most highly conserved regions in the coding region targeted for the respective virus. Potyvirus genus-specific primers were designed to target the phylogenetically related *Ipomoea*-specific lineage, which includes SPFMV, *Sweet potato virus C* (SPVC), *Sweet potato virus G* (SPVG), and *Sweet potato virus 2* (SPV2) (Untiveiros et al. 2008). The sequence for the potyvirus reverse primer was obtained from Li et al. 2012, and the coverage for all

potyvirus species was confirmed by aligning the primer sequences with the respective GenBank reference sequence. The primers specifications are summarized in Table 1.

Polymerase chain reaction (PCR) amplification. For the PCR amplification of sweepoviruses, 1X Jump start Red Taq (Sigma-Aldrich, St. Louis, MO, USA), 0.2uM of each primer, and ddH_2O were mixed to a final volume of 25uL. The thermal cycling conditions were: 1 cycle at 94°C for 2 min; 35 cycles of 94°C for 45 s, 55°C for 45 s and 72°C for 45s, and 1 cycle of 10 min at 72°C.

Amplicons were analyzed by agarose as described above, and cloned into the pGEM T-Easy plasmid vector transformed into *E. coli* strain DH5 alpha, and transformants were selected on LB media containing ampicillin, using standard molecular biology protocols (Sambrook and Rusell, 2001). Colony PCR of the resultant clones containing putative inserts of the expected size, 632 base pairs (bp), was performed on five colonies from each transformation event, in a reaction mix consisting of 1X Buffer E, 0.2mM dNTP mix, 0.2uM of M13 F and M13R primers, 2 units of Platinum Taq DNA polymerase, and nuclease free water to a final volume of 50uL (Invitrogen, Carlsbad, CA, USA).Colony PCR cycling conditions were: 1 cycle at 94°C for 10 min; 35 cycles at 94°C for 1 min, 53°C for 1 min and 72°C for 1min, followed by a final extension cycle at 72°C for 10 min. Gel electrophoresis was performed as described above.

Reverse transcription polymerase chain reaction (RT-PCR) amplification. AccessQuick RT-PCR system (Promega, Madison, Wisconsin) was used for amplification of the viral RNA samples. The reaction mix consisted of AccessQuick Master Mix 1X, 0.2uM of each primer, 2.5u of AMV reverse transcriptase, and nuclease free water in a total volume of 25uL. The thermal cycling conditions were: 45°C for 45 min, 94°C for 2 min; 35 cycles at 94°C for 45 s,

58°C for 45 s and 45 s at 72°C, with a final extension step of 5 min at 72°C. To visualize the size of the PCR products, the amplicons were analyzed by gel electrophoresis as described above.

The resultant RT-PCR amplicons were ligated to pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed transformed into JM109 chemically competent cells by 2 min heat shock, cells were incubated for 1h in 450uL of LB media and 200uL were plated on LB agar with 32ug/mL Xgal, 100ug/mL of Ampicillin, and 0.1mM isopropyl-beta-D-thiogalactopyranoside (IPTG). Colony PCR was performed as previously described, on five colonies from each transformation event using M13 forward and reverse primers to amplify the cloned insert. The presence of the expected insert size for each primer pair (Table 1) was confirmed by gel electrophoresis, as described above.

Sequencing. The colony PCR amplicons were purified using ExcelaPure PCR purification blocks (Edge Biosystems, Gaithersburg, MD, USA) and sequenced by capillary (Sanger) DNA sequencing at the University of Arizona Genetics Core (UAGC, http://uagc.arl.arizona.edu/ include the URL), in the forward and reverse directions.

Sequence assembly and alignment. Seqman Pro version 11 (DNASTAR, Madison, WI) software was used for sequence assembly. The full-length genomic and partial reference sequences for each groups of viruses, were downloaded from GenBank and aligned with the assembled sequences for each set of amplicons using MUSCLE (Edgar, R.C. 2004) algorithm in CLC Sequence Viewer (version 7.0.2, CLC Bio, Aarhus, Denmark). The aligned sequences were trimmed to remove all expect the amplified viral sequence, and haplotypes were collapsed by 100% similarity using FaBox (Villesen et al. 2007). The following sequences were used as outgroups: for sweepoviruses, *Bean golden yellow mosaic virus* (BGYMV, M10070), for

potyviruses, *Potato virus Y* (PVY, AJ223595), and for SPCSV, *Cucurbit yellow stunting disorder virus* (CYSDV, AY242078).

Sequence alignments were exported in the .nexus format using Mesquite (Maddison and Maddison 2011). The BLAST algorithm (Zhang et al. 2000, available at http://blast.ncbi.nlm.nih.gov/Blast.cgi), was used to determine the identity of the virus isolates based on sequence comparisons with all of the plant viral sequences available in the GenBank database.

Percentage pairwise identity. The percentage pairwise nucleotide (nt) sequence identity analysis was carried out following the alignment of sequences with MUSCLE, followed by analysis using the Sequence Demarcation Tool version 1.2 software (Muhire et al. 2004).

Phylogenetic analyses. Three different tree inference methods were used to assess the phylogenetic relationships among haplotypes from each group of viruses.
The NJ analysis (Saitou and Nei 1987) was performed with 1000 bootstrap replicates, and the p-distance method (Nei and Kumar 2000), in Mega 6 (Tamura et al. 2013).

The optimal DNA model testing for ML analysis was performed in Mega 6 (Tamura et al 2013). The ML phylogeny was reconstructed using the optimal DNA model for evolution, estimated using Model Test (Darriba et al. 2012) for 1, 000 bootstrap replicates.

The jModelTest 2 algorithm was used to determine the model that best fits the evolution inherent in the sequences, based on Akaike information criterion with correction (AICc, Akaike, 1973). MrBayes 3.2 (Ronquist et al. 2012) was used to carry out the Bayesian analyses over four independent runs with 10 million Markov Chain Monte Carlo (MCMC) generations each with

sampling at a frequency of every 1,000 trees. Both the model testing and Bayesian analyses were performed in CIPRES (Miller et al. 2010) using XSEDE resources (Town et al. 2014). Convergence of the result from MrBayes was evaluated with Tracer 1.6 (Rambaut et al. 2014), and convergent runs were used for the inference of the Bayesian consensus tree (eliminating the first 1,000 trees as burn-in parameter, constructed with 50% majority rule), using MrBayes3.2 (Ronquist et al. 2012). The trees produced by the NJ or phylogenetic analyses programs were edited using Fig3 version 1.4 (Rambaut et al. 2012).

RESULTS

Detection of sweet potato viruses by PCR and RT-PCR

The PCR and RT-PCR primers for the detection of sweepoviruses, potyviruses and SPCSV successfully amplified the respective targeted region for each virus group or species, respectively. The expected identity was determined using BLAST2GO, and then by phylogenetic analysis using virus reference sequences obtained from the GenBank database for each virus.

The total DNA/RNA isolated from the sweet potato leaf samples collected in Guatemala were screened for the presence of both RNA and DNA viruses. When only DNA viruses were detected, quantification of DNA and RNA for eight samples was performed, and an agarose electrophoresis was run to analyze the integrity of the nucleic acid preparations. The spectrophotometric readings indicated that the DNA and RNA concentration varied greatly, from 11.5 to 213.8ng/uL, respectively. When preparations were analyzed by gel electrophoresis, no

high molecular weight bands were observed, suggesting that nucleic acids had degradation. Given that only DNA viruses were detected, it was concluded that RNA quality was insufficient to screen samples obtained from Guatemala for potyviruses and SPCSV, as was initially planned.

The analysis of the samples from Guatemala's indicated that 12 of 49 samples (24%) were positive for sweepovirus presence, a result that was also confirmed by cloning and DNA sequencing. Sweepoviruses were the most abundant species of virus found in Escuintla, in 9 (82%) samples (Fig. 3B). In contrast, most of the samples analyzed in Sacatepéquez 15 (83%) samples, and all the samples in Baja Verapaz were negative for the presence of sweepoviruses.

The occurrence of different plant viruses detected in sweet potato samples from Honduras is shown in Figure 2, reported as the abundance of each virus, without considering whether they were associated with mixed infections (Fig. 2A.), and then as the occurrence of each possible single infection and combination of viruses found in mixed infections (Fig. 2B). The most abundant virus detected in Honduras was SPCSV, which was present in 63 of 104 samples (61%) of the samples, followed by potyviruses in 55 of 104 samples (53%), and then sweepoviruses in 43 of 104 samples (41%). Among the total samples that were screened 29% were negative for the all the primer pairs tested.

