STUDIES ON EPIDEMIOLOGY, MOLECULAR DETECTION AND GENETIC DIVERSITY OF SELECTED VIRUSES INFECTING CASSAVA AND WINE GRAPES

By

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To the Faculty of Washington State University:

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STUDIES ON EPIDEMIOLOGY, MOLECULAR DETECTION AND GENETIC DIVERSITY OF SELECTED VIRUSES INFECTING CASSAVA AND WINE GRAPES

Abstract

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Chair: Naidu A. Rayapati

Cassava (*Manihot esculenta* Crantz) is an important staple food crop for people in Africa and grapevines (*Vitis vinifera* L.) are cultivated worldwide for multiple purposes, including consumption of its berry and processed products such as raisins and wine. The focus of this research was on begomoviruses associated with cassava mosaic disease (CMD) in Nigeria and grapevine rupestris stem pitting disorder of wine grape cultivars in the Pacific Northwest (PNW) of the United States of America.

Molecular characterization of DNA A genomic component of begomoviruses associated with CMD confirmed the occurrence of *African cassava mosaic virus* (ACMV) and *East African cassava mosaic Cameroon virus* (EACMCV) in Nigeria. Nucleotide sequence analysis of DNA A of ACMV and EACMCV isolates collected from different plant species confirmed two weed hosts (*Senna occidentalis* and *Combretum confertum*) and three crop plants (*Ricinus communis*, *Leucana leucocephala*, and *Glycine max*) as potential alternative hosts for the two viruses. This information expanded our current knowledge of the ecology of cassava-infecting begomoviruses in Nigeria. A multiplex PCR assay in conjunction with simplified sample preparation method was developed for concurrent detection of ACMV and EACMCV in cassava infected with CMD.

This technique facilitated high throughput diagnosis of CMD in epidemiological studies, crop improvement and phytosanitary programs in Nigeria.

Molecular diversity of field isolates of *Grapevine rupestris stem pitting-associated virus* (GRSPaV; genus *Foveavirus*) in wine grape cultivars grown in the PNW region was evaluated relative to virus isolates from other grape-growing regions. Assessment of the coat protein and a portion of the helicase region of the replicase using phylogenomic and population genetics approaches showed that genetic diversity of GRSPaV in the PNW vineyards is considerably greater than reported in other regions. The results are useful for developing improved diagnosis of different genetic variants of the virus in 'clean' plant programs. The study also showed putative recombination events in GRSPaV that contributed to an increased understanding of molecular population genetics of viruses infecting woody perennials.

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DEDICATION

This dissertation is dedicated to the loving memory of my father, the late Michael Dele Alabi.

PREFACE

The research presented in this dissertation has been published in peer-reviewed scientific journals. The results on CMD described in Chapters Three and Four were published in Archives of Virology (2008, vol. 153, pp. 1743-1747) and Journal of Virological Methods (2008, vol. 154, pp.111-120), respectively. Molecular studies on GRSPaV described in Chapter Six have been accepted for publication in Journal of General Virology (2009, doi: 10.1099/vir.0.014423-0). Citations within each chapter are included in the "References" section of the corresponding chapter and formatted according to the specifications of the respective journals.

CHAPTER 1

GENERAL INTRODUCTION

Cassava (*Manihot esculenta* Crantz) and wine grapes (*Vitis vinifera* L.) are two economically important vegetatively propagated crops grown in different parts of the world. While cassava is a staple food crop in Sub-Saharan Africa (SSA), wine grapes are grown in the Pacific Northwest (PNW) region of the USA for premium wine production. Indeed, grapes are the most widely cultivated fruit crop worldwide, encompassing about 8 million hectares of arable land (Vivier and Pretorius, 2002). The two crops represent contrasting scenarios - cassava is largely used as a food crop in developing countries whereas grapes are used for multiple purposes with wine and distilled liquor having the highest economic value. Thus, cassava is a crop associated with livelihood in Africa and grape has been aligned with prosperity and luxury in many countries. Nevertheless, both crops are vulnerable to diseases caused by viruses with distinct genome organization and replication strategies.

Cassava in SSA is affected by CMD caused by single-stranded DNA viruses termed cassava mosaic begomoviruses (CMBs). In contrast, grapevine in the PNW is impacted by single-stranded RNA viruses that include *Grapevine rupestris stem pitting-associated virus* (GRSPaV). CMBs are transmitted by whiteflies (*Bemisia tabaci*) but no biological vector has been reported for GRSPaV. However, CMBs and GRSPaV are disseminated through vegetative propagation of cassava and grape, respectively. Thus, prevention and sanitation have become important strategies for the management of viruses in both crops.

While management of CMBs requires a two-pronged strategy consisting of prevention of virus dissemination via cuttings and control tactics to avoid spread of CMBs by the whitefly

vector, prevention of virus dissemination via cuttings is the principal strategy for management of GRSPaV. Therefore, an understanding of disease epidemiology and development of high throughput diagnostic methods is critical for vector-borne diseases like CMD in order to develop effective integrated disease management strategies in Africa. Since spread of GRSPaV occurs exclusively through the distribution of vegetative cuttings, sensitive and specific diagnosis of the virus is vital for distribution of grapevines in 'Clean' plant programs in the PNW. Due to their perennial nature, mixed virus infections are common in cassava and grapevine, and vegetative propagation of these hosts provide opportunities for recombination to occur between different strains of a virus thereby increasing the diversity within virus populations. Detailed information on genetic diversity of viruses in their host plants is a prerequisite for developing reliable and robust diagnostic assays for control measures to prevent their spread.

The research work presented in this dissertation addressed specific aspects related to viruses associated with CMD in cassava and GRSPaV in wine grapes. As a graduate student from Africa, it is critical for me to gain broader experiences in various aspects of plant virology in order to be able to address a wide range of virus disease problems beyond CMD impacting agriculture in Nigeria. Hence, I have chosen research projects on viruses infecting cassava and grapevine to gain competency in dealing with both DNA and RNA viruses. Experience with disparate viruses is essential for a successful professional career as a plant virologist in Nigeria, where a scientist is expected to address an array of virus disease problems in many different crops.

Cassava:

A review of literature on cassava mosaic disease is presented in Chapter 2 as a prelude to the research on CMD described in Chapter 3 and 4. The main objectives of this research presented in these chapters were to:

i. Confirm identity of cassava mosaic begomoviruses associated with CMD in Nigeria by carryout molecular analysis of their DNA A components.
ii. Confirm identity of cassava mosaic begomoviruses in alternative host species and determine their genetic relationship with corresponding sequences in cassava infected with CMD.
iii. Develop high throughput multiplex PCR assay for simultaneous detection of cassava mosaic begomoviruses associated with CMD in Nigeria.

Grapes:

Information on viruses and their genetic variants will aid the development of strategies for mitigating their negative impact on sustainability of the wine grape industry in the PNW. A review of literature on Rugose Wood complex was presented in Chapter 5 as a prelude to the research on GRSPaV described in Chapter 6. The objective of this research was to assess genetic diversity of field isolates of GRSPaV in wine grape cultivars grown in the PNW region relative to virus isolates from other grape-growing regions.

CONTRIBUTIONS OF THE CANDIDATE AND HIS COLLABORATORS TO THE PRESENT STUDIES

CHAPTER 3: Alternative hosts of *African cassava mosaic virus* and *East African cassava mosaic Cameroon virus* in Nigeria.

Olufemi J. Alabi contributed to the development of the project and he designed and performed all experiments and wrote the chapter for publication. Dr. F. O. Ogbe, Dr. R. Bandyopadhyay, Dr. P. Lava Kumar, Dr. A. G. O. Dixon and Dr. J. d'A. Hughes contributed in planning some of the experiments and facilitated sample collection from Nigeria. Dr. Naidu A. Rayapati developed the project and contributed to research experiments, interpretation of results and writing of the manuscript leading to its publication.

CHAPTER 4: Multiplex PCR for the detection of *African cassava mosaic virus* and *East African cassava mosaic Cameroon virus* in Nigeria.

Olufemi J. Alabi contributed to the development of the project and he designed and performed all experiments and wrote the chapter for publication. Dr. P. Lava Kumar facilitated sample collection from Nigeria. Dr. Naidu A. Rayapati developed the project and contributed to research experiments, interpretation of results and writing of the manuscript leading to its publication.

CHAPTER 6: Sequence diversity, population genetics and potential recombination events in *Grapevine rupestris stem pitting-associated virus* in Pacific Northwest vineyards.

Olufemi J. Alabi contributed to the development of the project and he designed and performed all experiments and wrote the chapter for publication. Dr. R. R. Martin contributed samples inform Oregon and in preparation of the manuscript. Dr. Naidu A. Rayapati developed the project and contributed to research experiments, interpretation of results and writing of the manuscript leading to its publication.

CHAPTER 2

CASSAVA AND THE CASSAVA MOSAIC DISEASE

Cassava and its importance in Nigeria

Cassava (*Manihot esculenta* Crantz, family *Euphorbaiceae*, synonyms: yucca, manioc and mandioca), a native to South America (Abraham, 1956; Karakacha, 2001), is believed to have been introduced into Sub-Saharan Africa (SSA) by Portuguese traders during the 16th century (Lebot, 2009, Fauquet and Fargette, 1990; Carter *et al.*, 1995). Currently, it is an important staple food crop in many countries of the subcontinent (FAOSTAT, 2009) and provides an affordable source of carbohydrate for over 200 million people around the world (Nweke and Lyman, 1997). Among the cassava-growing countries, Nigeria accounts for nearly 20 % of the global cassava production of 214.52 million metric tons (FAOSTAT, 2009). Farmers grow cassava successfully under a wide range of agro-ecological zones where cereals and other crops cannot thrive due to its adaptability to marginal environments (Thresh, 2006). In addition, cassava produces higher yields per unit of land than other crops for example yams, wheat, rice and maize (Nweke *et al.*, 2002). The significance of cassava that sustained life in Nigeria, including during the civil war period of the late 1960s, was described by a famous Nigerian novelist and poet, Flora Nwapa. In a poem in the book titled "Cassava Song and Rice Song" (Nwapa, 1986), she eloquently praised "Mother Cassava" as a staple food for the vast majority as opposed to rice that is viewed as an expensive, imported food not affordable by the poor.

Cassava is primarily cultivated for its tuberous roots, which are a major source of starch for food. The tubers are eaten fresh and/or in various forms of processed food. Cassava is grown by resource-poor farmers as an intercrop with vegetables, plantation crops (coconut, oil palm and

coffee), yams, melon, sweet potato, maize, cowpea, groundnut and other legumes for food security and assured household income,. Cassava leaves are also consumed as a vegetable, especially in East Africa, to provide an important source of proteins, minerals and vitamins (Smith, 1988). In recent years, cassava has been increasingly used as raw material in various industrial products such as starch and flour. The recent prospect of ethanol for biofuels has driven the cultivation of cassava from subsistence to a more commercially-oriented farming enterprise (Asiedu, 1989, Vera *et al.*, 1997). Consequently, cassava hectarage has been increasing in Nigeria.

Cassava mosaic disease

Cassava is vulnerable to a broad range of virus diseases (Table 1). Among them, cassava mosaic disease (CMD) is the most severe and widespread, limiting the production of cassava in Nigeria and indeed in the entire SSA region. CMD produces a variety of foliar symptoms that include mosaic, mottling and deformation or distortion leading to misshapen and twisted leaflets with reduced size (Fig. 1). CMD-affected cassava plants produce few or no tubers (Fig.1).

Etiology of CMD

CMD was first reported from the Usambaras Mountains range in North-East Tanzania (Warbug, 1894). Warbug called the disease "Kräuselkrankheit" which translates to "rippling/crinkling illness". Although the disease was suspected to be caused by a 'virus' (Zimmermann, 1906), its transmission from cassava to cassava by whiteflies was the first evidence that the causal agent is transmissible by an insect vector (Chant, 1958). The presence of small, quasi-isometric particles, mostly occurring as twins, and hence called geminate particles,

in affected plants provided the first evidence for the possible association of a virus with CMD (Harrison *et al.*, 1977, Bock *et al.*, 1978, 1981).

Further evidence for viral nature of the causal agent was supported by the observation that manual inoculation of sap from CMD-affected cassava plants could elicit symptoms in herbaceous test plants such as *Nicotiana clevelandii* Gray and *N. benthaminana* Domin. (Bock *et al.*, 1978). However, back-inoculation from symptomatic tobacco plants failed to infect healthy cassava and therefore, the causal agent was tentatively named as *Cassava latent virus* (CLV, Bock *et al.*, 1978).