The analysis of sweet potato viruses by location (Fig 3A), show that none of the viruses tested in this study were detected in one location, Copan Ruinas. The samples collected in San Pedro Sula contained a similar abundance of sweepovirus and SPCSV, but no potyvirus was detected. The location with the higher infection frequency was Francisco Morazán, where at least one virus

was detected in each sample. The locales Comayagua and La Paz presented similar virus distribution. Viruses were detected in 24 (100%) of the samples obtained from Francisco Morazán, 25 (82%) in La Paz, 18 (75%) in Comayagua, and 10 (67%) in San Pedro Sula.

Among the samples from Honduras, the virus combination found to be most abundant was potyvirus, SPCSV, and sweepovirus in 24(23%) samples (Fig. 2B.), closely followed by co-infection by potyvirus and SPCSV in 23(22%) samples. Double infections that involved sweepoviruses and other viruses were the least frequent at 8 of 104 samples (8%), with the proportion of sweepovirus-potyvirus and potyvirus-SPCSV being the same. In addition, potyviruses were never detected without the presence of at least one other virus. .

Pairwise distances among sequences of each group of viruses

Sweepoviruses. Sweepovirus sequences (230) were collapsed by 100% nucleotide similarity in 185 haplotypes (Villensen et al. 2007). The pairwise nt identity of the haplotypes varied from ~88~100% and isolates formed two major groups, referred to as Group A and B, at 9% divergence. Group A included all of the haplotypes from Guatemala, whereas, the isolates from Honduras were represented in both groups, with the exception of San Pedro Sula isolates that were only clustered in group A. Based on the demarcation criteria for full-length begomovirus sequences, at the threshold of 91% shared nt identity (Brown et al. 2015), groups A and B each represented distinct sweepovirus species. Among the isolates that grouped with each species, only one strain was detected that diverged by 94% or higher.

The SDT analysis, which included GenBank reference sequences and sweepovirus sequences obtained in this study (n=329 haplotypes) (Fig. 4) indicated that group A shared its highest percentage nt identity with *Merremia leaf curl virus* (MeLCV) from Puerto Rico, SPLCV from Venezuela, United States, Puerto Rico, Mexico, Kenya, Argentina; *Sweet potato golden vein associated virus* (SPGVaV) from Brazil; SPLCV and *Ipomoea yellow vein virus* (IYVV) from Spain. The isolates that were placed in group B were most closely related to the following reference sequences: *Sweet potato leaf curl Lanzarote virus* (SPLCLaV) from Spain, IYVV from Spain and Italy, SPLCV from Spain, China, Japan, South Korea, Brazil, United States, Peru, Kenya, India, and SPGVaV from the United States,

To determine if the 91% or greater threshold could be applied to obtain a phylogenetic result that was congruent between trees reconstructed from the partial CP sequences obtained in this study aligned with the analogous portion of the CP sequences from GB references, and the corresponding full-length genomic reference sequence (Fig 5 A and B). In both analyses, a large number of sequences shared 91% or greater identity, thus, there was no clear separation of sequences that would be indicative of more than one species. In contrast, at the lower threshold of 79-80%, as is revealed by the SDT analyses of CP and full-length genomic sequences, shown in Fig. 5B, all known sweepoviruses group as a single species.

Potyviruses. The sequences for Honduran isolates, were collapsed into 212 haplotypes. The SDT pairwise sequence analysis indicated that most isolates belonged to the species, SPFMV (Fig. 6.). A single sequence from the sample, HNLP14277e, was most closely related to members of the SPVG group, whereas, sequences obtained from the sample HNFM142712

shared the highest nt identity with the SPVC GenBank reference sequences. One of the potyvirus species targeted in this study, SPV2, was not detected on field samples, however PCR primers PvF and SPFCG2-R2 (Table 1) were able to amplify the SPV2 positive control. The taxonomic criteria for demarcation of species in the genus, *Potyvirus*, uses the established threshold of 76 nt identity for either the CP of full length sequence (Adams et al. 2012). Sequences belonging to the 4 different species groups targeted in this study shared nt sequence identities of greater than 76%, indicating that the partial CP sequence and three prime untranslated region (3' UTR) amplified in this study shared greater conservation than the full CP sequence by 15%. The SPFMV partial CP sequence shows 87-89% identity with SPVC, 79-82% pairwise identity with SPV2, and 81-84% identity with SPVG. The SPVC partial CP sequences shared 81-82% pairwise nt identity, compared to SPVG at 79-81% identity with SPV2, and SPVG compared with SPV2 showed 87-89% pairwise nt identity (Fig. 7.).

Sweet potato chlorotic stunt virus. For SPCSV, 299 sequences were obtained from Honduras samples and collapsed in 140 haplotypes. The SDT pairwise identities (Fig.8.) for the HSP70 sequences from Honduras and Guatemala shared the highest nt identity ranging from 97.5 to 100%. Phylogenetic analyses (ML and Bayesian) indicated that the isolates clustered together in the same clade, which included the West Africa positive control, and the GenBank reference sequences. In comparison, for the SPCSV East Africa strain control, the pairwise nt identity was only 78-79% indicating greater divergence with this isolate (Fig.9.). One GenBank reference sequence, corresponding to an isolate from Argentina (AY729021) was distantly related to the East Africa isolates at 76% nt identity, and shared 87% nt identity with West Africa strain. The PCR primer pair designed in this study successfully amplified the SPCSV East Africa positive

control, and therefore is considered useful for the detection of both viral strains. Based on the species demarcation threshold of 75% at the amino acid level (Martelli et al. 2012), all of the isolates in this study shared greater than 75% amino acid sequence similarity, indicating they are members of the same species.

Phylogenetic analyses

Sweepoviruses. The sweepovirus ML and Bayesian analyses show that group A delineated by the pairwise SDT analysis is part of a single clade, whereas group B is part of a polytomy. However, the support values were not significant for group A clade (significant at >70% bootstrap for ML, and >95% posterior probability for Bayesian (Fig. 10).

Potyviruses. The phylogenetic analysis of the potyvirus sequences revealed four main clades, each one containing a different species: SPFMV, SPVC, SPV2 and SPVG (Fig. 11). All sequences with the exception of those for two samples grouped in the SPFMV clade. In the Bayesian phylogeny, the sequences of all the isolates from Honduras clustered together in a subgroup within SPFMV clade, at 98% posterior probability. However this relationship was not well-supported for the ML and NJ trees at 48 and 46% bootstrap values, respectively. The closest relatives of the SPFMV isolates from Honduras were reference isolates the Pacific Rim countries, including Japan, Korea, Peru, Australia, and China. The sample that contained SPVC isolates that grouped in the same clade as the isolates Australia, New Zealand, Easter Island, French Polynesia, South Africa, Uganda, Zimbabwe, Argentina, and Korea, grouped as a polytomy (Fig. 11). In contrast, one SPVG isolate, HNLP14277e, was most closely related to the Argentina, China, and Korea isolates (Fig. 11). None of the isolates from Honduras were found to cluster within SPV2 clade.

Phylogenetic analyses of SPCSV amplicons (unpublished data) showed 100% bootstrap support for the clades that grouped with other viral strains from East Africa and West Africa. No well-supported groups were identified within those clades. All the isolates collected from sweet potato plants in Honduras grouped together with West Africa reference sequences.

DISCUSSION

Detection of sweet potato viruses by PCR and RT-PCR

The three primer pairs (Table 1) designed and used in the study successfully amplified and yielded the expected size amplicon size for the respective groups of viruses. The identity of all of the amplicons was confirmed by DNA sequencing of cloned amplicons, comparative sequence alignment, and SDT analysis with sequences obtained for the positive viral PCR control samples, and from the selected GenBank reference sequences.

Twenty-four percent of the samples from Guatemala were identified positive for sweepoviruses. The locale in which sweepoviruses were most abundant was Escuintla, for which 82% of the samples were infected (Fig. 3B). In contrast, sweepoviruses were not detected in most of the samples analyzed in Sacatepequez (83%) and all of the samples in Baja Verapaz. The number of samples from Guatemala in which sweepoviruses were detected, at 24%, was lower than that found in sweet potato samples from Honduras, at 41% (Fig. 2A).

Given that the samples from Guatemala appeared to be degraded to some extent, based on the spectrophotometer readings and agarose gel electrophoresis carried out to detect high-molecular weight nucleic acids, it may not be surprising that no RNA viruses were detected in those samples. Thus it is also possible that the percentage of sweepoviruses that was detected by PCR is underrepresented due either to a low concentration because of the partial degradation of total and also viral DNA. Consequently, future sampling and analysis is needed to more accurately to obtain a more reliable estimation of the occurrence and identity of both RNA and DNA viruses infecting sweet potatoes in Guatemala.