Table 1. Viruses infecting cassava

Virus	Genus/Family	Reference isolate[†]	Symptoms	Vector	Distribution[‡]
African cassava mosaic virus (ACMV)	Begomovirus/Geminiviridae	X17095, X17096	Mosaic	Whitefly	SSA
Cassava American latent virus (CsALV)	*Nepovirus*/*Comoviridae*.	NA	Symptomless	Unknown	Brazil and Guyana
Cassava brown streak virus (CBSV)	Ipomovirus/Potyviridae	FJ039520	brown, elongate necrotic leaf lesions	Whitefly	East Africa
Cassava Colombian symptomless virus (syn. *Cassava Caribbean mosaic virus*)	tentative Potexvirus/Flexiviridae	NA	Symptomless	Unknown	Columbia
Cassava common mosaic virus (CsCMV)	Potexvirus/Flexiviridae	U23414	mild mosaic	Unknown	South America
Cassava green mottle virus (CsGMV)	*Nepovirus*/*Comoviridae*.	NA	local and systemic mottle	Unknown	Australasia and Pacific Islands, Solomon Islands
Cassava Ivorian bacilliform virus (CsIBV)	Unassigned Ourmiavirus	NA	Symptomless	Unknown	Cote d'Ivoire
Cassava symptomless virus (CsSLV)	*unassigned Nucleorhabdovirus*/*Rhabdoviridae*	NA	Symptomless	Unknown	Brazil
Cassava vein mosaic virus (CSVMV)	*Cavemovirus*/*Caulimoviridae*.	NC001648, U59751, U20341	*Vein mosaic*	Unknown	Brazil
Cassava virus C (CsVC) syn. *Cassava Q virus*	Ourmiavirus/Unassigned	FJ157981-83	pronounced leaf fleck	Unknown	Cote d'Ivoire
Cassava virus X (CsVX)	Potexvirus/Flexiviridae	NA		Unknown	Columbia
East African cassava mosaic Cameroon virus (EACMCV)	Begomovirus/Geminiviridae	AF112354, AF112355	Mosaic	Whitefly	West Africa, Tanzania
East African cassava mosaic Kenya virus (EACMKV)	Begomovirus/Geminiviridae	AJ717580, AJ704965	Mosaic	Whitefly	East Africa
East African cassava mosaic Malawi virus (EACMMV)	Begomovirus/Geminiviridae	AJ006460, N/A	Mosaic	Whitefly	Malawi
East African cassava mosaic virus (EACMV)	Begomovirus/Geminiviridae	AJ717542, AJ704949	Mosaic	Whitefly	East Africa
East African cassava mosaic virus-Ugandan Variant (EACMV-UG)	Begomovirus/Geminiviridae	AF126804-7	Mosaic	Whitefly	SSA

[‡]SSA = Sub-Saharan Africa
[‡]NA = Not available

Table 1 (contd). Viruses infecting cassava

Virus	Genus/Family	Reference isolate‡	Symptoms	Vector	Distribution
East African cassava mosaic Zanzibar virus (EACMZV)	Begomovirus/Geminiviridae	AJ717562, AJ704942	Mosaic	Whitefly	Zanzibar, Madagascar
Indian cassava mosaic virus (ICMV)	Begomovirus/Geminiviridae	Z24758, Z24759	Mosaic	Whitefly	India and Sri Lanka
Kumi viruses A and B	Uncharacterized	NA	pronounced leaf mottle	Unknown	Kumi district of Uganda
South African cassava mosaic virus (SACMV)	Begomovirus/Geminiviridae	AF155806, AF155807	Mosaic	Whitefly	South Africa, Madagascar, Zimbabwe
Sri Lankan cassava mosaic virus (SLCMV)	Begomovirus/Geminiviridae	AJ579307, AJ579308	Mosaic	Whitefly	India and Sri Lanka

‡NA = Not available

Fig. 1. Symptoms of cassava mosaic disease on cassava plants (B & E) in comparison to a healthy plant (A). Plants affected by CMD often produce small or no tuber (D) in contrast to large tubers produced by disease-free plants (C).

Information on the etiology of CMD began emerging nearly 80 years after its first report in Tanzania. Pioneering studies conducted at the Scottish Crop Research Institute, UK, by Prof. Harrison and his group (Harrison *et al.*, 1977) and subsequently by other groups (Bock and Woods, 1983; Rossel *et al.*, 1987; Briddon *et al.*, 1998) led to the molecular characterization of viruses associated with CMD. The first virus characterized from CMD-affected plants was named as *African cassava mosaic virus* (ACMV) since it was found in several Africa countries, even though it was first identified in East Africa. Further molecular characterization of ACMV isolates from CMD-affected plants in Kenya revealed the presence of a second virus with similar genome organization as ACMV but with distinct serological properties. Based on criteria established by International Committee on Taxonomy of Viruses (ICTV; Davies and Stanley, 1989; Lazarowitz, 1992; Padidam *et al.*, 1995; van Regenmortel *et al.*, 2000), the second virus was named as *East African cassava mosaic virus* (EACMV; Hong *et al.*, 1993). Subsequently, the virus associated with CMD in India was characterized and named as *Indian cassava mosaic virus* (ICMV; Hong *et al.*, 1993). The three viruses are serologically distinct from each other based on their reactions with a panel of monoclonal antibodies to ACMV coat protein (Swanson and Harrison, 1994).

Currently, the ICTV has placed cassava-infecting viruses with geminate particles in the genus *Begomovirus*, the largest genus in the family *Geminiviridae*. These viruses are also referred in the literature as cassava mosaic begomoviruses (CMBs; Thottappilly *et al.*, 2003; Ariyo *et al.*, 2005; Ogbe *et al.*, 2006) or cassava mosaic geminiviruses (CMGs; Patil and Fauquet, 2009). To date, nine distinct CMBs have been reported worldwide and seven of them are native to SSA. They are: ACMV, EACMV, *East African cassava mosaic Cameroon virus* (EACMCV, Fondong *et al.*, 2000), *East African cassava mosaic Kenya virus* (EACMKV, Bull *et*

al., 2006), *East African cassava mosaic Malawi virus* (EACMMV, Zhou *et al.*, 1998), *East African cassava mosaic Zanzibar virus* (EACMZV, Maruthi *et al.*, 2004), and *South African cassava mosaic* (SACMV, Berrie *et al.*, 1998). The other two CMBs, namely, ICMV and *Sri Lankan cassava mosaic virus* (SLCMV, Saunders *et al.*, 2002) were reported from the Indian sub-continent.

Distribution of CMBs

Previously, CMBs were thought to show geographic structuring with ACMV limited to West and Central African countries towards the west of the Rift Valley and in South Africa. EACMV is confined to the eastern part of the Rift Valley in coastal Kenya, Tanzania, Malawi, Zimbabwe and Madagascar, and ICMV is confined to India and Sri Lanka (Swanson and Harrison, 1994; Harrison *et al.*, 1997). Subsequent studies (Swanson and Harrison, 1994; Berrie *et al.*, 1997; Ogbe *et al.*, 1997a,b; 1998; 1999; Fondong *et al.*, 1998; Winter, 1998; Offei *et al.*,1999; Legg *et al.*, 2001; Neuenschwander *et al.*, 2002; Bigirimana *et al.*, 2004; Legg *et al.*, 2004) have shown that most of the seven CMBs reported from SSA are widespread across the sub-continent, while ICMV and SLCMV are confined to cassava-growing regions of India and Sri Lanka, respectively (Thottappilly *et al.*, 2003).

It is interesting to note that there is no report to date of CMD from South America, the center of origin for cassava. This suggests that the causal agent(s) of CMD are 'native' to Africa perpetuating in indigenous hosts, and that cassava became an 'accidental' host after its introduction into the continent. This type of 'new encounter phenomenon' (Buddenhagen, 1977), where a pathogen that co-evolved with indigenous host plant species in a given geographic area,

and becomes a serious pathogen of an introduced plant species, has been reported in several crops that have been introduced to Africa (Thresh and Fargette, 2001).

Impact of CMD on cassava

At the cellular level, it has been shown that ACMV induces cytological modifications in cassava (Horvat and Verhoyen, 1981) as well as in the experimental host *N. benthamiana* (Adejare and Coutts, 1982b). Such modifications include irregular distribution of patches of chromatin at the periphery of the nuclear membrane, presence of electron-dense ring-shaped inclusions of granulofibrillar materials in the nucleoplasm, and small patches of virus-like granular material (Atiri *et al.*, 2000).

CMD symptoms and accompanying cellular modifications depend on whether cassava is infected with single virus or concurrent infection of two or more CMBs causing synergistic interactions (Harrison *et al.* 1997a; Fondong *et al.*, 2000). Characteristic symptoms of CMD on singly-infected plants range from green to yellow mosaic of affected leaves, coupled with leaf distortion (Fig. 1a), regardless of the associated CMB.Co-infection of CMBs may significantly reduce stem girth, plant height and petiole length (Thankappan and Chacko, 1976; Kaiser and Teemba, 1979). It has also been shown that ACMV infection through clonal propagation causes greater yield losses than external infection by whitefly vectors (Fargette *et al.*, 1988). Although symptom severity correlated negatively in many cases with shoot weight, storage root yield and harvest index (Fauquet and Fargette, 1990; Otim-Nape *et al.*, 1994), some resistant genotypes showed significant storage root yield losses even with mild or no symptoms (Seif *et al.*, 1982) indicating lack of a general correlation between symptom severity and yield loss. In general, storage root yield reduction due to CMD may range from 20 to 95 %, depending on the genotype

(Thankappan and Chacko, 1976; Terry and Hahn, 1980; Seif, 1982; Fargette *et al.*, 1988) and the overall storage root yield loss across SSA was estimated to be between 15 and 24 % annually, equivalent to 12-23 million tons. At US$ 100 per ton in the late 1990's, annual losses due to CMD were estimated to be between US$ 1.2 and 2.3 billion (Thresh *et al.*, 1997).

Morphology and genome organization of CMBs

CMBs are characterized by their geminate particles measuring 30 x 20 nm in size and their circular, bipartite, single-stranded DNA (ssDNA) genomes are encapsidated in protein coats of about 30 kDa (Stanley *et al.*, 2005). The two genomic components of CMBs are referred to as DNA A and DNA B (Fig. 2a; Harrison *et al.*, 1977; Stanley, 1983; Stanley and Gay, 1983). The organization of both genomic components is distinct except that they share a stretch of ~ 200 nucleotide-long sequences referred to as the common region (CR). The CR encompasses a conserved stem-loop structure and contains several regulatory elements including the nonanucleotide TAATATTA↓C sequence (arrow denotes the nicking site for initiation of virion-sense DNA replication), the TATA box and iterons which act as binding sites for the replication-associated protein (Hanley-Bowdoin *et al.*, 1999). By far, the most informative of both genomic components is the DNA A. It encodes two overlapping virion-sense open reading frames (ORFs), AV2 and AV1, and at least four overlapping complementary-sense ORFs, AC1, AC2, AC3 and AC4 (Fig. 2a; Hong *et al.*, 1993; Haley *et al.*, 1995). A putative ORF, AC5 that is encoded in the complimentary sense and embedded within the CP gene, has also been reported for some CMBs (Hong *et al.*, 1993) but it is yet to be proven that this ORF is transcribed and translated.

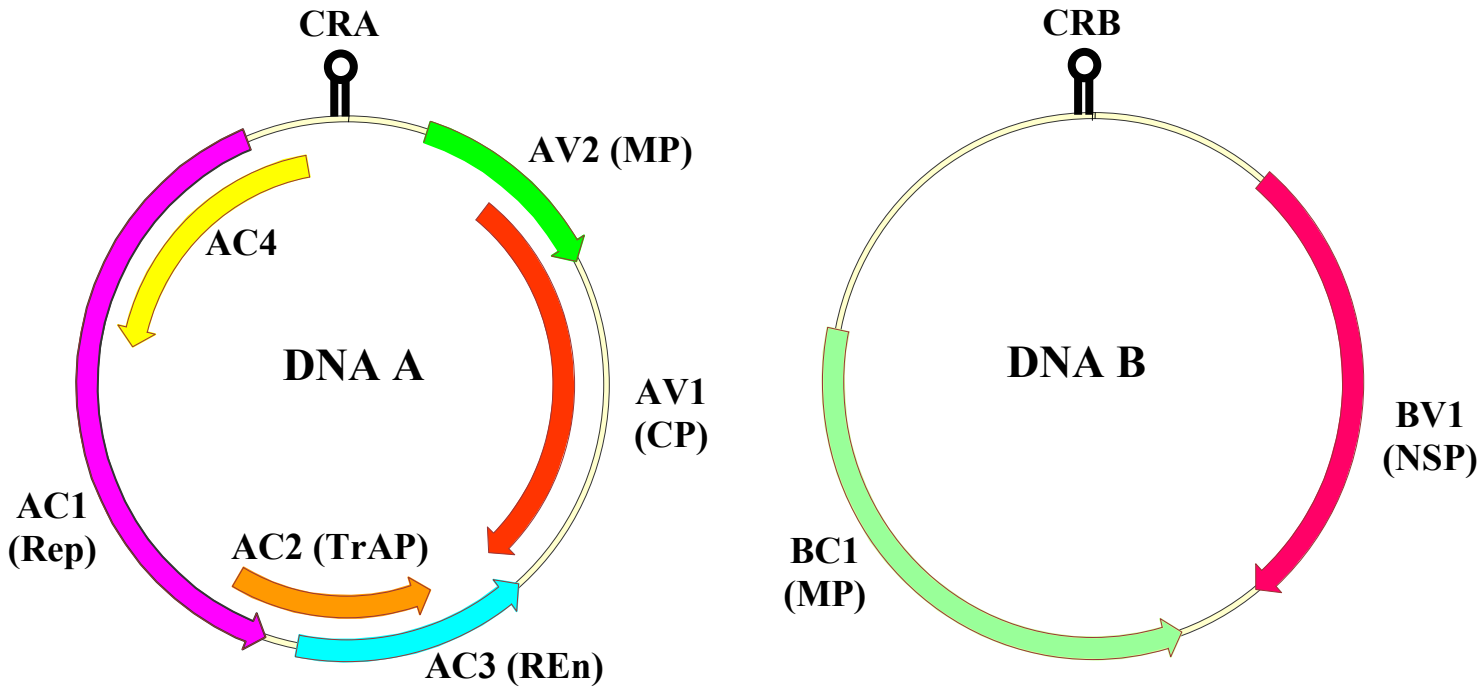

Fig. 2. Genome organization of DNA A and DNA B components of cassava-infecting begomoviruses. The genomic maps were drawn based on DNA A (GenBank Accession No. X17095) and B (GenBank Accession No. X17096) sequences of *African cassava mosaic virus*. Open reading frames (ORFs) are denoted as either being encoded in the virion-sense (V) or complementary-sense (C) strand, preceded by component designation (A or B). CRA, common region A; CRB, common region B; CP, coat protein; MP, movement protein; Rep, replication-associated protein; TrAP, transcriptional activator protein; REn, replication enhancer protein.

AV2 codes for the pre-coat gene, a signature of Old World begomoviruses (Rybicki, 1994), that functions as a movement protein. AV1 encodes the coat protein gene (CP) and it is the determinant of vector transmission (Harrison *et al.*, 2002), in addition to its role in genome encapsidation. As depicted by their names, the complementary-sense genes, individually and in concert, are implicated in the replication of CMBs within the host cell. Hence, ORFs AC1, AC2 andAC3 code for a replication-associated protein (Rep), transcriptional activator protein (TrAP) and replication enhancer protein (REn), respectively. Recently, it has been shown that AC4 ORF plays a role as a host activation protein, in that it serves as an important symptom determinant implicated in cell-cycle control, and may counter a host response to Rep gene expression (Stanley *et al.*, 2005). The TrAP of ACMV can act as a trans-activator of several plant genes (Trinks *et al.*, 2005; Lacatus and Sunter, 2008), besides its role as a trans-activator of the late viral genes AV1 and BV1 (Sunter and Bisaro, 1992). TrAP also functions in the suppression of post-transcriptional gene silencing (Vanitharani *et al.*, 2004; Stanley *et al.*, 2005). The two ORFs of the DNA B component are BV1 and BC1 and they encode the nuclear shuttle protein and the movement protein, respectively. BV1 and BC1 genes are non-overlapping and they play a role in intra- and inter-cellular movement, respectively, of virions within the host plant cell (Hull, 2002; Jeske, 2009).

Recombinant strains and subviral agents associated with CMD

In the early to mid 1990's, an epidemic of CMD with unusually severe symptoms broke out in Uganda (Gibson *et al.*, 1996; Otim-Nape *et al.*, 1997; Legg and Ogwal, 1998). Subsequently, the epidemic spread to other countries in East Africa (Kenya, Gibson *et al.*, 1996; Tanzania, Legg and Okao-Okuja, 1999) leading to a pandemic of CMD. The CMBs associated

with this 'unusual' form of CMD were found to be ACMV and a novel form of EACMV, designated as a Ugandan variant (Zhou *et al.*, 1997) or EACMV-UG (Deng *et al.*, 1997). Molecular characterization of EACMV-UG has shown that this strain originated as a consequence of recombination between sequences of ACMV and EACMV. The DNA A genome of EACMV-UG was 16 % and 84 % similar to that of ACMV and EACMV, respectively (Zhou *et al.*, 1997). The epidemic caused by EACMV-UG devastated many cassava farms forcing thousands of subsistence farmers to abandon the crop (Thresh *et al.*, 1994; Otim-Nape *et al.*, 1997) and leading to famine-related deaths (Otim-Nape *et al.*, 1998). Since then, EACMV-UG has been reported from Sudan (Harrison *et al.*, 1997), Rwanda (Legg *et al.*, 2001), the Democratic Republic of Congo (Neuenschwander *et al.*, 2002), Burundi (Bigirimana *et al.*, 2004) and Gabon (Legg *et al.*, 2004). More recently, EACMV-UG has been reported from Burkina Faso (Triendrebeogo *et al.*, 2009), Cameroon and Nigeria (P. Lava Kumar, pers. comm.), thereby representing the westward movement of the virus within the African continent. The rapid spread of this recombinant virus could be due to indiscriminate dissemination of virus-infected vegetative cuttings of cassava across SSA, probably as a consequence of poor sanitation and inefficient quarantine programs in many African countries. Although other recombinant CMBs, such as SACMV (Berrie *et al.*, 1997), EACMCV (Fondong *et al.*, 2000), EACMMV (Zhou *et al.*, 1998), EACMZV (Maruthi *et al.*, 2004), EACMKV (Bull *et al.*, 2006, 2007), SLCMV (Saunders *et al.*, 2002) and ICMV (Malathi *et al.*, 1987; Mathew and Muniyappa, 1992, 1993) have been identified, they appear to be localized in their distribution relative to EACMV-UG. Factors that could contribute to molecular diversity among EACMV-type viruses have been recently reviewed (Patil and Fauquet, 2009). It is interesting to note that many of the recombinant CMBs have EACMV lineage, whereas no recombinant from ACMV lineage has so

far been observed, despite the wide distribution of ACMV across Africa and its frequent existence as mixed virus infections with other CMBs (Patil and Fauquet, 2009).

More recently, subviral agents, called DNA satellites, have been found associated with CMD. These subviral agents are of two types, the DNA B-like DNA β and nanovirus-like DNA 1. DNA β components are a group of symptom-modulating, small circular ssDNA satellite molecules that are dependent upon the CP of associated begomovirus DNA A for their encapsidation (Dijkstra and Khan, 2006). In particular, small circular ssDNA agents of approximately 1.3 kb in size, typically associated with some Old World monopartite viruses (Saunders *et al.*, 2000; Mansoor *et al.*, 1999; Briddon *et al.*, 2004; Stanley *et al.*, 2005; Briddon and Stanley, 2006), have been found in CMD-affected cassava. DNA 1 components are satellite-like, single-stranded DNA molecules associated with begomoviruses that require the satellite molecule DNA β to induce authentic disease symptoms in some hosts (Briddon *et al.*, 2004). Similar to their DNA β counterpart, DNA 1 components shares no significant homology with their helper viruses and are dependent on them (helper viruses) for encapsidation and movement (Mayo *et al.*, 2005). However, unlike DNA β, DNA 1 components are capable of autonomous replication (Briddon *et al.*, 2006). More intriguing is the discovery of resistance-breaking satellite molecules (approximately 1000 and 1200 nucleotides) found associated with CMD in Tanzania (Patil and Fauquet, 2009). Preliminary data revealed that these satellite molecules can exacerbate symptoms in cassava caused by ACMV, EACMV and EACMCV, leading to disease symptoms in an otherwise resistant landrace TME3 (Briddon and Stanley, 2006). In general, DNA satellites associated with CMD have been shown to play a role in symptoms and pathogenicity, and the suppression of gene silencing (Mansoor *et al.*, 2006).

Transmission of CMD and its viruses

Since cassava is a vegetatively propagated crop, CMBs and their DNA satellites can be disseminated vertically through infected stem cuttings. CMBs have also been shown to be transmissible by grafting infected budwood to healthy cassava plants (Seif, 1982; Adejare and Coutts, 1982a; Ogbe *et al.*, 2001). The whitefly vector, *Bemisia tabaci* Gennadius (Aleyrodidae, Hemiptera), causes secondary spread of CMBs (Chant, 1958; Dubern, 1994; Maruthi *et al.*, 2002). Among different biotypes of *B. tabaci* (Bedford *et al.*, 1994), the highly fecund biotype B appears to be a more efficient and highly successful vector of CMBs (Legg and Thresh, 2001). While a single whitefly was capable of transmitting any of the CMBs, an optimum rate of transmission in controlled experiments was achieved with 10 viruliferous whiteflies per cassava plant (Chant, 1958; Seif, 1981; Dubern, 1994). Non-starved insects acquired the virus 5 h after acquisition feeding from infected cassava, while the starved individuals acquired the virus within 3.5 h indicating that starvation accelerated virus acquisition (Dubern, 1994). The latent period is about 6-8 h in the insect and a period of 10-30 min inoculation access period is required for inoculation into healthy cassava plants. Post-acquisition, ACMV can be retained by an infectious whitefly for about 9 days (Dubern, 1994). The virus is transtadially (Chant, 1958; Harrison, 1987; Dubern, 1994) but not transovarially (Dubern, 1994) transmitted. Other species of whitefly, such as *B. afer*, can transmit CMD, albeit poorly (Fishpool and Burban, 1994; Akano *et al.*, 1995; Palaniswami *et al.*, 1996). CMBs are neither transmissible from cassava to cassava by mechanical inoculations nor through seed (Storey and Nichols, 1938; Malathi *et al.*, 1987).

Diagnosis of CMBs

The successful purification of CMBs (Bock *et al.*, 1977) paved way for the development of antibody-based diagnoses of viruses associated with CMD. Polyclonal antibodies have been used for the detection of ACMV in cassava leaf samples by the double antibody sandwich (DAS) method of enzyme-linked immunosorbent assay (ELISA; Sequeira and Harrison, 1982) and immunosorbent electron microscopy (Roberts *et al.*, 1984). The availability of a panel of monoclonal antibodies (Thomas *et al.*, 1986) gave impetus for rapid detection and discrimination of CMBs using triple antibody sandwich-ELISA (Swanson and Harrison, 1994; Harrison *et al.*, 2002; Thomas *et al.*, 1986). Although diagnosis of CMBs by ELISA is versatile and can be used for large-scale testing of field samples in diagnostic surveys (Ogbe *et al.*, 1996, 1997), its major limitation lies in its inability to distinguish different CMBs in mixed virus infections (Thottappilly *et al.*, 2003). In addition, it is not possible to discriminate recombinant EACMV-UG, SACMV and EACMV from ACMV by ELISA due to similarity in their coat protein epitopes (Thottappilly *et al.*, 2003). Nevertheless, ELISA is still a valuable diagnostic tool for large scale testing of cassava samples in disease surveys conducted by national programs in SSA.

The advent of polymerase chain reaction (PCR) technique has advanced molecular diagnosis of CMBs in several African countries including Cameroon, Nigeria, Tanzania, Kenya, South Africa, etc. (Fondong *et al.*, 2000; Ndunguru *et al.*, 2005; Ogbe *et al.*, 2003a, 2006; Okao-Okuja *et al.*, 2004; Pita *et al.*, 2001; Berry and Rey, 2001; Sseruwagi *et al.*, 2004; Were *et al.*, 2004; Zhou *et al.*, 1997). In addition, a heteroduplex mobility assay has been used to differentiate four different CMBs and their strains (Berry and Rey, 2001). These assays were developed using oligonucleotide primers specific to the DNA A component of CMBs in most

cases. Amplified DNA fragments were then analyzed using restriction enzymes (Legg and Thresh, 2001) or sequenced for profiling CMBs (Thottappilly *et al.*, 2003).

Epidemiology of CMD

Studies have shown that the spread of CMD to new fields can occur much more rapidly from external sources than within field spread (Fargette *et al.*, 1985; Fargette *et al.*, 1990). It was suggested that the direction of prevailing wind (Thresh and Cooter, 2005) and the orientation of the farm (Thresh and Otim-Nape, 1994) could influence the extent of primary infection in the crop. Since population of the whitefly vector fluctuates during the growing season in different agroecologies (Leuschner, 1977), this information was used to manipulate new cassava plantings at periods of low vector population counts to reduce CMD incidence in the early stages of the crop (Thottappilly *et al.*, 2003). It was also found that immigrant whitefly populations tended to preferentially alight on plants in the outer rows (Fargette *et al.*, 1985; Fishpool *et al.*, 1995; Colvin *et al.*, 1998) leading to greater CMD incidence at the margins of plantings (Fargette *et al.*, 1985; Fauquet and Fargette, 1990). The genotype of cassava, the climatic conditions under which it is grown and 'genotype x environment' interactions can also significantly influence whitefly vector populations and CMD spread (Abdullahi *et al.*, 2003). Overall, CMD spread is more rapid in susceptible than in resistant genotypes (Hahn *et al.*, 1980; Thresh *et al.*, 1994; Otim-Nape *et al.*, 1998), indicating that the type of cassava genotype being planted can influence the rate of CMD spread.

Experimental and alternative hosts of CMBs

The experimental host range of CMBs is restricted to members of the family *Solanaceae*, especially those belonging to the genus *Nicotiana* and *Datura* (Bock and Woods, 1983). In addition, SLCMV was shown to infect *Ageratum conyzoides* L. (Saunders *et al.*, 2002; family *Asteraceae*) and *Arabidopsis* (Mittal *et al.*, 2008; family *Brassicaceae*). ACMV was detected in *Jatropha multifida* L. (*Euphorbiaceae*; Fauquet and Fargette, 1990) and suspected to infect *Hewittia sublobata* (L.f) Kuntze (*Convolvulaceae*) in Kenya (Bock *et al.*, 1981) and *Laportea* (*Fluerya*) *aestuans* (*Urticaceae*) in Nigeria (Rossel *et al.*, 1987). In a recent study, Ogbe *et al.* (2006) documented both ACMV and EACMV in *M. glaziovii* (a wild relative of cassava), a weed *Combretum confertum* (Benth.) M.A. Lawson (*Combretaceae*), a leguminous plant, *Senna occidentalis* (L.) Link (*Fabaceae*), and only ACMV in *Ricinus communis* L. (*Euphorbiaceae*) in the Humid Forest and Derived/Coastal Savannah agroecological zones of Nigeria. Subsequently, ACMV and EACMCV were detected in the common hedge plant *Leucana leucocephala* (Lam.) De Witt (*Fabaceae*; Alabi *et al.*, 2007) in the Derived/Coastal Savannah zone and ACMV in cultivated soybean (*Glycine max* L. Merr.; family *Fabaceae*) in Ibadan and Benue in the Derived/Coastal Savannah agroecological zone of Nigeria (Mgbechi-Ezeri *et al.*, 2008). However, the genetic relationship of CMBs from these non-cassava plant species with those from cassava was not ascertained. Therefore, samples from cassava and non-cassava plant species at specific locations in Nigeria (Fig. 3) were collected for the study described in Chapter 2.