A limited survey of sweet potato viruses in Honduras and Guatemala (Kashif et al. 2012) showed that three of 12 (25%) analyzed samples were positive for sweepovirus infection. Previous studies carried out in Costa Rica reported the presence of SPLCV in two of 42 samples, or approximately 5% of them (Valverde et al. 2004). Also, 10 to 20% of the sweet potato accessions originating from different countries and maintained in the germplasm collection at CIP tested positive for sweepovirus (Clark et al. 2012), as did more than 50% of the plants that were tested from Spain (Lozano et al. 2009), 12% of asymptomatic samples from Latin America and East Africa (Cuellar et al. 2014), and 84% of the plants in a Brazilian germplasm collection (Paprotka et al. 2010). The percent detection of sweepoviruses in the samples collected in Guatemala and Honduras was found to be higher than what has been reported for recent studies carried out in Central America (Valverde et al. 2004, Kashif et al. 2012).

With respect to the RNA viruses detected and identified, the frequency of their detection in sweet potato samples from Honduras had a higher detection frequency of SPCSV (61%, Fig. 2A),

compared with potyviruses and sweepoviruses. This is consistent with previous findings reported for SPCSV detection in sweet potato samples from Honduras and Guatemala, at 58% (Kashif et al. 2012). Detection of RNA viruses in sweet potato samples from Costa Rica indicated that 42% of the plants were infected (Valverde et al. 2004), whereas, in Peru, 19% detection has been reported (Gutiérrez et al.2003), and in China the virus was detected in 15% of the samples tested (Qin 2013). Reports from studies carried out in the US indicate that SPCSV is not widely distributed (Abad et al. 2007). In contrast, high disease incidence at 90% have been reported for Uganda (Aritua et al. 2007). In addition several studies report that SPCSV interacts synergistically with other viruses, including SPFMV, certain potyviruses (Untiveiros et al. 2007), sweepoviruses (Cuellar 2014), and members of genera, *Cavemovirus* (Cuellar et al. 2011), *Ipomovirus*, and *Carlavirus* (Untiveiros 2007).

Approximately 53% of the sweet potato samples from Honduras were infected with potyviruses, based on the primers implemented here, which were designed to detect the closely related *Ipomoea*-infecting potyviruses, including SPFMV, SPVC, SPVG, and SPV2 (Fig.2A). Most studies report a higher incidence of potyviruses, compared to SPCSV (Valverde et al. 2004, Clark et al. 2010, Gutiérrez et al. 2003, Qin et al. 2013), with the exception of one study from Honduras and Guatemala that detected SPFMV in 2 of 12 (16%) of the samples (Kashif et al. 2012). Lower detection levels than were observed herein, have been reported in sweet potato samples from Peru, at 24% (Gutierrez et al. 2003). In contrast samples from Uganda (Aritua et al. 2007) and Costa Rica showed high potyvirus frequency, 90% and 71% respectively (Valverde et al. 2004). In addition, in the US, 84% of second generation planting material was infected with

potyviruses, suggesting that this viruses are rapidly transmitted by aphids under field conditions (Clark et al. 2010).

In this study, 29% of the samples from Honduras were found to be negative in all locations for all of the plant viruses targeted, except for Francisco Morazán, where all the samples presented at least one virus isolate. Samples analyzed in this study were primarily collected because they showed virus-like symptoms, however, some of the symptoms could be caused by nutrient deficiencies, or perhaps to infection, by other viruses not tested for in this study. The use of next generation sequencing technology, such as High-throughput Illumina sequencing, for the analysis of negative samples, could perhaps provide additional information regarding the presence of additional viruses that were not assayed for in this study (Kashiff et al. 2012).

Among the samples collected in Honduras, multiple virus infections were more frequent in plants than were single infections (Fig 3B). This suggests that the primers designed in this study were effective in detecting multiple viruses from the same plant sample. Interestingly, the detection of infections in sweet potato plants caused by three viruses was the most common scenario, followed by co-infection of SPFMV and SPCSV. Given these findings, the potential importance of these combinations warrants future research in this area. The presence of SPFMV and SPCSV that cause the SPVD has been reported in sweet potato plants in Costa Rica (Valverde et al. 2004). In the latter study all potyviruses were detected in mixed infections. The presence of the two co-infecting viruses, SPFMV and SPCSV, has been related to severe symptoms of SPVD (Guttierez et al. 2003, Aritua et al. 2007), however, studies relating symptoms and yield loss

have not been carried out to evaluate the impact of multiple infections in sweet potato crops in Central America.

The results of molecular detection assays indicated that sweet potato viruses infecting the crop in Honduras were not strictly distributed by location (Fig 3.A), however, in one location, Copan Ruinas, in which the fields were planted with primary seed material, no viruses were detected. This indicated that the seed pieces used to plant the fields were virus-free (for at least, those viruses included in this survey), as has been the practice in other locations in Honduras. After that most growers will save some of the progeny from the primary seed mother plants, and replant it the following year, often after increasing it either in the subsequent planting (near or in the same field) or in a location at some distance from the commercial production fields. Also, although no potyviruses were detected in the San Pedro Sula samples, both sweepoviruses and SPCSV were detected in the samples from this location. The location having the highest virus infection frequency was Francisco Morazán, at which at least one of the viruses assayed for was detected in each sample tested. Whiteflies were observed in the fields when samples were collected in these locations. It is notable that the same frequencies of infection by sweet potato-infecting viruses were found for the Comayagua and La Paz locations. This could have been due to the minimal geographical distance between collection sites (Fig.1), which might suggest that both locations experience similar planting times and length of growing season, and environmental conditions which produce similar vector abundance and exposure to sweet potato viruses either from already infected crops in the area, and/or from wild host species common to both commercial locations.

The viruses SPCSV, SPFMV and sweepoviruses were detected in 67% to 100% of samples in locations in Honduras, except for Copan Ruinas, where no viruses were detected in the samples assessed during in this study. Because yield losses due to the viruses detected in this study have been reported in previous studies for other locations (Clark et al. 2010, Clark et al. 2012, Gutierrez et al. 2003, Aritua et al. 2007), it is important to address disease management strategies, such as vector control. Vector control should be focused on the aphids, *Myzus persicae* (Sulzer) and *Aphis gossypii* (Glover), which spread potyviruses (Byamukama et al. 2004, Adams et al. 2012; Atreya et al. 1995), and *B. tabaci* (Genn.) which transmits sweepoviruses and SPCSV (Simmons et al. 2009, Cohen et al. 1992). Other management strategies can involve starting with clean seed periodically (no more than two generations from same seed) and isolate sources of clean seed area to prevent re-infection before planting should be considered.

Pairwise distances and phylogenetic analyses

In previous study, which considered 89% identity for species demarcation criteria on complete sweepovirus genomes, 17 species were identified (Albuquerque et al. 2012). In contrast, using new guidelines for begomovirus species demarcation, which considers 91% demarcation threshold, 8 species were identified (Brown et al. 2015). Using a different dataset for pairwise analysis and 91% similarity criteria, indicated the existence of 20 different, but species are not supported by Bayesian phylogenetic analysis (Brown et.al. publication in progress). The presence of outliers and guidelines followed to interpret such sequences as well as the set of samples chosen for the analysis clearly show that different outcomes can be obtained depending

on which species demarcation criteria are used for the sweepoviruses. The absence of well-supported phylogenetic clades as additional sequences are added to analyses, show that different criteria need to be established for this group of viruses. Based on full length sequence analysis (Brown et al., publication in progress) implementing a phylogenetically supported 77% threshold (as predicted by SDT analysis) will minimize the number of outliers, and will groups all sweepoviruses in a single supported clade. The same conclusion can be drawn from an analysis of the partial CP sequences obtained herein, and all from the available GenBank reference sequences (Fig. 5 and Fig. 10**)**. Given that sweepovirus CP is a highly conserved sequence, the threshold for sweepovirus demarcation based on the partial CP sequence would be slightly higher than 77%, at 80-81%, in relation to that for the full-length sequence. This result indicates that the location of the CP gene targeted by the primers designed in this study provide a useful preliminary prediction of sweepovirus species status (phylogenetically and by SDT), and also appear to amplify most if not all sweepoviruses known to date.

Potyvirus pairwise comparissons and phylogenetic analysis show 4 main groups, each one grouping with a different species: SPFMV, SPVC, SPV2 and SPVG (Figs. 6 and 11). All but sequences from two samples were present in SPFMV clade. The closest virus relatives for Honduras isolates are not related geographically, which might be explained by the global exchange of germplasm, which can easily occur in the case of SPFMV that can be present in asymptomatic plants (Li et al. 2012, Karyeija et al. 1998). Both SPFMV and SPVC were previously reported in Guatemala and Honduras (Kashif et al. 2012), and SPFMV in Costa Rica (Valverde et al. 2004), but to our knowledge, this is the first report of SPVG in Honduras. Given that universal primers for potyviruses were used, it is possible that the high presence of SPFMV

masked the presence of other potyviruses (Valverde et al. 2007), such as SPV2, which remains undetected in Guatemala and Honduras. The primers used in this study were able to amplify members of all the intended targets among the *Potyvirus* genus. In order to determine species status, based on the fragment amplified by this study, a 91% pairwise identity threshold is recommended.