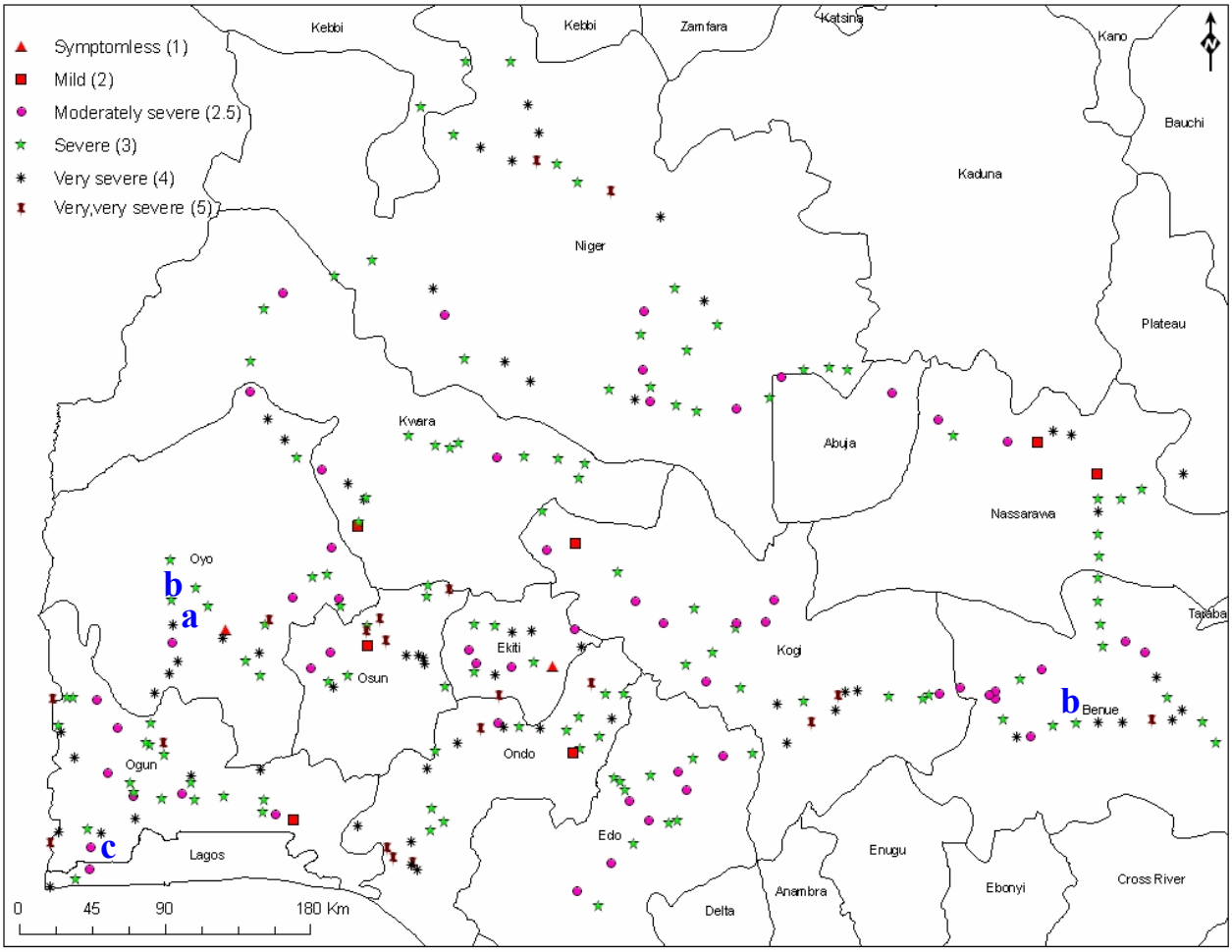

Fig. 3. Abridged country map of Nigeria showing locations where samples from cassava and non-cassava plants were collected. Symbols indicate fields from which samples were collected during diagnostic surveys conducted between 2005 and 2007. *Leucana leucocephala* was obtained from location **a**, *Glycine max* from location **b** and *Manihot glaziovii* was collected from location **c**. Samples from *Ricinus communis*, *Combretum confertum* and *Senna occidentalis* were obtained from other locations are not shown in this map, but can be obtained from Ogbe et al. (2006).

Management of CMD

A broad range of approaches have been developed for the management of CMD. These have been discussed thoroughly in several review articles (Thresh and Cooter, 2005; Thresh, 2006; Vanderschuren *et al.*, 2007). Such strategies include heat treatment to eliminate ACMV and EACMV from growing shoots (Chant, 1959; Kaiser and Teemba, 1979; Kaiser and Louie, 1982), use of meristem-tip culture (Kartha and Gamborg, 1975; Kaiser and Teemba, 1979; Adejare and Coutts, 1981; Ng *et al.*, 1992), roguing infected plants during early stages of plant growth (Bock and Guthrie, 1982; Malathi *et al.*, 1987; Bock, 1994; Thresh and Otim-Nape, 1994), and the use of cultivars with appreciable resistance to both the virus (Fargette *et al.*, 1996; Jennings, 1960; Otim-Nape *et al.*, 1998; Ogbe *et al.*, 2003b) and the vector (Leuschner, 1977; Fauquet *et al.*, 1988; Otim-Nape *et al.*, 1998). Chemical control of the whitefly vector has seldom been practiced by farmers in Africa for economic reasons. In addition, pesticides are least effective in controlling arthropod-borne viruses if the main spread is from external sources and not within crops (Thresh and Cooter, 2005). The negative impact of pesticides on the environment and risks to beneficial organisms including natural enemies and farmers' health makes pesticidal use less appealing (Thottappilly *et al.*, 2003). The potential of biological control of the whitefly vector remains to be explored although studies in this direction have been initiated recently (J. P. Legg, pers. comm.). Cultural practices have also been evaluated for the control of CMD. The observation that disease incidences were highest at the upwind edges of cassava fields (Fargette *et al.*, 1985) led to the recommendation that elongated fields should be oriented along rather than across wind direction to control CMD (Thresh and Otim-Nape, 1994). However, such recommendations are impractical due to limited land available for farmers in SSA. Intercropping cassava with crops such as maize and cowpea did not yield appreciable

benefits for controlling CMD (Fondong *et al.*, 1997, 2000, Fargette and Fauquet, 1988). Some studies have shown that varietal mixtures involving a combination of susceptibe and resistant cultivars is effective in CMD management. Hence this approach has been widely advocated for use by resource-poor farmers (Otim-Nape *et al.*, 2001; Sserubombwe *et al.*, 2001). One limitation of resistant varieties lies in the fact that some of them may accommodate moderate to high levels of virus inoculum (Ogbe *et al.*, 2003b). Such varieties could actually be tolerant, rather than resistant to virus infection in that they permit virus replication within their tissue although no visible symptoms of the infection are apparent. From an epidemiological standpoint, such materials could serve as sources of inoculum if conditions for vector spread are favorable. The use of transgenic resistance against CMD has been explored in recent years (Patil and Fauquet, 2009). Although the technology has shown some promising results under controlled conditions, the performance of transgenic cassava plants under field conditions of Africa remains to be assessed. Besides, the African perception of genetically modified organisms and their perceived negative impact on small farmers in Africa could be limiting factors in exploring this technology for cassava improvement against CMD (Kuyek, 2002).

Among the various strategies evaluated for CMD management, planting virus-free cuttings was shown to be the most effective in minimizing spread of the disease in Africa (Thresh and Otim-Nape, 1994), since infected stem cuttings are the primary sources of virus inoculum (Fargette *et al.*, 1994). The phenomenon of "reversion" due to uneven distribution of virus in CMD-resistant cultivars has been exploited in selecting virus-free cuttings in Cote d'Ivoire (Fargette *et al.*, 1985; Fargette *et al.*, 1988). Also, an often overlooked factor in the management of CMD management is the role of volunteer cassava plants and other plant species as alternative hosts of CMBs. Such plants could serve as reservoirs for the perpetuation of CMBs

during off-season and could provide sources of inoculum for primary spread of CMBs to new plantings by the whitefly vector.

Conclusions and perspectives

Despite many advances, CMD continues to be a significant threat to sustainable production of cassava in SSA. Although molecular studies on CMBs and host-virus interactions are advancing our knowledge on various aspects of CMD, there are still many outstanding questions that need to be addressed for mitigating the negative impact of the disease on cassava production. One of the key gaps in knowledge on the epidemiology of CMD in Nigeria, and indeed on a regional scale, is the potential role of non-cassava plant species as alternative/reservoir hosts in the perpetuation of CMBs. Since cassava was introduced from South America in the 16th Century, it is likely that CMBs endemic to Africa infecting indigenous African plant species have become adapted to cassava upon its introduction. Thus, it is plausible that native plant species could act as alternative and/or reservoir hosts for CMBs and contribute to continued virus evolution and disease epidemics. Since cassava is a vegetatively propagated crop, distribution of vegetative cuttings largely contributes to long distance dissemination of CMBs. Hence, concerted efforts are needed to enforce quarantine regulations in African countries to prevent introduction of CMBs through propagation materials. A prerequisite to this effort is the availability of sensitive, reliable and rapid diagnostic tools for specific detection and discrimination of CMBs in a timely fashion. Given that CMBs often occur as co-infections in CMD-affected cassava plants (Thottappilly *et al.*, 2003; Ogbe *et al.*, 2006), high throughput diagnosis of these viruses will facilitate a comprehensive understanding of the

epidemiology of CMD at the regional scale and give thrust for crop improvement and robust phytosanitary programs in African countries.

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CHAPTER 3

ALTERNATIVE HOSTS OF *AFRICAN CASSAVA MOSAIC VIRUS* AND *EAST AFRICAN CASSAVA MOSAIC CAMEROON VIRUS* IN NIGERIA

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ABSTRACT

Cassava mosaic disease (CMD) caused by *African cassava mosaic virus* (ACMV) and *East African cassava mosaic Cameroon virus* (EACMCV) is the major constraint to cassava production in Nigeria. Sequences of the DNA A component of ACMV and EACMCV isolates from leguminous plant species (*Senna occidentalis*, *Leucana leucocephala* and *Glycine max*), castor oil plant (*Ricinus communis*), a weed host (*Combretum confertum*) and a wild species of cassava (*Manihot glaziovii*) were determined. All ACMV isolates from these hosts showed 96-98 % nucleotide sequence identity with cassava isolates from West Africa. EACMCV was found only in four hosts (*S. occidentalis*, *L. leucocephala*, *C. confertum*, *M. glaziovii*), and sequences of these isolates showed 96–99 % identity with cassava isolates from West Africa. These results provide definitive evidence for the natural occurrence of ACMV and EACMCV in plant species besides cassava.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz.) is a staple food crop grown by subsistence farmers in Sub-Saharan Africa and contributes significantly to the household food security in the region.

Cassava mosaic disease (CMD) is an important constraint to cassava production in Sub-Saharan Africa, with an estimated annual yield loss of US $1.5 billion [9, 18]. Six distinct cassava mosaic begomoviruses (CMBs, family *Geminiviridae*, genus *Begomovirus*) have been found occurring in CMD-affected cassava plants in the region [4]. Among them, only *African cassava mosaic virus* (ACMV) and *East African cassava mosaic Cameroon virus* (EACMCV) have so far been documented in CMD-affected cassava plants in West Africa [2]. The whitefly vector, *Bemisia tabaci* (Gennadius) (*Aleyrodidae*, Hemiptera) transmits CMBs from plant-to-plant. Long-distance spread of CMD occurs by the distribution of infected stem cuttings.

One of the key gaps in knowledge on the epidemiology of CMD in Nigeria, and indeed on a regional scale, is the potential role of non-cassava plant species as alternative/reservoir hosts in the perpetuation of CMBs. Since cassava was introduced from South America in the 16th Century, it is likely that CMBs endemic to Africa infecting indigenous African plant species have become adapted to cassava upon its introduction [16]. Thus, it is plausible that native plant species could act as alternative and/or reservoir hosts for CMBs and contribute to virus evolution and disease epidemics. Available information on the natural host range of CMBs indicates that they are largely restricted to cassava and a few of its wild relatives such as *Manihot glaziovii* Müll. Arg. Previously, ACMV was detected in *Jatropha multifida* L. (*Euphorbiaceae*) [6] and suspected to infect *Hewittia sublobata* (*Convolvulaceae*) in Kenya [3] and *Laportea* (=*Fluerya*) *aestuans* (*Urticaceae*) in Nigeria [13]. Recently, Ogbe *et al.* [12] documented both ACMV and EACMV in *M. glaziovii*, a leguminous plant, *Senna occidentalis* (L.) Link (*Fabaceae*), a weed plant *Combretum confertum* (Benth.) M.A. Lawson (*Combretaceae*) and only ACMV in *Ricinus communis* L. (*Euphorbiaceae*) in the Humid Forest and Derived/Coastal Savannah agroecological zones in Nigeria. Subsequently, ACMV and EACMCV were detected in the

common hedge plant *Leucana leucocephala* (Lam.) De Witt (*Fabaceae*) [1] (Fig. 1) in the Derived/Coastal Savannah zone and ACMV in *Glycine max* L. Merr.(*Fabaceae*) in Ibadan and Benue in the Derived/Coastal Savannah zone [10]. Since these reports were based on PCR detection, further studies were undertaken to confirm the identity of ACMV and EACMCV by nucleotide sequence analysis of the DNA A component to determine relationships with corresponding sequences from cassava.

MATERIALS AND METHODS

DNA was extracted from field-collected leaf tissues of cassava and other plant species according to Dellaporta *et al.* [5]. Final DNA pellet was resuspended in 200 µl of Tris–EDTA (TE; 50 mM Tris–HCl, pH 8.0 and 10 mM EDTA). PCR assays were performed in a final reaction volume of 15 µl containing 1X PCR buffer (Roche Applied Sciences, IN, USA), 0.2 mM each dNTP, 0.267 µM each of upstream and downstream primer, 0.8 units Taq DNA polymerase (Roche Applied Sciences, IN, USA) and 2.5 ng μl^{-1} of plant DNA. Separate PCR assays for individual viruses were performed in a GeneAmp PCR System 9700 (Applied Biosystems, CA, USA). Cycling conditions were: one cycle consisting of 94 ºC for 1 min, 52 ºC for 2 min and 72 ºC for 3 min, followed by 36 cycles with each cycle consisting of 94 ºC at 1 min, 52 ºC for 2 min and 72 ºC for 1.33 min. This was followed by a final extension at 72 ºC for 5 min. Virus-specific primers used in PCR assays were designed based on published sequences of ACMV and EACMV isolates available in the GenBank database. Primer sequences specific for each virus and the expected sizes of amplicons are indicated in Table 1.

The virus-specific amplicons obtained from each plant species (Fig. 2) were cloned separately into pCR2.1 vector and transformed into *Echerichia coli* following the manufacturer's

instructions (Invitrogen Corp, Carlsbad, CA). Recombinant DNA isolated from three independent colonies of *E. coli* representing each amplicon was sequenced in both directions. DNA sequences were edited, and a consensus sequence was derived for each amplicon (Vector NTI Advance 10 program, Invitrogen Corp, Carlsbad, CA). Sequences were assembled to obtain a full-length DNA A sequence specific to ACMV and EACMCV isolates from each host. A total of six ACMV and four EACMCV sequences were obtained from non-cassava hosts. In addition, a DNA A sequence of another EACMCV isolate from cassava was obtained in this study. Pair-wise comparisons of ACMV and EACMCV sequences were made with corresponding sequences in GenBank using the Vector NTI Advance10 program (Invitrogen Corp., USA). Multiple sequence alignments and phylogenetic analyses were performed by the neighbor-joining method using molecular evolutionary genetics analysis (MEGA) software version 4.0 [17], and a consensus tree was generated using the same program.

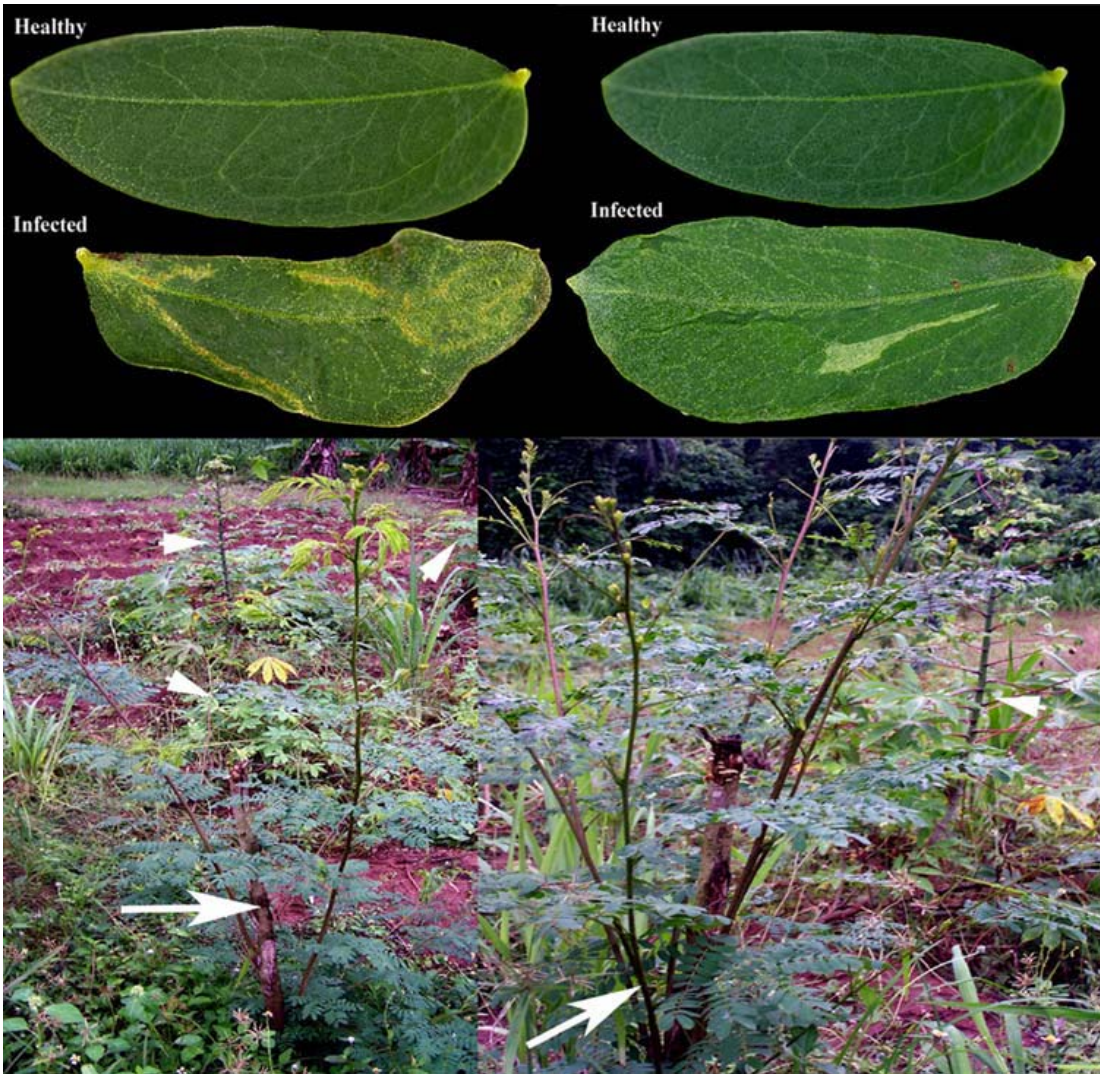

Fig. 1. Symptoms on *Leucana leucocephala* infected with a mixture of *African cassava mosaic virus* (ACMV) and *East African cassava mosaic Cameroon virus* (EACMCV). Top Infected leaves show chlorotic streaks and deformation, Bottom *L. leucocephala* (shown by white arrows) is grown in borders of cassava (shown by white arrow heads) fields for fodder by farmers in Nigeria.

Table 1. Primers used for amplifying the overlapping fragments of DNA A component of ACMV and EACMCV from cassava and alternative hosts

Primer name[a]	Sequence on DNA A (5′-3′)[b]	Target virus	Orientation	Position in DNA A[c]	Size of amplicon (bp)
ACMV-AL1/F	GCGGAATCCCTAACATTATC	ACMV	Sense	1,987-2,006	1,030
ACMV-ARO/R	GCTCGTATGTATCCTCTAAGGCCTG	ACMV	Antisense	211-235	
UV-AL3/F	TACACATGCCTCRAATCCTG	EACMV	Sense	1,036-1,055	1,087
UV-AL1/R2	CTCCGCCACAAACTTACGTT	EACMV	Antisense	2,103-2,122	
OJA001F	GCTAGTGCGCAATGTGGGATC	ACMV	Sense	125-145	1,945
OJA002R	GTTTCTCCCTTCCCATGTTC	ACMV	Antisense	2,050-2,069	
OJA003F	CGRCTATCACCTTCKAGAA	EACMCV	Sense	1,975-1,993	2,158
OJA004R	GGGGATQCACAAGTGTTTT	EACMCV	Antisense	1,312-1,330	
OJA005F	AGAACGATTTGAGGGATAGG	ACMV	Sense	807-826	728
OJA006R	CGTAGGAGAGTGGATCTTGTC	ACMV	Antisense	1,514-1,534	
OJA007F	ATTKGCTGTCGTTTTGKA	EACMCV	Sense	2,646-2,663	1,258
OJA008R	GTCACTGMATCATARAAATAGRT	EACMCV	Antisense	1,079-1,101	

[a]Primers ACMV-AL1/F, ACMV-ARO/R, UV-AL3/F and UV-AL1/R2 were designed in previous studies [8, 19]. All other primers were designed in this study
[b]The following IUB Group codes were used to identify redundancies: R = A + G, M = A + C, K = G + T, Q = G + T
[c]Relative positions of the ACMV primers were based on ACMV-[NG], A*frican cassava mosaic virus*-[Nigeria] (GenBank Accession # X17095) and those of EACMCV were based on EACMCV-CM[CM:98], *East African cassava mosaic Cameroon virus*-Cameroon[Cameroon:1998] (GenBank Accession # AF112354)

RESULTS

PCR assays using virus-specific primers ACMV-AL1/F & ACMV-ARO/R and UV-AL3/F & UV-AL1/R2 amplified DNA fragments of the expected size, confirming the presence of ACMV and EACMV-like particles in symptomatic leaves of *S. occidentalis*, *C. confertum*, *L. leucocephala*, and *M. glaziovii*. The presence of both viruses in these hosts indicates mixed virus infections. In contrast, only an ACMV-specific fragment was amplified from symptomatic leaves of *R. communis* [12] and G. max [10], indicating a single virus infection. Subsequently, full-length DNA A sequences of ACMV isolates from *S. occidentalis* [2,781 nucleotides (nts)], *R. communis* (2,780 nts), *L. leucocephala* (2,781 nts), *M. glaziovii* (2,781 nts) and *G. max* (2,781 nts) were obtained. Similarly, full-length DNA A sequences of EACMCV isolates from *S. occidentalis* (2,800 nts), *L. leucocephala* (2,800 nts) and *M. glaziovii* (2,800 nts) were obtained. Only partial sequences of ACMV (1,717 nts) and EACMCV (1,089 nts) were obtained from *C. confertum* owing to the lack of adequate sample. A pair-wise comparison of ACMV DNA A sequences obtained in this study with corresponding sequences of cassava isolates from West Africa showed nucleotide sequence identities from 96 to 98 %. Similarly, DNA A sequences of EACMCV isolates from different plant species showed 96-99 % nucleotide sequence identity with corresponding EACMCV isolates from West Africa and 92 to 93 % with another EACMCV isolate reported from Tanzania [(EACMCV-TZ(TZ:1:01)]. A comparison of nucleotide sequences of individual genes of ACMV and EACMCV isolates from non-cassava hosts with corresponding gene sequences of cassava isolates showed sequence identities in the same range as the values obtained with full-length DNA A sequences (data not shown).

Phylogenetic analyses revealed that ACMV and EACMCV isolates obtained from different plant species in Nigeria clustered with the respective ACMV and EACMCV isolates

available in GenBank (Fig. 3). These results indicate that ACMV and EACMCV isolates from non-cassava hosts are closely related to the corresponding virus isolates infecting cassava. Further analysis of phylograms revealed that EACMCV isolates from Nigeria are more closely related to an isolate from neighboring Cameroon than to another West African isolate from a geographically distant country (Côte d'Ivoire, Fig. 3). This observation is in agreement with a previous study reported by Ariyo *et al.* [2].

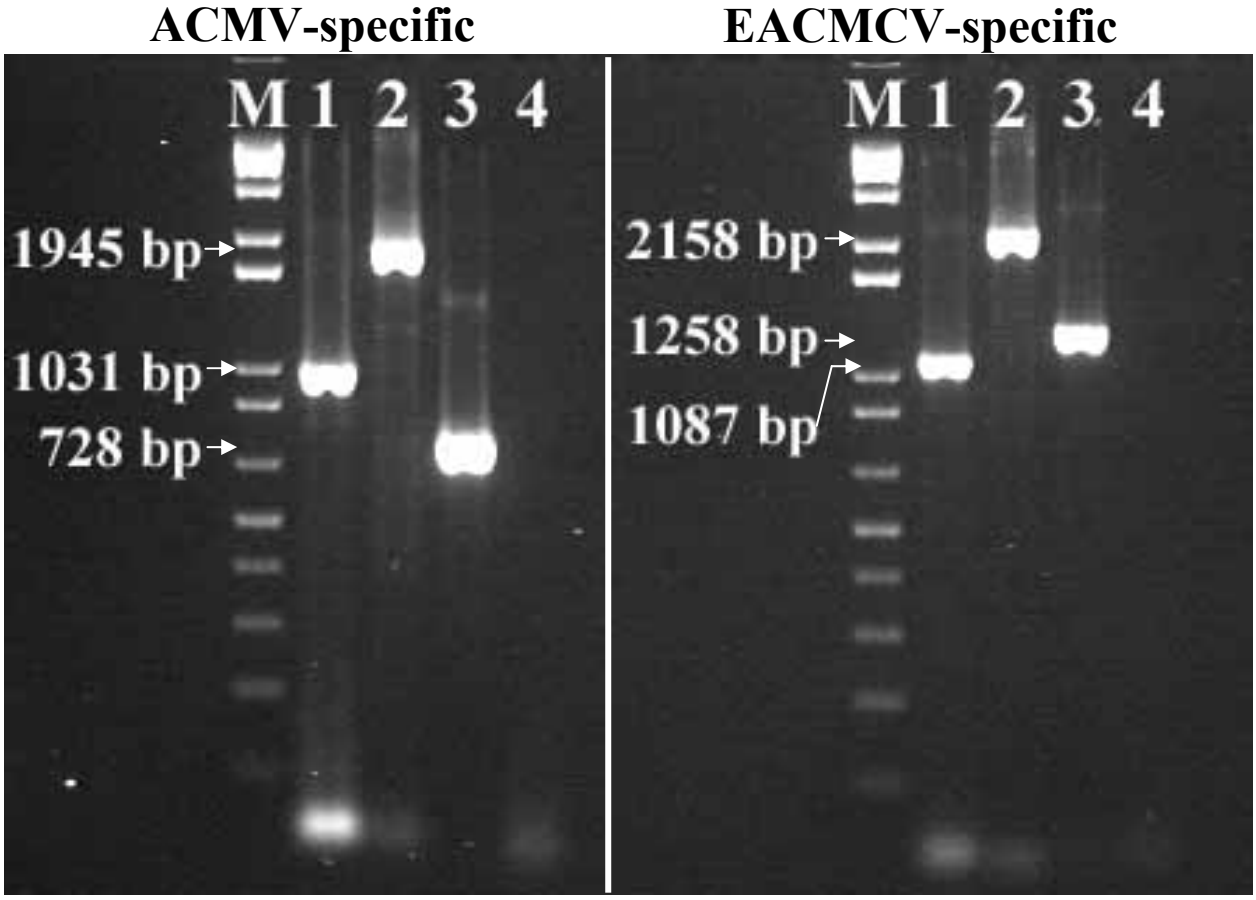

Fig. 2. Amplification of the DNA A genome of cassava mosaic begomoviruses (CMBs) from alternative hosts species infected with both *African cassava mosaic virus* (ACMV) and *East African cassava mosaic Cameroon virus* (EACMCV). Amplicons were obtained using ACMV-specific primers 1, ACMV-AL1/F & ACMV-ARO/R; 2, OJA001 & OJA002; 3, OJA005 & OJA006 and EACMV-type virus specific primers UV-AL3/F & UV-AL1/R2; 2, OJA003 & OJA004; 3, OJA007 & OJA008; 4, Healthy control; M = 1 kb Plus DNA ladder (Invitrogen Corp., Carlsbad, CA) . The sizes of virus-specific DNA fragments are indicated on the left hand side.