Both pairwise distances and phylogenetic analysis show that SPCSV detected in this study belong to West Africa strain. Presence of West Africa strain in Guatemala and Honduras has been previously reported, and further sequence analysis showed that identities among the two sequences analyzed were higher than 98% in nucleotide and amino acid sequence of RNA endoribonuclease III and p7 ORFs (Kashif et al. 2012, Tugume et al. 2013). This is consistent with the nt identities predicted for isolates in this study (97.5-100%) based on analysis of the HSP70. Presence of West Africa strain in samples from this study is expected given its known worldwide distributed, except in East Africa, where East African strain is prevalent. East Africa strain was originally thought to be restricted to East African countries, but it has also been reported in Peru, Tanzania and China (Qin et al. 2013, Tugume et al. 2013). One outlier, represented by an isolate from Argentina (AY729021), was observed to be distantly related with East Africa isolates (about 76% pairwise identity), and did not cluster with West African isolates either, at 87% pairwise identity. This isolate might indicate the presence of a third strain of SPCSV. This strain was identified in sweet potato plants analyzed in the CIP study (unpublished) and reported by Clark et al. 2012, but no other reports of it have been published to date, suggesting it may be somewhat rare or not predominant at this time. Finally, the primers designed and implemented in this study successfully detected these two SPCSV strains, and

identification of each of them was made possible by sequencing the amplicon. A 94% threshold is recommended for the identification of SPCSV strains using the primers from this study.

In summary, the PCR and RT-PCR primers designed herein for amplification of plant viral species that are commonly recognized to infect sweet potato, effectively detected the target viruses, both in single and mixed infections from symptomatic sweet potato plants. Further, the amplified gene regions were shown to be phylogenetically informative at the species or strain levels, depending on the virus or virus group. Given the knowledge that these viruses occur widely in mixtures and that they are prevalent in sweet potato throughout the Americas, the design of multiplex molecular assays would provide the most cost-effective diagnostics for their detection in sweet potato plants.

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Table legends

Table 1. PCR primers used in this study. CP: Coat protein, 3'UTR: 3 prime untranslated region, HSP70: Heat shock protein 70 homologue.

Figure legends

Fig. 1. Sample collection locations in Guatemala and Honduras. BV: Baja Verapaz, CM: Comayagua, CP: Copán Ruinas, ES: Escuintla, FM: Francisco Morazán, SA: Sacatepéquez, SAP: San Pedro Sula.

Fig. 2. Occurrence of the viruses detected in Honduras samples. **A,** Overall percentage occurrence of the viruses screened in Honduras samples. **B,** Percentage occurrence of the viruses detected in Honduras samples, detailing the proportion of single infections and different possible combinations among the types of viruses. Numbers in parenthesis show the number of samples that corresponds to the shown percentage. 104 samples were analyzed.

Fig. 3. Percentage occurrence of different type of viruses in the different sampling locations. **A,** occurrence of different types of viruses in Honduras locations. **B,** percentage occurrence of sweepoviruses in different locations in Guatemala. Numbers in parenthesis show the number of samples that were analyzed per location.

Fig. 4. Pairwise identity among sweepoviruses nucleotide sequences of samples from Guatemala and Honduras. Analysis was performed in SDT software, and was based on Muscle alignment, which includes 185 haplotypes. Following the most recent species demarcation

criteria (Brown et al. 2015), different species show pairwise identity lower than 91% (blue), between 91 and 94% pairwise identity isolates belong to the same species and a different strain (green), and above 94%, sequences belong to the same species and strain (red). Brackets A and B show the 2 main groups that were observed.

Fig. 5. Pairwise comparison plot for partial CP of sweepoviruses. Analyses were performed in SDT software and was based on Muscle Alignment and BGYMV_M10070 was used as outgroup. A, includes sequences obtained in this study and GenBank references, and B, GenBank references.

Fig. 6. Pairwise identity among potyvirus nt sequences of samples from Honduras. Analysis was performed in SDT software, and was based on Muscle alignment that included 212 haplotypes. Red indicates the presence of sequences from the same species, whereas blue indicates different species. Section of the pairwise identity matrix that includes the sequences that did not cluster with SPFMV GenBank reference sequences was magnified.

Fig. 7. Pairwise comparison plot for potyvirus partial region of CP and 3'UTR, including sequences obtained in this study and GenBank references. PVY_AJ223595 was used as an outgroup.

Fig. 8. Pairwise identity among SPCSV nt sequences of samples from Honduras. Analysis was performed in SDT software, and was based on Muscle alignment, which includes 140 haplotypes. Sequences in red show pairwise identities from 98 to 100%, and sequences with a

similarity of 78% are shown in blue. WA and EA refer to West and East Africa strain, respectively.

Fig. 9. Partial HSP70 pairwise identity analysis for SPCSV obtained in this study and GenBank references. CYSDV_AY242078 was used as an outgroup.

Fig. 10. Molecular phylogenetic analysis of sweepovirus amplified CP region. Phylogeny was inferred by Bayesian analysis, with 10, 000, 000 replicates and 4 independent runs. The tree was collapsed to include clades supported by at least 50% posterior probability values. 329 haplotypes were included in the analysis, taxa in blue represent sequences obtained in this study, taxa in red sequences from positive controls infected with known sweepovirus species, and taxa in black represent GenBank references. Numbers on the nodes indicate posterior probability values.

Fig. 11. Potyvirus phylogeny based on the region amplified by the primers used in the present study. Phylogeny was inferred by Bayesian analysis, with 10, 000, 000 replicates and 4 independent runs. The tree was collapsed to include clades supported by at least 50% posterior probability values. 375 haplotypes were included in the analysis, taxa in blue represent sequences obtained in this study, taxa in red sequences from positive controls infected with known potyvirus species, and taxa in black represent GenBank references. Numbers on the nodes indicate posterior probability values.

Tables

Table 1.

Virus	Primer	Sequence	PCR target	Annealing temperature (°C)
Sweepovirus	SpvF SpvR	AATGCTGTCCCAATTGCTG CTGCAACGCAGGGTCTGATA	CP, 394-1026	55
Potyvirus	PvF SPFCG2-R2	GTTTGGTGYATHGARAATGG TCGGACTGAARGAYACGAATTTAA (Li et al.2012)	CP and 3' UTR 10141-10679	58
SPCSV	SPCSVF	GACGGTGGTACTATGAAAGTCCT	HSP70, 915-1331	58

Figures

Fig. 1.

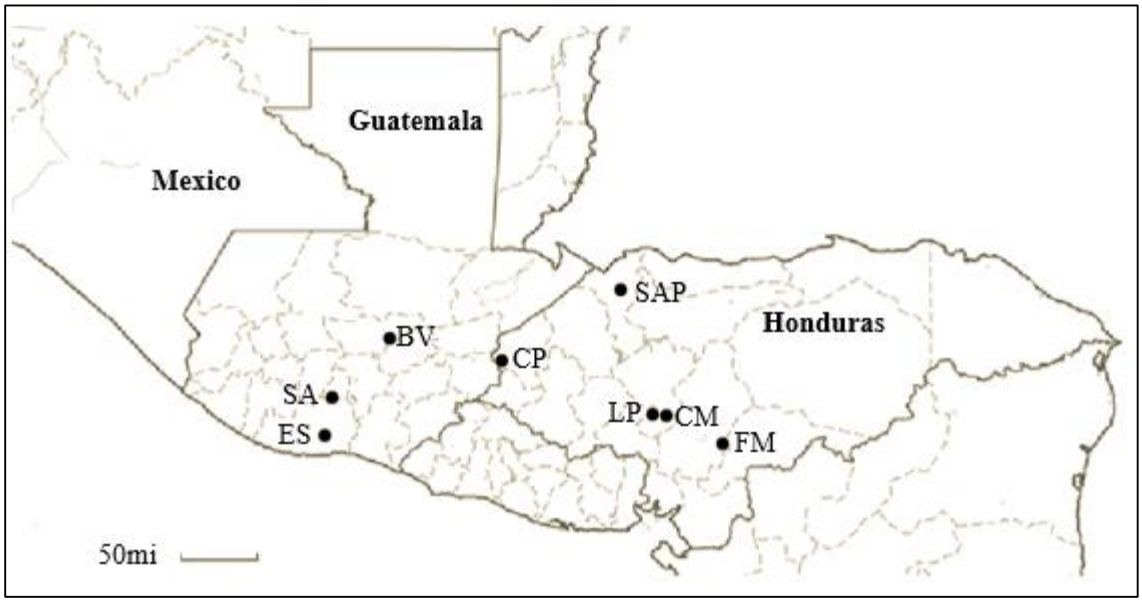

Fig. 2.