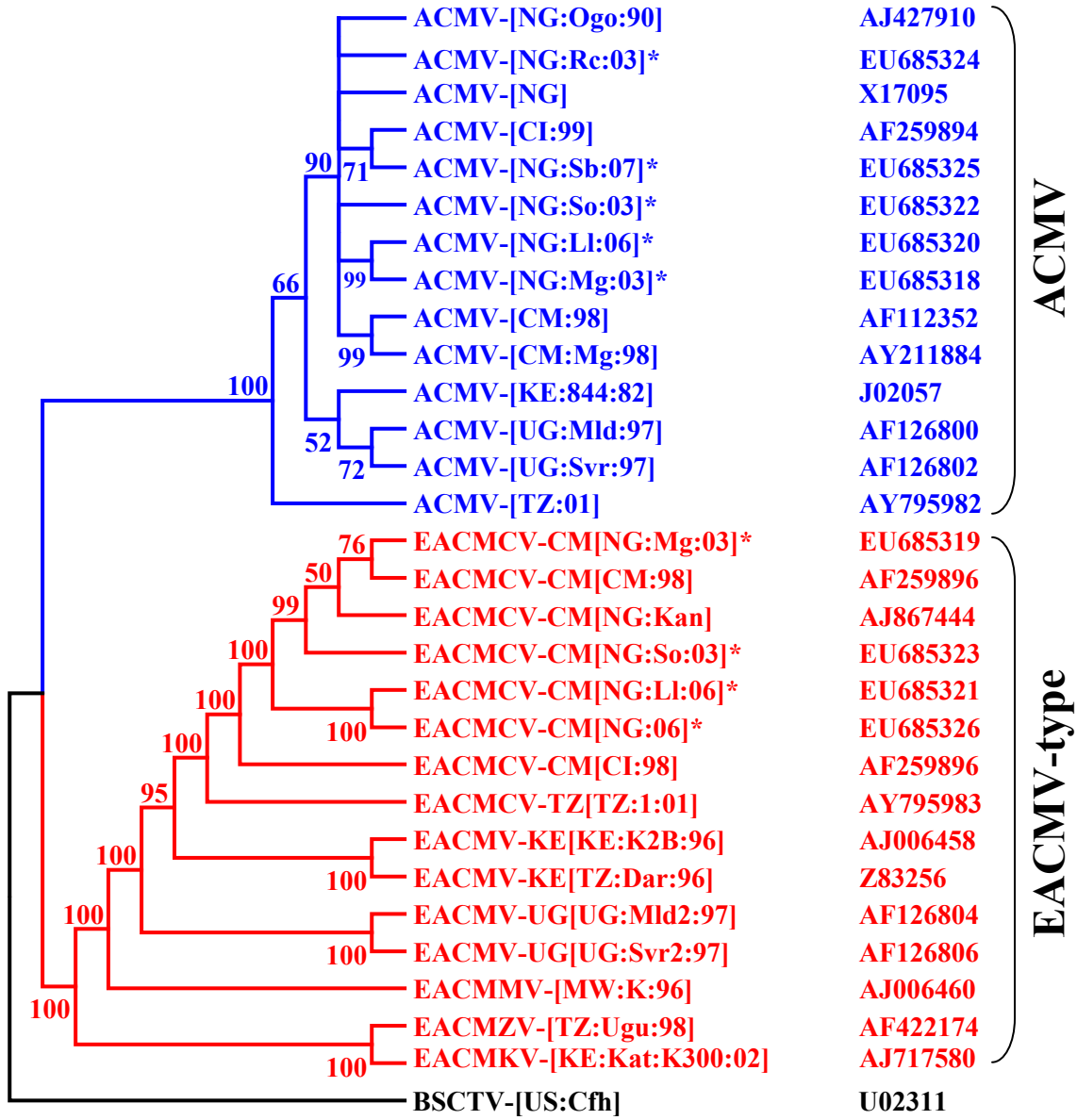

Fig. 3. Phylogenetic tree (cladogram) inferred from the neighbor-joining method using MEGA4 with complete deletion option. Bootstrap values are shown as percentages at the branch points (1,000 replications). ACMV and EACMCV isolates from cassava and alternate host species are indicated in bold font and isolates whose sequences were determined in this study are indicated with asterisk. *Beet severe curly top virus*-[United States of America:Cfh] (BSCTV-[US:Cfh]) was used as the outgroup. Abbreviations and accession numbers are: Rc, *Ricinus communis*; So, *Senna occidentalis*; Ll, *Leucana leucocephala*; Mg, *Manihot glaziovii*; ACMV-[NG:Rc:03], *African cassava mosaic virus*-[Nigeria:Rc:2003]; ACMV-[NG:Ogo:90], *African cassava mosaic virus*-[Nigeria:Ogoroco:1990]; ACMV-[NG], *African cassava mosaic virus*-[Nigeria]; ACMV-[CI:99], *African cassava mosaic virus*-[Côte d'Ivoire:1999]; ACMV-[NG:Sb:07], *African cassava mosaic virus*-[Nigeria:Soybean:2007]; ACMV-[NG:So:03], *African cassava mosaic*

virus-[Nigeria:So:2003]; ACMV-[NG:Ll:06], *African cassava mosaic virus*-[Nigeria:Ll:2006]; ACMV-[NG:Mg:03], *African cassava mosaic virus*-[Nigeria:Mg:2003]; ACMV-[CM:98], *African cassava mosaic virus*-[Cameroon:1998]; ACMV-[CM:Mg:98], *African cassava mosaic virus*-[Cameroon:Mg:1998]; ACMV-[KE:844:82], *African cassava mosaic virus*-[Kenya:844:1982]; ACMV- [UG:Mld:97], *African cassava mosaic virus*-[Uganda Mild:1997]; ACMV-[UG:Svr:97], *African cassava mosaic virus*-[Uganda:Severe:1997]; ACMV-[TZ:01], *African cassava mosaic virus*-[Tanzania:2001]; EACMCV-CM[NG:Mg:03], *East African cassava mosaic Cameroon virus*-Cameroon[Nigeria:Mg:2003]; EACMCV-CM[CM:98], *East African cassava mosaic Cameroon virus*-Cameroon[Cameroon:1998]; EACMCV-CM[NG:Kan], *East African cassava mosaic Cameroon virus*-Cameroon[Nigeria:Kano]; EACMCV-CM[NG:So:03], *East African cassava mosaic Cameroon virus*-Cameroon[Nigeria:So:2003]; EACMCV-CM[NG:Ll:06], *East African cassava mosaic Cameroon virus*-Cameroon[Nigeria:Ll:2006]; EACMCV-CM[NG:Iba:06], *East African cassava mosaic Cameroon virus*-Cameroon[Nigeria:Ibadan:2006]; EACMCV-CM[CI], *East African cassava mosaic Cameroon virus*-[Côte d'Ivoire]; EACMCV-TZ[TZ:1:01], *East African cassava mosaic Cameroon virus*-Tanzania[Tanzania:1:2001]; EACMV-KE[KE:K2B:96], *East African cassava mosaic virus*-Kenya[Kenya:K2B:1996]; EACMV-KE[TZ:Dar:96], *East African cassava mosaic virus*-Kenya[Tanzania:Dar Es Salaam:1996]; EACMVUG[UG:Mld2:97], *East African cassava mosaic virus*-Uganda[Uganda:Mild2:1997]; EACMV-UG[UG:Svr2:97], *East African cassava mosaic virus*-Uganda[Uganda:Severe2:1997]; EACMMV-[MW:K:96], *East African cassava mosaic Malawi virus*-[Malawi:K:1996]; EACMZV-[TZ:Ugu:98], *East African cassava mosaic Zanzibar virus*[Tanzania:Uguja:1998]; EACMKV-[KE:Kat:K300:02], *East African cassava mosaic Kenya virus*-[Kenya: Kathiana:K300:2002]. Naming of isolates/strains is based on Fauquet *et al.* [7]

DISCUSSION

By carrying out nucleotide sequence analysis of the entire DNA A genomic components, we have confirmed previous reports [1, 10, 12] that ACMV and EACMCV can infect non-cassava plant species including both weed hosts (*S. occidentalis* and *C. confertum*) and crop plants (*R. communis*, *L. leucocephala*, and *G. max*). In addition, our study clarified the inconsistency with regard to misidentification of EACMCV as EACMV by Ogbe *et al.* [12] in PCR assays using virus-specific primers. This type of misdiagnosis underscores the need to carry out nucleotide sequence analysis of PCR fragments to alleviate problems associated with diagnosis of CMBs using only PCR. The presence of only ACMV in *R. communis* and *G. max* indicates that these two plant species may be non-hosts for EACMCV. The occurrence of only ACMV in *R. communis* confirms an earlier report by Ogbe *et al.* [12]. Together with our study, these findings are in contrast with an earlier report by Shoyinka *et al.* [14] indicating natural infection of castor oil plant (*R. communis*) with both ACMV and EACMV in Nigeria. The difference in results could be that this study was based on serological reactions and not corroborated further by PCR and sequence analysis.

Since nucleotide sequences of ACMV and EACMCV isolates from different non-cassava host plant species reported in this study are highly similar to those from cassava, it can be concluded that non-cassava hosts could play an important role in the epidemiology of CMD in Nigeria. It is likely that ACMV and EACMCV isolates can be transmitted between cassava and non-cassava hosts by the whitefly vector, thereby facilitating the survival of viruses. It is interesting to note that all of the natural hosts of ACMV and EACMCV documented so far in Nigeria occur in the Humid Forest and Derived/Coastal Savannah agroecological zones of the country. These two zones have been documented as hot spots for CMD with high whitefly

population [11]. Recent studies conducted in Uganda have shown that the whitefly vector could colonize different plant species besides cassava [15]. A wide host range of CMBs and whitefly vectors could facilitate the survival of both CMBs and their vector during periods when cassava is not available. Many farmers in Nigeria use *L. leucocephala* as a hedge crop around cassava fields because of its value as fodder for livestock. The finding that *L. leucocephala* is a host for ACMV and EACMCV and an observation of whiteflies on this plant species (data not shown) would indicate the potential of *L. leucocephala* as a reservoir host for CMBs. Due to its perennial nature, this plant species could offer a constant source of inoculum for the whitefly vector to spread both viruses to new plantings of cassava in the vicinity. Similarly, the perennial weed hosts (*S. occidentalis* and *C. confertum*) widely present in cassava-growing regions would provide a reservoir for CMBs year round. Although soybean (*G. max*) is identified as a host for ACMV, it may be a 'dead-end' host and might not play any significant role in the epidemiology of CMD due to its annual life cycle.

In summary, this study underscores the importance of documenting the natural host range of different CMBs to better understand the role of indigenous non-cassava plant species in the epidemiology of CMD. Studies on the ability of *B. tabaci* to transmit CMBs to non-cassava hosts and vice versa would complement these findings to provide a foundation for a detailed understanding of CMD epidemiology and help to develop sustainable strategies for the management of the disease in Nigeria and in the wider Sub-Saharan Africa region.

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CHAPTER 4

MULTIPLEX PCR FOR THE DETECTION OF *AFRICAN CASSAVA MOSAIC VIRUS* AND *EAST AFRICAN CASSAVA MOSAIC CAMEROON VIRUS* IN NIGERIA

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ABSTRACT

A multiplex PCR was developed for simultaneous detection of *African cassava mosaic virus* (ACMV) and *East African cassava mosaic Cameroon virus* (EACMCV) in cassava affected with cassava mosaic disease (CMD). One set of three primers consisting of an upstream primer common for both viruses and two down stream virus-specific primers were designed for simultaneous amplification of 368 base pair (bp) and 650 bp DNA fragments specific to the replicase gene of ACMV and EACMCV, respectively. Similarly, a second set of three primerswere designed for simultaneous amplification of 540 bp and 655 bp fragments specific to the coat protein gene of EACMCV and ACMV, respectively. Primers that can amplify a 171 bp fragment of the large subunit of ribulose bisphosphate carboxylase oxygenase L were included as an internal control in these assays to determine the reliability of multiplex PCR. A simplified, cost-effective and rapid sample preparation method was adapted in place of the conventional plant DNA extraction procedure for multiplex PCR detection of ACMV and EACMCV. The method was validated using CMD-affected cassava samples obtained from farmers' fields in Nigeria. The multiplex PCR is useful for reliable assessment of the prevalence of CMBs in epidemiological studies and for crop improvement and phytosanitary programs in African countries.

1. INTRODUCTION

Cassava mosaic disease (CMD) is the most economically important viral disease of cassava in Sub-Saharan Africa (Legg *et al.*, 2006). The disease is wide spread in many cassava-growing countries of the region (Fauquet and Stanley, 2003; Sseruwagi *et al.*, 2004a, 2005). CMD epidemics are frequent in subsistence agriculture with crop losses throughout Sub-Saharan Africa between 19 and 27 metric tonnes (Legg and Thresh, 2004) and an estimated economic loss of over US$1.5 billion per year (Thresh *et al.*, 1997). CMD-affected plants are stunted with conspicuous foliar symptoms and produce no or greatly diminished tuberous root yield (Otim-Nape *et al.*, 2000).

Eight begomoviruses (genus *Begomovirus*, family *Geminiviridae*) have so far been documented in cassava infected with CMD worldwide (Fauquet and Stanley, 2003). Only six of these cassava infecting begomoviruses (CMBs) have been reported to occur in Sub-Saharan Africa. They are: *African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV), *East African cassava mosaic Cameroon virus* (EACMCV), *East African cassava mosaic Malawi virus* (EACMMV), *East African cassava mosaic Zanzibar virus* (EACMZV) and *South African cassava mosaic virus* (SACMV) (Fauquet *et al.*, 2008). In addition, *East African cassava mosaic virus-Uganda2* (EACMV-UG2, Ugandan variant), a recombinant virus between ACMV and EACMV has been associated with a pandemic in East Africa (Harrison *et al.*, 1997; Pita *et al.*, 2001). CMBs can occur in an infected plant either alone or as mixed infections of different combinations (Berry and Rey, 2001; Bull *et al.*, 2006; Ogbe *et al.*, 2003; Were *et al.*, 2004). Among the six African CMBs, only ACMV and EACMCV have been reported in CMD-infected cassava plants in West Africa (Ariyo *et al.*, 2005; Thottappilly *et al.*, 2003). CMBs are vectored by the whitefly, *Bemisia tabaci* (Gennadius) (*Aleyrodidae*, Hemiptera) (Maruthi *et al.*,

2002) and are also disseminated through infected stem cuttings used for new plantings, since cassava is a crop propagated vegetatively.

Enzyme-linked immunosorbent assays (ELISA), using a panel of monoclonal antibodies, have been used for the discrimination of ACMV and EACMV (Swanson and Harrison, 1994). Although ELISA is practical due to its simplicity and cost-effectiveness, the technique has the disadvantage of being less reliable for detection of these viruses in plants with low virus titer or in asymptomatic plants. ELISA also fails to identify other CMBs and distinguish ACMV from the recombinant EACMV-UG2 in mixed virus infections due to their similar epitope profiles (Thottappilly *et al.*, 2003). Consequently, the polymerase chain reaction (PCR) has been used for specific detection of different CMBs occurring in several African countries (Fondong *et al.*, 2000; Ndunguru *et al.*, 2005; Ogbe *et al.*, 2003, 2006; Okao-Okuja *et al.*, 2004; Pita *et al.*, 2001; Sseruwagi *et al.*, 2004b; Were *et al.*, 2004; Zhou *et al.*, 1997). In addition, a heteroduplex mobility assay (HMA) has been used to differentiate four different CMBs and their strains (Berry and Rey, 2001).

In all these instances, DNA was extracted from CMD-affected cassava plants (Dellaporta *et al.*, 1983) and individual CMBs were detected by separate PCRs (uniplex PCR) using species-specific primers. In this study, a multiplex-PCR assay was developed using new primers targeting replicase and coat protein regions of DNA A component for specific and simultaneous detection of ACMV and EACMCV. In addition, a simplified and rapid sample preparation method was sought for use in PCR since DNA extraction is a time-consuming, multi-step process and has the inherent risk of cross-contamination while processing large numbers of samples. The protocol was validated by including a house keeping gene of host origin as an internal control for increased reliability of virus diagnosis in CMD-affected cassava leaves. The

ability to diagnose different viruses accurately and simultaneously by multiplex PCR, as opposed to uniplex PCRs, facilitates high throughput diagnosis of CMBs in epidemiological studies and for crop improvement and phytosanitary programs in many African countries.

2. MATERIALS AND METHODS

2.1. Plant materials

Leaf samples from healthy and ACMV- and EACMCV-infected cassava plants maintained in an insect proof screen house at the International Institute of tropical Agriculture (IITA), Ibadan, Nigeria, were used for developing the multiplex PCR. Cassava leaves infected with EACMV-UG2 were kindly provided by Dr. M.N. Maruthi (Natural Resources Institute, Chatam Maritime, Kent, UK). In addition, leaf samples from asymptomatic and symptomatic cassava plants were collected from farmers' fields in Nigeria. All samples used in this study were imported under the USDA-APHIS-PPQ permit number P526P-07-06707.

2.2. Sample preparation

Total plant DNA was extracted from cassava leaf tissue using the protocol described by Dellaporta *et al.* (1983). The DNA was used at a concentration of 2.5 ngμl^{-1} in all PCRs. Leaf extracts were prepared as described previously (Rowhani *et al.*, 2000). Briefly, leaf tissue (250 mg fresh weight) was macerated in filter sterilized extraction buffer (GEB buffer: 1.59 g/l Na2CO3, 2.93 g/l NaHCO3, pH 9.6, 2 % PVP-40, 0.2 % bovine serum albumin, and 0.05 % Tween 20) at a ratio of 1:20 (w/v). The extract was either used immediately or distributed into aliquots which were stored at −80 ºC for subsequent use in PCR. In either case, 2µl of the extract (stored extracts were thawed and mixed before use) was mixed with 25µl of denaturing buffer (GES buffer: 0.1M glycine, pH 9.0, 50mM NaCl, 1mM EDTA, 0.5 % Triton X-100 and 1 % 2-

mercaptoehtanol, which was added just before use), denatured at 95 ºC for 10 min and kept on ice until subsequent use in PCR.

2.3. Primers

In initial experiments, primers ACMV-AL1/F (5′-GCGGAATCCCTAACATTATC) & ACMV-ARO/R (5′-GCTCGTATGTATCCTCTAAGGCCTG) and UV-AL3/F (5′-TACACATGCCTCRAATCCTG) & UVAL1/R2 (5′-CTCCGCCACAAACTTACGTT) designed earlier (Harrison *et al.*, 1997; Zhou *et al.*, 1997) were used for detection by PCR of ACMV and EACMV, respectively. New primers were designed based on multiple alignments of full-length DNA A sequences of ACMV (13 isolates) and EACMV (14 isolates) from cassava and non-cassava hosts available in the NCBI GenBank (Table 1) using the ClustalW multiple sequence alignment program (Vector NTI Advance 10, Invitrogen Corp, Carlsbad, CA). Two sets of primers were identified, one targeting the Rep gene and the other targeting the CP gene of ACMV and EACMCV (Fig. 1). In both cases, the primers were designed so that a single upstream primer represents a conserved sequence in both viruses and two downstream species-specific primers. The primer sets were designed to have similar melting temperatures (T_m) to minimize disproportionate yields of amplification products due to a marked difference in T_m between primers in multiplex PCR assays (Atlas and Bej, 1994). They were analyzed by NetPrimer software (Premier Biosoft, CA, USA) to avoid secondary structure and to prevent the formation of "primer-dimers" during multiplex PCR assays.

Table 1. List of *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus* (EACMV) isolates used to design primers for multiplex PCR

Virus[a]	Country of origin	Specific host	GenBank Accession No.	Position of the primers on the DNA A[b]			
				CMBRep/F	ACMVRep/R	CMBCP/F	ACMCP/R
ACMV							
ACMV-[CM:98]	Cameroon	*Manihot esculenta*	AF112352	1829-1847	2179-2196	304-322	935-955
ACMV-[CM:Mg:98]	Cameroon	*Manihot glaziovii*	AY211884	1833-1851	2183-2200	305-323	939-959
ACMV-[CI:99]	Côte d'Ivoire	*M. esculenta*	AF259894	1833-1851	2183-2200	305-323	939-959
ACMV-[NG]	Nigeria	*M. esculenta*	X17095	1833-1851	2183-2200	305-323	939-959
ACMV-[NG:Ogo:90]	Nigeria	*M. esculenta*	AJ427910	1833-1851	2183-2200	305-323	939-959
ACMV-[NG:Ll:06]	Nigeria	*Leucana leucocephala*	EU685320	1833-1851	2183-2200	305-323	939-959
ACMV-[NG:Mg:03]	Nigeria	*M. glaziovii*	EU685318	1833-1851	2183-2200	305-323	939-959
ACMV-[NG:So:03]	Nigeria	*Senna occidentalis*	EU685322	1833-1851	2183-2200	305-323	939-959
ACMV-[NG:Rc:03]	Nigeria	*Ricinus communis*	EU685324	1832-1850	2182-2199	304-322	938-958
ACMV-[KE:844:82]	Kenya	*M. esculenta*	J02057	1831-1849	2181-2198	303-321	937-957
ACMV-[TZ:01]	Tanzania	*M. esculenta*	AY795982	1834-1852	2184-2201	306-324	940-960
ACMV-[UG:Mld:97]	Uganda	*M. esculenta*	AF126800	1832-1850	2182-2199	304-322	938-958
ACMV-[UG:Svr:97]	Uganda	*M. esculenta*	AF126802	1834-1852	2184-2201	306-324	940-960

[a]Isolated from cassava (*Manihot esculenta*).
[b]Location of primer sequences in the virus genome. The numbering of the genome begins at the conserved nonanucleotide in the hairpin-loop, TAATATTAC, characteristic of the members of the family *Geminiviridae*.

Table 1 (contd.). List of *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus* (EACMV) isolates used to design primers for multiplex PCR

Virus[a]	Country of origin	Specific host	GenBank Accession No.	Position of the primers on the DNA A[b]			
				CMBRep/F	ACMVRep/R	CMBCP/F	ACMCP/R
EACMV							
EACMCV-CM[CM:98]	Cameroon	*M. esculenta*	AF112354	1869-1887	2499-2518	344-362	845-867
EACMCV-CM[CI:98]	Côte d'Ivoire	*M. esculenta*	AF259896	1867-1885	2497-2516	342-360	843-865
EACMCV-CM[NG:Kan]	Nigeria	*M. esculenta*	AJ867444	1867-1885	2497-2516	342-360	843-865
EACMCV-CM[NG:Iba:06]	Nigeria	*M. esculenta*	EU685326	1867-1885	2497-2516	342-360	843-865
EACMCV-CM[NG:Ll:06]	Nigeria	*L. leucocephala*	EU685321	1867-1885	2497-2516	342-360	843-865
EACMCV-CM[NG:Mg:03]	Nigeria	*M. glaziovii*	EU685319	1867-1885	2497-2516	342-360	843-865
EACMCV-CM[NG:So:03]	Nigeria	*S. occidentalis*	EU685323	1867-1885	2497-2516	342-360	843-865
EACMCV-TZ[TZ:1:01]	Tanzania	*M. esculenta*	AY795983	1868-1886	2498-2517	343-361	Variable
EACMV-KE[TZ:Dar:96]	Tanzania	*M. esculenta*	Z83256	1868-1886	2498-2517	343-361	844-866
EACMV-KE[KE:K2B:96]	Kenya	*M. esculenta*	AJ006458	1868-1886	2498-2517	343-361	844-866
EACMV-UG[UG:Mld2:97]	Uganda	*M. esculenta*	AF126804	1865-1883	2495-2514	340-358	Variable
EACMV- UG[UG:Svr2:97]	Uganda	*M. esculenta*	AF126806	1866-1884	2496-2515	341-359	Variable
EACMMV-[MW:K:96]	Malawi	*M. esculenta*	AJ006460	1871-1889	2501-2520	343-361	Variable
EACMZV-[TZ:Ugu:98]	Zanzibar	*M. esculenta*	AF422174	1868-1886	Variable	343-361	844-866

[a]Isolated from cassava (*Manihot esculenta*).
[b]Location of primer sequences in the virus genome. The numbering of the genome begins at the conserved nonanucleotide in the hairpin-loop, TAATATTAC, characteristic of the members of the family *Geminiviridae*.

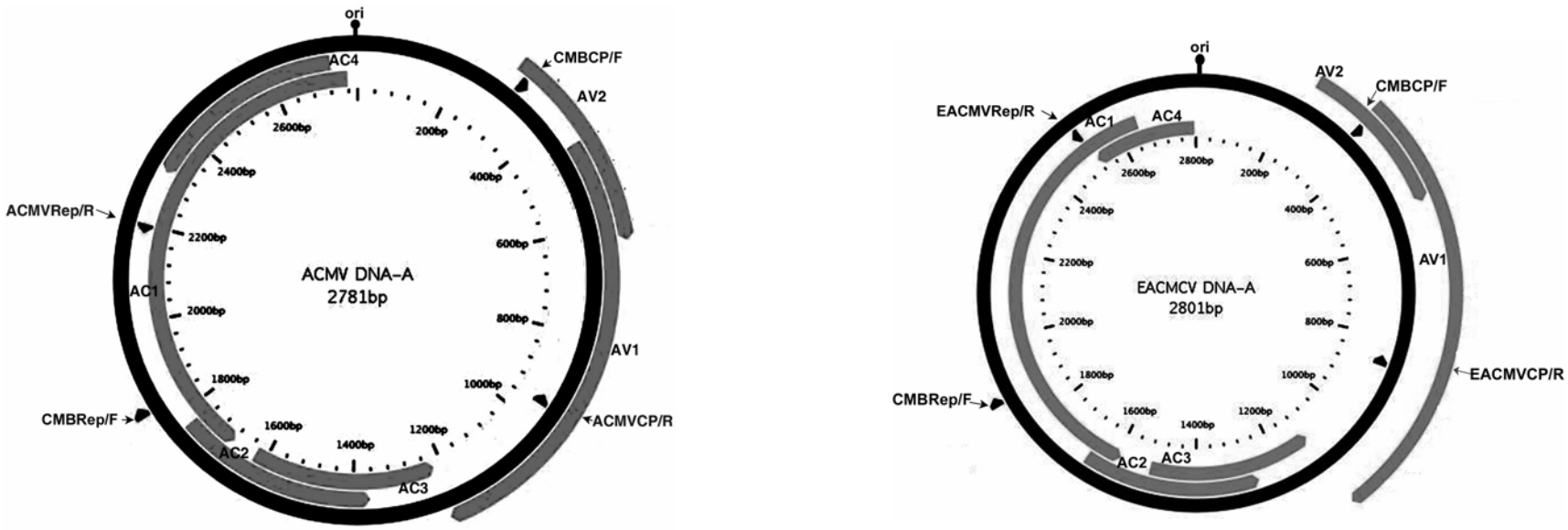

Fig. 1. Circular genomic map of (a) ACMV DNA A (X17095) and (b) EACMCV DNA A (AF112354). The location of replicase (Rep) and coat protein (CP) gene specific primers are shown by arrow heads. The location of different genes encoded by DNA A is indicated by grey arcs. For Rep gene, ACMV-specific primers CMBRep/F (5′-CRTCAATGACGTTGTACCA) and ACMVRep/R (5′-CAGCGGMAGTAAGTCMGA) amplify 368 bp fragment, while EACMCV-specific primers CMBRep/F and EACMVRep/R (5′-GGTTTGCAGAGAACTACATC) amplify 650 bp fragment. For CP gene, ACMV-specific primers CMBCP/F (5′-GKCGAAGCGACCAGGAGAT) and CMVCP/R (5′-CCCTGYCTCCTGATGATTATA) amplify 655 bp fragment while EACMCV-specific primers CMBCP/F and EACMVCP/R (5′-GGTCCCTAWRATCATTCTTCACR) amplify 524 bp fragment.

2.4. Primers for amplification of house keeping gene sequences from cassava

Primers specific to the large subunit of ribulose bisphosphate carboxylase oxygenase (RubiscoL) and NADH dehydrogenase subunit 5 (*nad5*) were made as described by Nassuth *et al.* (2000) and Menzel *et al.* (2002), respectively. The RubiscoL specific primers (RBCL-F535: 5′-CTTTCCAAGGCCCGCCTCA and RBCL-R705: 5′-CATCATCTTTGGTAAAATCAAGTCCA) would amplify DNA fragment of 171 base pairs (bp) and *nad5*-specific primers (nad5-s: 5′-GATGCTTCTTGGGGCTTCTTGTT and nad5-as: 5′-CTCCAGTCACCAACATTGGCATAA) would amplify DNA fragment of 181 bp.

2.5. Uniplex and multiplex PCR

PCR detection of ACMV and EACMCV in cassava samples was carried out using virus-specific primers (ACMV-AL1/F and ACMVARO/R and UV-AL3/F and UV-AL1/R2) as described earlier (Ogbe *et al.*, 2006). The specificity of newly designed CP- and Rep specific primers for uniplex and multiplex PCR detection ACMV and EACMCV were optimized by modulating the assay conditions (concentration of primers, reaction buffer concentration and annealing temperature) using plant DNA as well as leaf extracts. Optimized conditions for multiplex PCR detection of these viruses are listed in Table 2. The PCR amplified products were resolved by agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light using a gel documentation system (Biorad Universal Hood, Biorad Laboratories, Milan, Italy). A 100 bp DNA molecular weight marker (Invitrogen Life Technologies, CA) was run in each gel as a reference to estimate the size of virus-specific DNA band in the PCR amplified products.