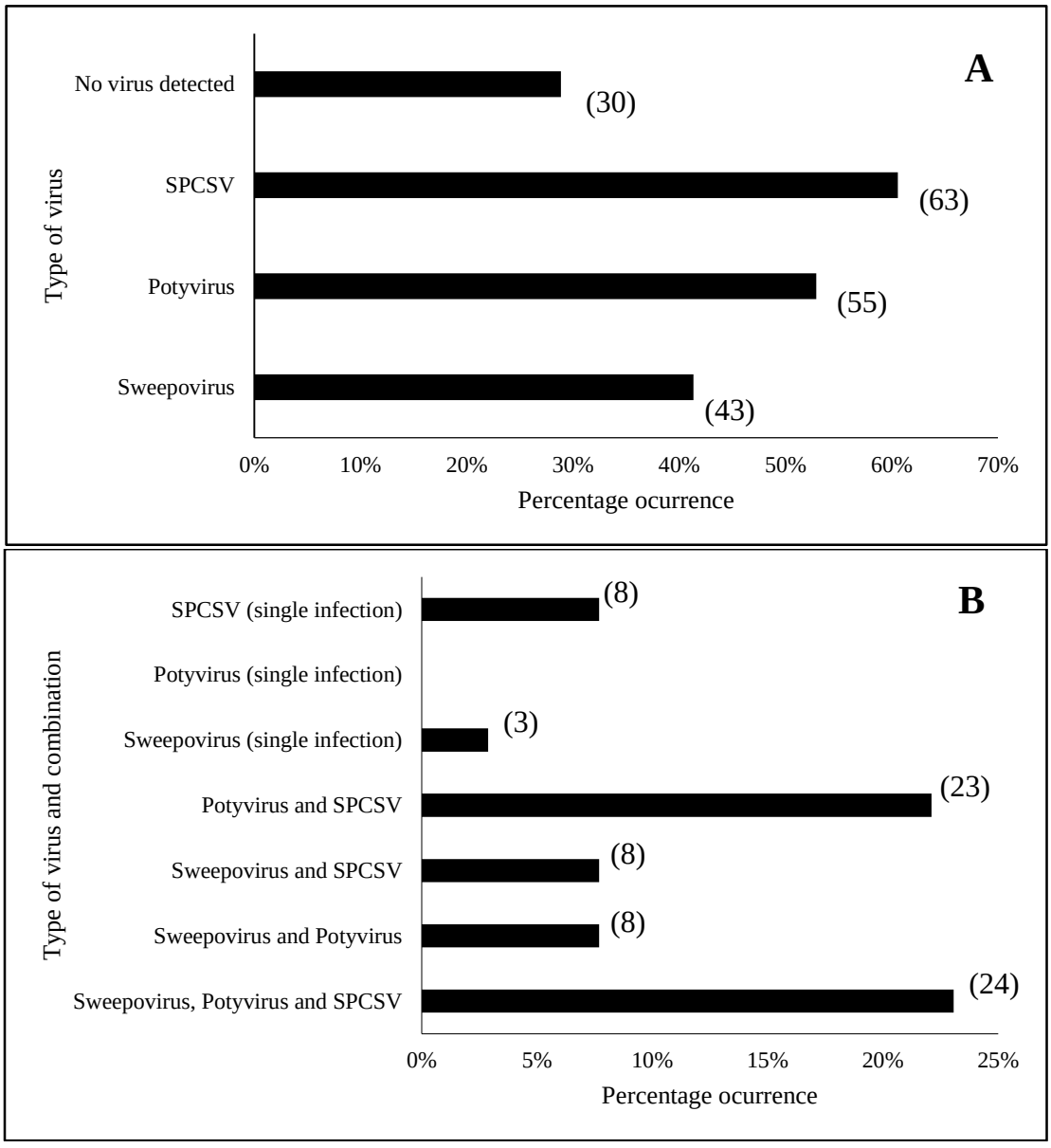
A
No virus detected
SPCSV
Potyvirus
Sweepovirus
(30)
(63)
(55)
(43)
Type of virus
0%
10%
20%
30%
40%
50%
60%
70%
Percentage ocurrence
B
SPCSV (single infection)
Potyvirus (single infection)
Sweepovirus (single infection)
Potyvirus and SPCSV
Sweepovirus and SPCSV
Sweepovirus and Potyvirus
Sweepovirus, Potyvirus and SPCSV
(8)
(3)
(23)
(8)
(8)
(24)
Type of virus and combination
0%
5%
10%
15%
20%
25%
Percentage ocurrence

Fig. 3.

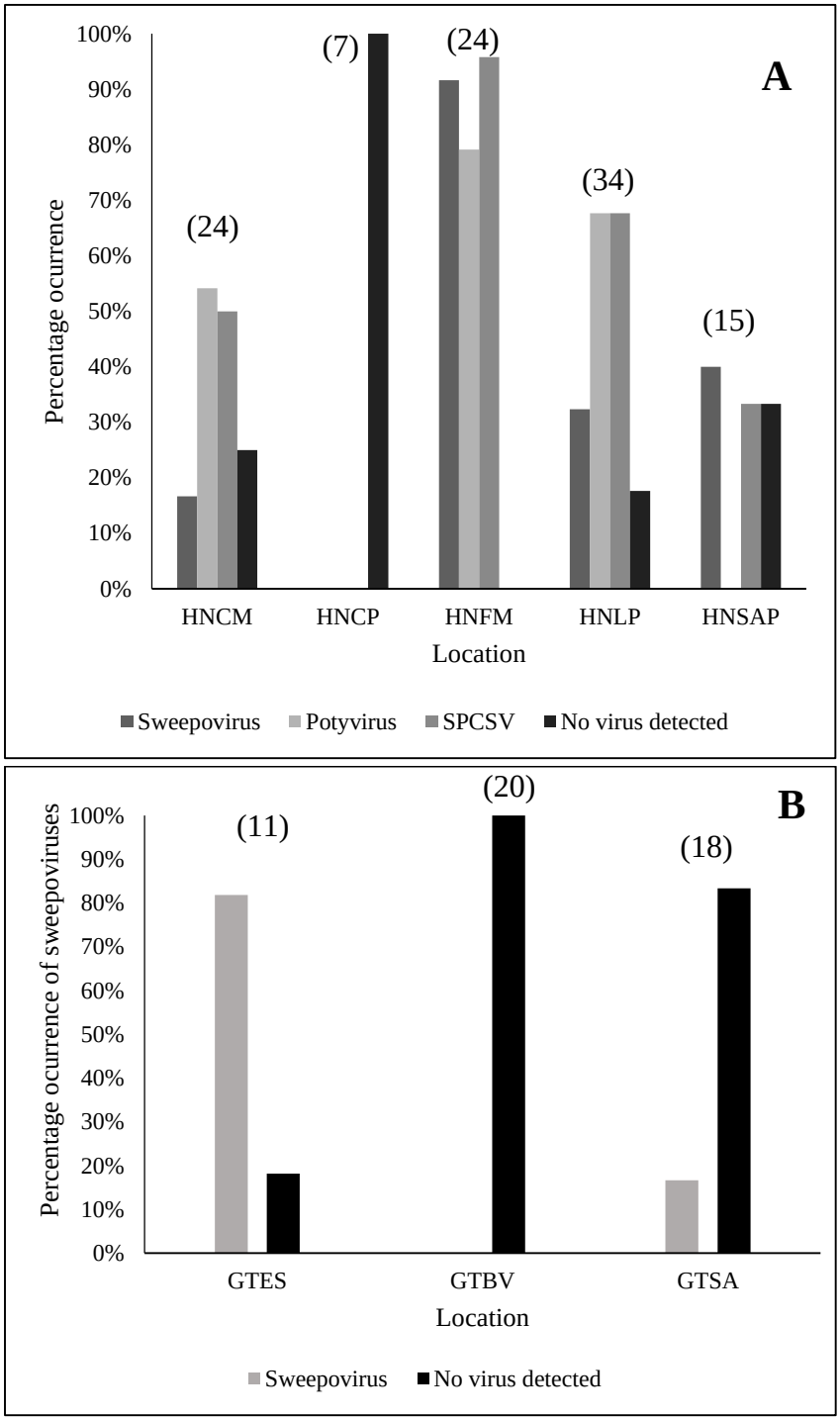
A
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Percentage ocurrence
(24)
(7)
(24)
(34)
(15)
HNCM
HNCP
HNFM
HNLP
HNSAP
Location
Sweepovirus
Potyvirus
SPCSV
No virus detected
B
Percentage ocurrence of sweepoviruses
(11)
(20)
(18)
GTES
GTBV
GTSA
Location
Sweepovirus
No virus detected

Fig.4.

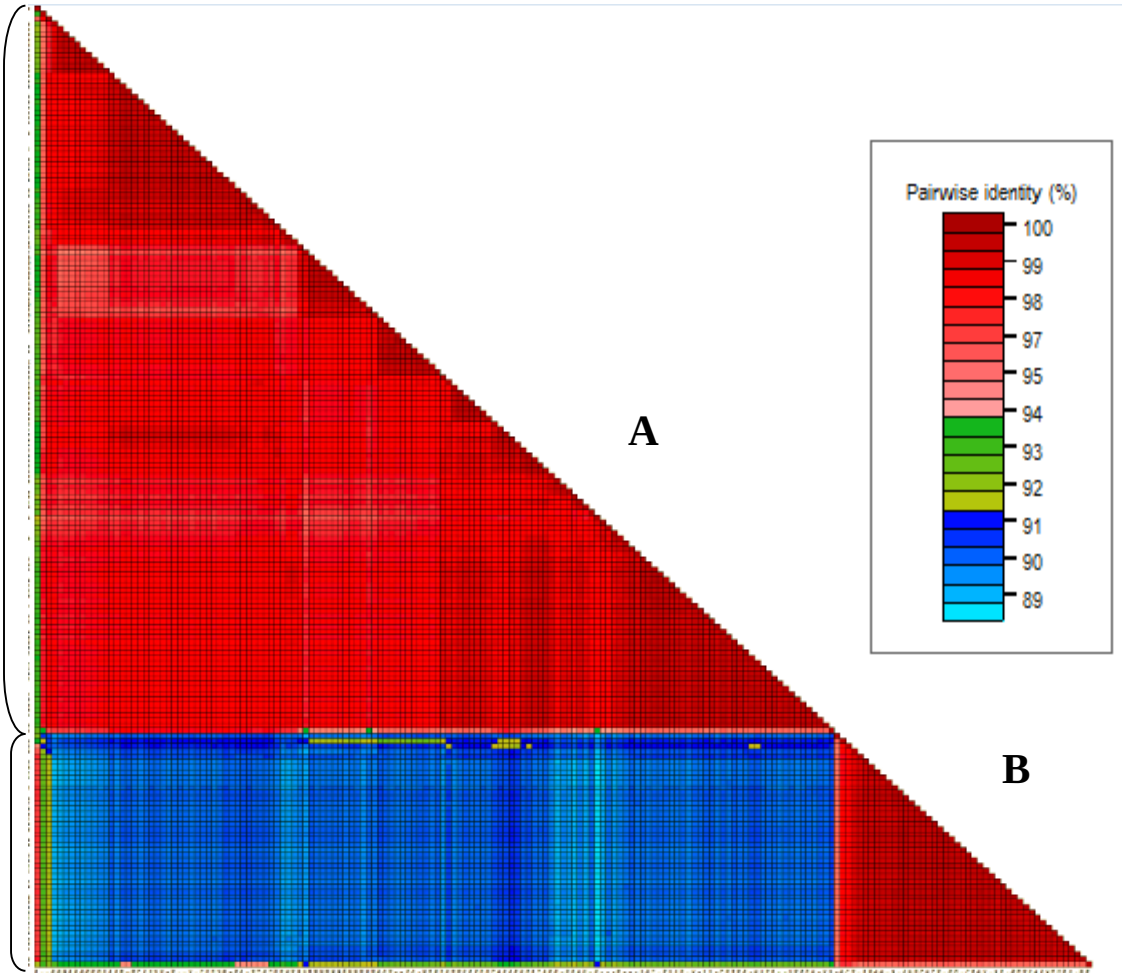

Fig. 5.

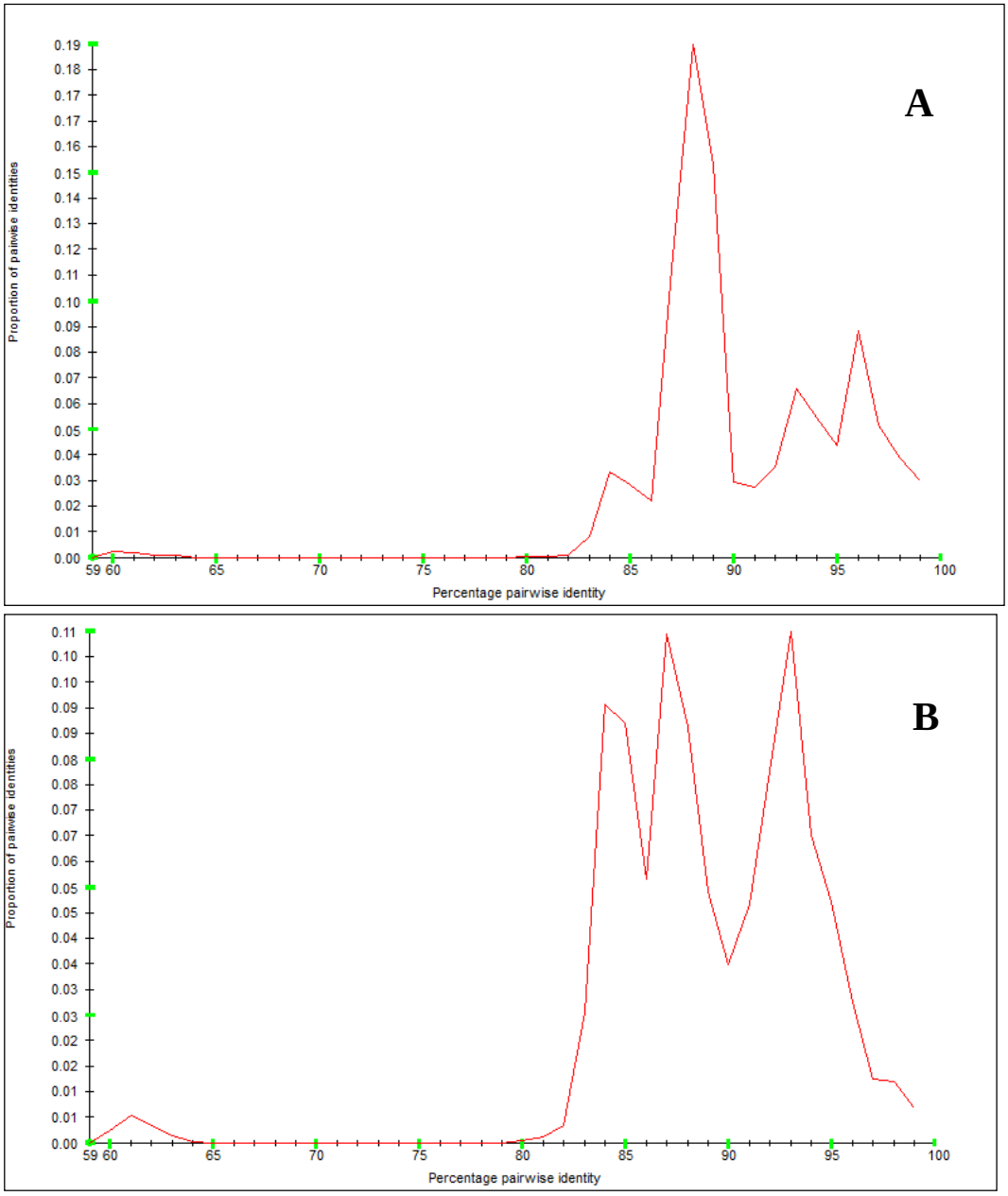
A
Proportion of pairwise identities
0.19
0.18
0.17
0.17
0.16
0.15
0.14
0.13
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
59 60
65
70
75
80
85
90
95
100
Percentage pairwise identity
B
Proportion of pairwise identities
0.11
0.10
0.10
0.09
0.09
0.08
0.08
0.07
0.07
0.06
0.05
0.05
0.04
0.04
0.03
0.03
0.02
0.02
0.01
0.01
0.00
59 60
65
70
75
80
85
90
95
100
Percentage pairwise identity

Fig. 6.

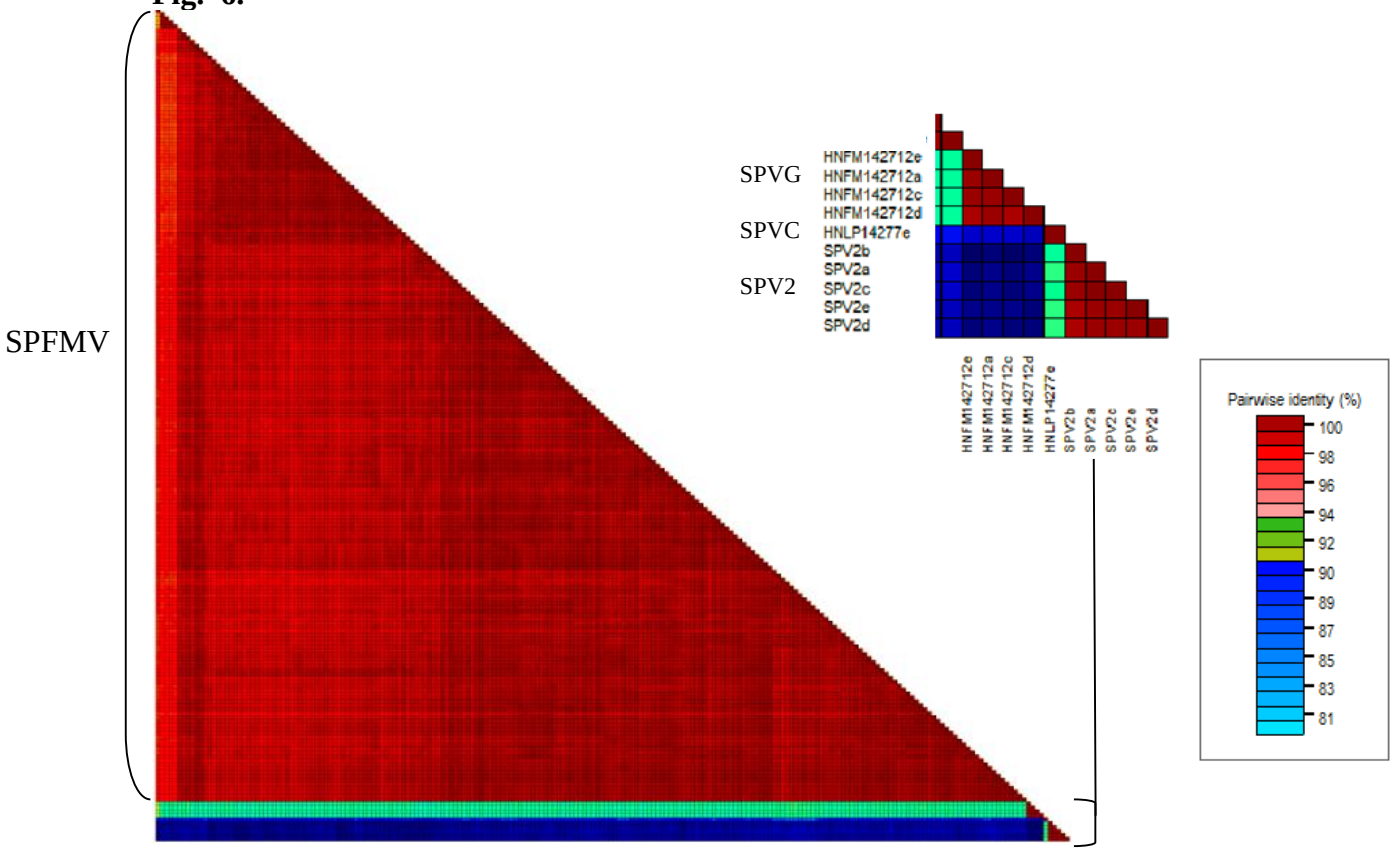
SPFMV
SPVG
SPVC
SPV2
HNFM142712e
HNFM142712a
HNFM142712c
HNFM142712d
HNLP14277e
SPV2b
SPV2a
SPV2c
SPV2e
SPV2d
Pairwise identity (%)
100
98
96
94
92
90
89
87
85
83
81

Fig. 7.

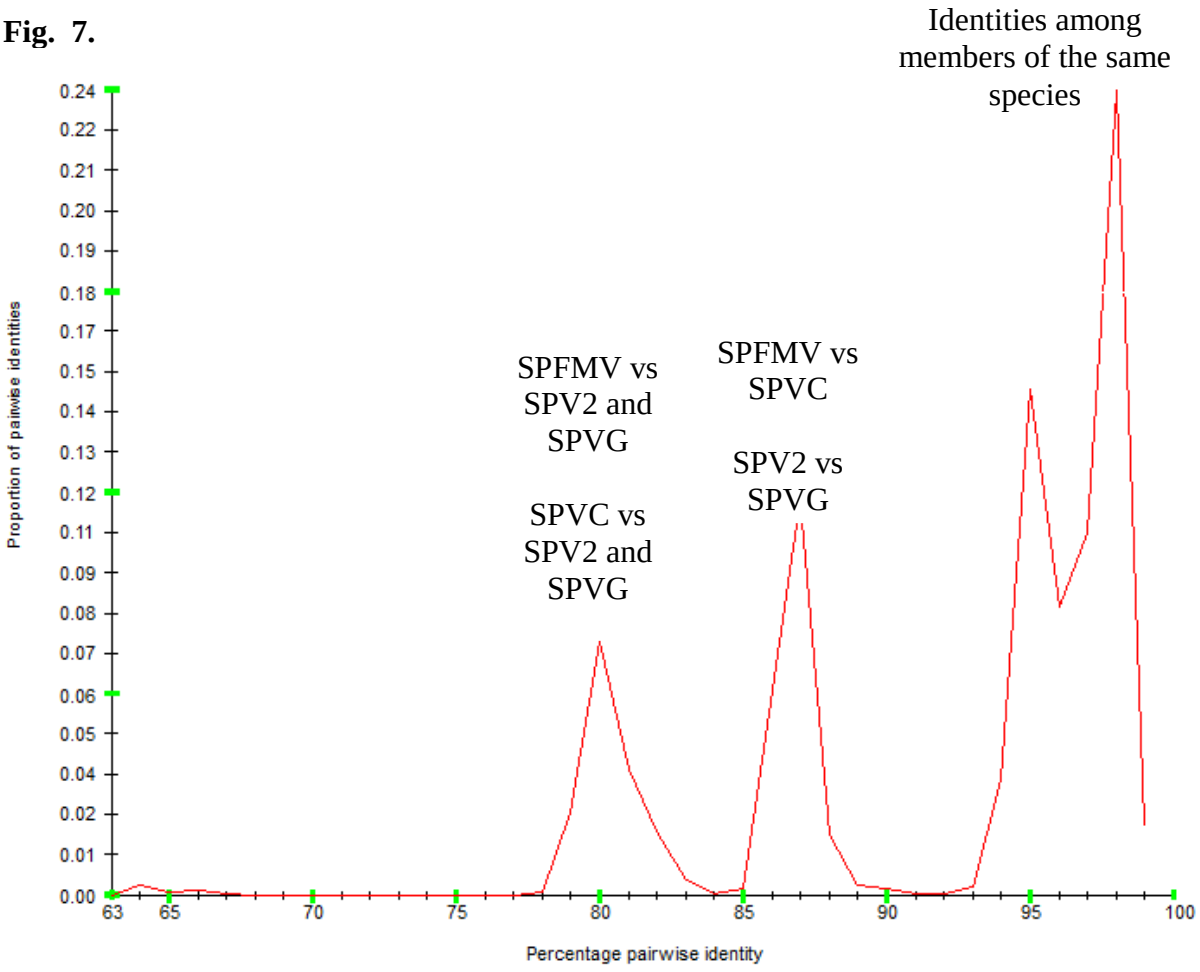
Identities among members of the same species
SPFMV vs SPV2 and SPVG
SPFMV vs SPVC
SPV2 vs SPVG
SPVC vs SPV2 and SPVG
Proportion of pairwise identities
0.24
0.22
0.21
0.20
0.19
0.18
0.17
0.15
0.14
0.13
0.12
0.11
0.09
0.08
0.07
0.06
0.05
0.04
0.02
0.01
0.00
63
65
70
75
80
85
90
95
100
Percentage pairwise identity

Fig. 8.

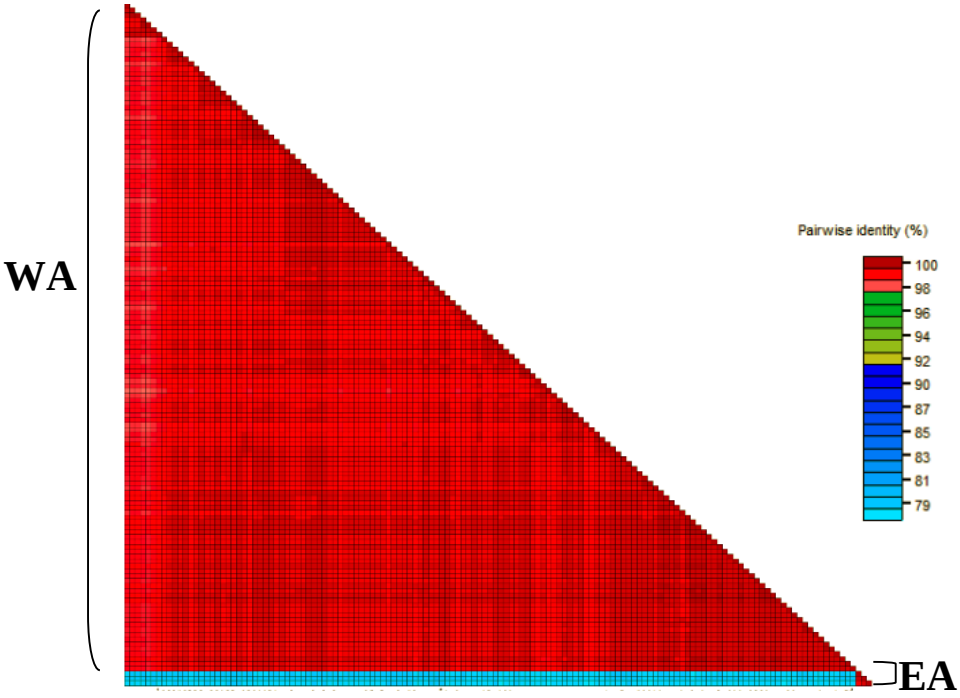

Fig. 9.

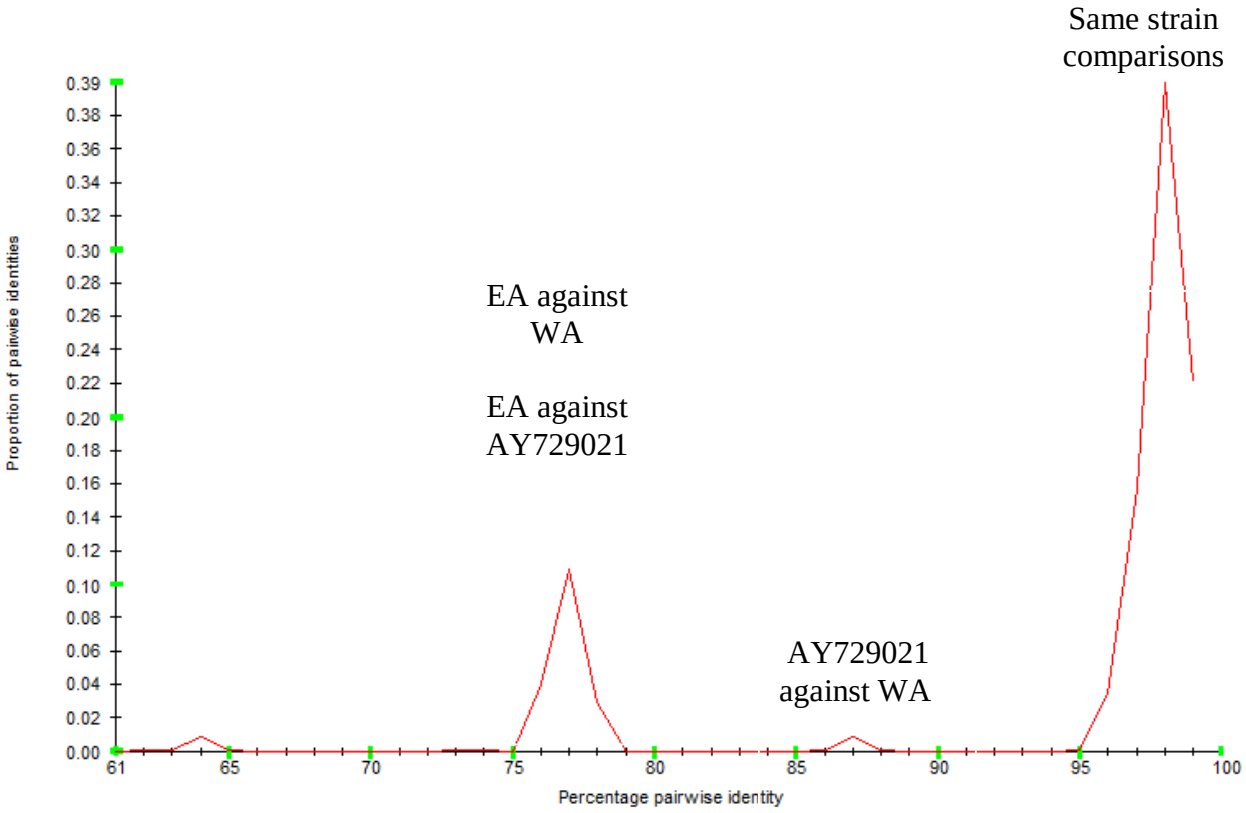
Same strain comparisons
EA against WA
EA against AY729021
AY729021 against WA
Proportion of pairwise identities
0.39
0.38
0.36
0.34
0.32
0.30
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
61
65
70
75
80
85
90
95
100
Percentage pairwise identity

Fig.10.

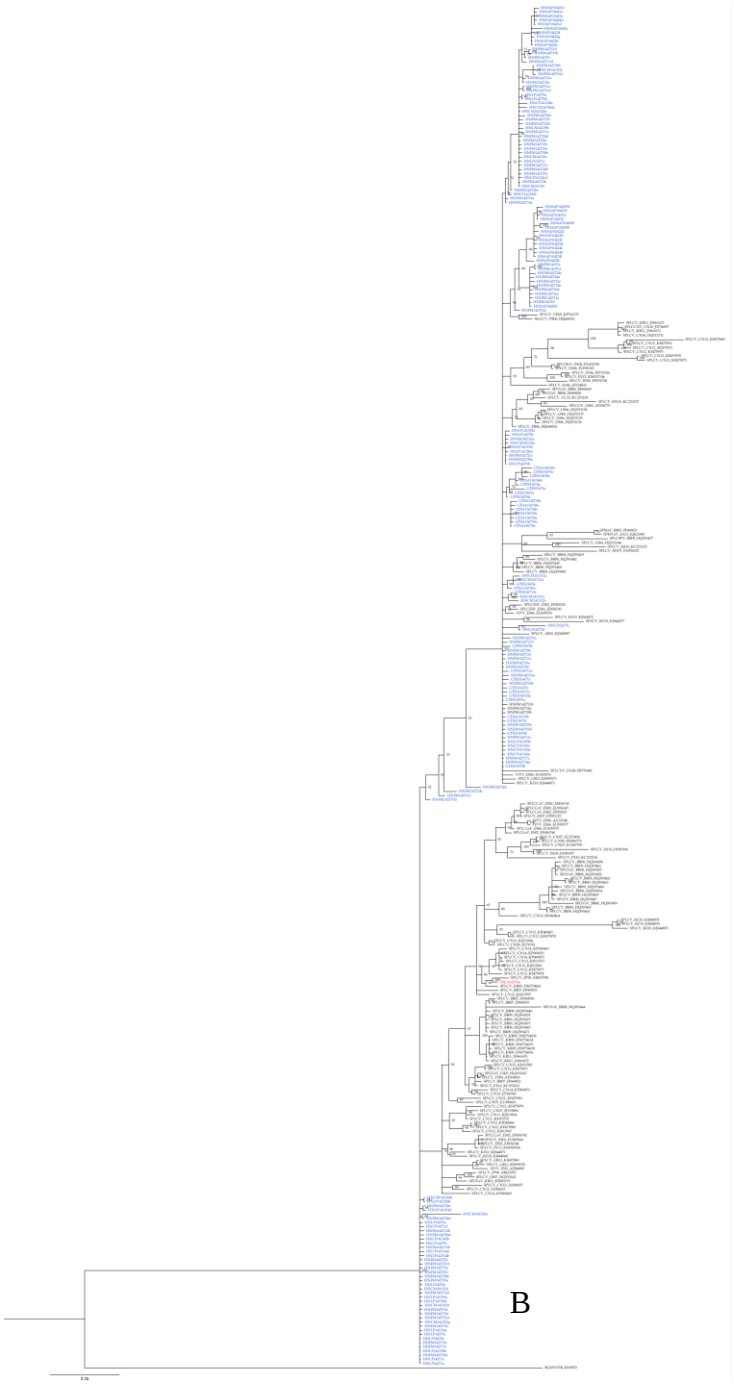

Fig. 11.