Table 2. Components of PCR and cycling conditions for multiplex detection of *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus* (EACMCV)[a]

Template	10X PCR buffer[b]	CMBRep/F	ACMVRep/R	EACMVRep/R	RBCL-F535	RBCL-R705	Annealing temperature[c]
Rep gene specific primers							
DNA[d,f]	2.2X	0.533µM	0.533µM	0.533µM	–	–	48 ºC
Leaf extract[e,f]	1.4X	0.533µM	0.533µM	0.533µM	–	–	48 ºC
DNA[d]	2.2X	0.533µM	0.533µM	0.533µM	0.033µM	0.033µM	56 ºC
Leaf extract[e]	1.4X	0.533µM	0.533µM	0.533µM	0.033µM	0.033µM	56 ºC
Template	**10X PCR buffer[b]**	**CMBCP/F**	**ACMVCP/R**	**EACMVCP/R**	**RBCL-F535**	**RBCL-R705**	**Annealing temperature[c]**
CP gene specific primers							
DNA[d,f]	1X	0.533µM	0.4µM	0.533µM	–	–	52 ºC
Leaf extract[e,f]	1X	0.533µM	0.4µM	0.533µM	–	–	52 ºC
DNA[d]	1X	0.533µM	0.4µM	0.533µM	0.067µM	0.067µM	52 ºC
Leaf extract[e]	1X	0.533µM	0.4µM	0.533µM	0.067µM	0.067µM	52 ºC

[a]PCR components are based on a total reaction volume of 15µl, consisting of 0.8U of Taq polymerase and 0.2mM of each dNTP. All components with the exception of primers were manufactured by Roche Applied Science (Roche Applied Sciences, IN, USA).
[b]10X buffer consists of 100 mM Tris–HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3.
[c]Cycling conditions were: one cycle consisting of denaturation at 94 ºC for 1min, annealing at 48/52/56 ºC for 30 s and extension at 72 ºC for 1min, followed by 36 cycles with each cycle consisting of denaturation at 94 ºC at 1 min, annealing at 48/52/56 ºC for 30 s and extension at 72 ºC for 1min and a final extension at 72 ºC for 5min.
[d]DNA isolated from cassava leaves according to Dellaporta *et al.* (1983).
[e]DNA template prepared according to Rowhani *et al.* (2000) and described in Section 2.2.
[f]Multiplex PCR without house keeping gene sequences as internal control.

2.6. Cloning and sequencing of PCR-amplified products

To confirm the specificity of DNA bands amplified by multiplex PCR, the amplicons from select samples were cloned separately into a plasmid vector (pCR2.1, Invitrogen Corp., Carlsbad, CA). Three independent clones specific for each DNA fragment were sequenced from both orientations using M13 universal forward and reverse primers. The sequences were verified by BLAST search (NCBI, Bethesda, MD) to confirm the specificity of PCR amplified DNA fragments.

3. RESULTS

3.1. Specificity of the new primers for the detection of ACMV and EACMCV

DNA extracts from CMD-affected cassava leaf tissue were tested by PCR for the presence of ACMV and EACMCV using primer pairs ACMV-AL1/F and ACMV-ARO/R for ACMV and UV-AL3/F and UVAL1/R2 for EACMCV (Harrison *et al.*, 1997; Zhou *et al.*, 1997). Samples that were positive for the two viruses were used to optimize multiplex PCR conditions using the new primers. The primer pair CMBRep/F and ACMVRep/R amplified 368 bp fragment specific to ACMV and the primer pair CMBRep/F and EACMVRep/R amplified 650 bp fragment specific to EACMCV from DNA extracts prepared from CMD-affected leaves (Fig. 2a). Similarly, the primer pair CMBCP/F and ACMVCP/R amplified 655 bp fragment and the primer pair CMBCP/F and EACMVCP/R amplified 524 bp fragment specific for ACMV and EACMCV, respectively, from the same DNA extracts used for testing Rep-specific primers (Fig. 2b). The nucleotide sequence of these amplicons showed 98 % and 99 % sequence identity with respective sequences of ACMV (GenBank Accession No. X17095) and EACMCV (GenBank Accession No. AF112354). These results ascertain specificity of the newly designed primers in

amplifying Rep- and CP-specific sequences of ACMV and EACMV from CMD-affected cassava leaves.

3.2. Comparative assessment between newly designed primers and primers used previously

A comparison was made between primers designed earlier (Harrison *et al.*, 1997; Zhou *et al.*, 1997) with the primers designed in this study for the detection of ACMV and EACMCV. As shown in Fig. 3, 8 of the 16 samples tested positive for ACMV using primer pair ACMV-AL1/F and ACMV-ARO/R, whereas 10 of the 16 samples were positive for ACMV using the primer pairs CMBRep/F and ACMVRep/R or CMBCP/F and ACMVCP/R. Primers pair UV-AL3/F and UV-AL1/R2 detected EACMCV in two of the 16 samples, whereas the primer pair CMBRep/F and EACMVRep/R detected the virus in three samples and CMBCP/F and EACMVCP/R primer pair detected the virus in two samples. However, UV-AL3/F and UV-AL1/R2 primers gave non-specific products in many of the samples tested when compared to the results obtained with Rep- or CP-specific primers. This could be due to considerable degeneration of UV-AL3/F and UV-AL1/R2 primers. These results suggest that the new primers are more reliable for the detection of ACMV and EACMCV.

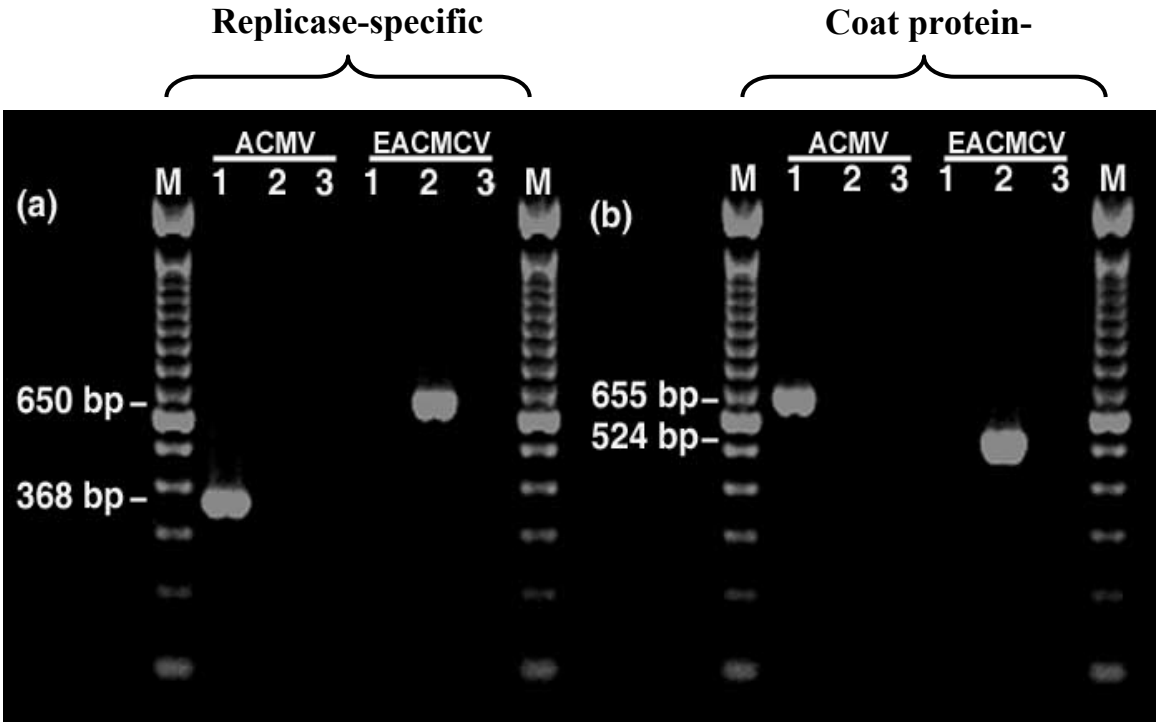

Fig. 2. Specificity of primers in amplifying (a) replicase- and (b) coat protein-specific DNA fragments from samples with mixed infections of ACMV and EACMCV. DNA fragments specific to ACMV (lane 1) and EACMCV (lane 2) were amplified in the presence of ACMV-specific primer pairs CMBRep/F & ACMVRep/R and CMBCP/F & ACMVCP/R and EACMCV-specific primer pairs CMBRep/F & EACMVRep/R and CMBCP/F & EACMVCP/R. No bands were amplified in healthy samples (lane 3). Lane M represents 100 bp DNA ladder (Invitrogen). The sizes of virus-specific DNA fragments are indicated on the left hand side.

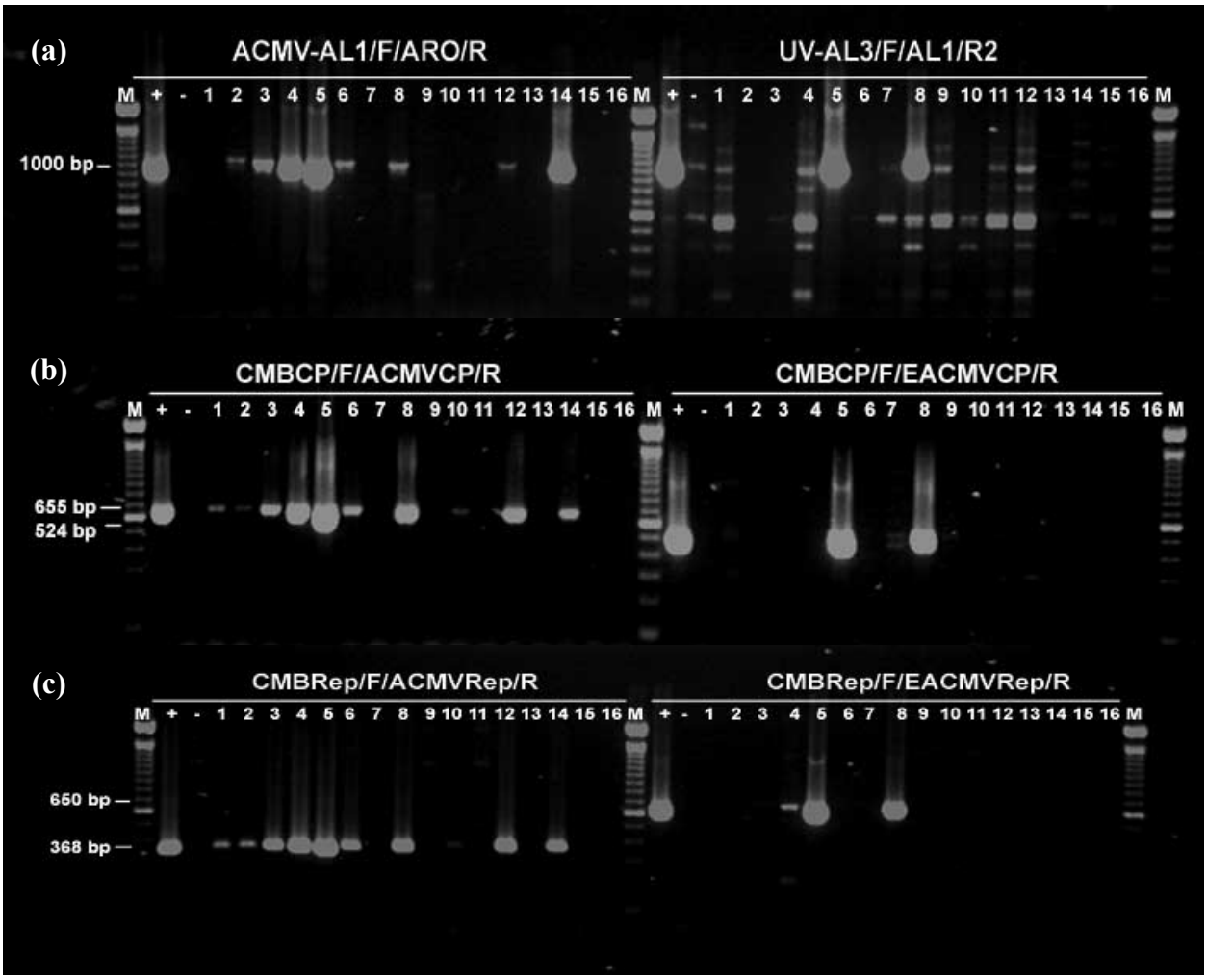

Fig. 3. Comparative advantage of new ACMV- and EACMCV-specific primers (b and c) over the primers designed previously (a). Virus-specific DNA fragments from virus infected cassava (lane +), healthy cassava (lane -) and field samples (lanes 1-16) were amplified in the presence of specific primer pairs indicated in the figure. Lane M represents 100 bp DNA ladder (Invitrogen). The sizes of virus-specific DNA fragments are indicated on the left hand side.

3.3. Specificity of primers for amplification of house keeping gene sequences in cassava

The reliability of multiplex PCR detection of ACMV and EACMCV in field-grown cassava plants can be influenced by the presence of inhibitory compounds, especially phenolic and polysaccharides compounds in sample preparations. In order to avoid false negative results by diagnostic PCR due to interference from such inhibitors, RubiscoL- and nad5-specific primers were tested for their ability to amplify gene-specific DNA from cassava. Primers RBCL-H535 and RBCL-C705amplified 171 bp DNA fragment from cassava, grapevine and tobacco samples in both RT-PCR and PCR (Fig. 4). In contrast, primers nad5-s and nad5-as amplified 181 bp fragment from all samples in RT-PCR but not in PCR (Fig. 4). These results indicate that both RubiscoL- and nad5-specific fragments can be amplified in RT-PCR and only RubiscoL-specific fragments can be amplified in PCR using extracts from cassava, grapevine and tobacco. Sequences of two independent clones of RubiscoL-specific amplicons from cassava showed 98 % identity with corresponding sequence of *Manihot esculenta* chloroplast RubiscoL (GenBank accession no. AB233880) confirming the specificity of amplified DNA fragments.

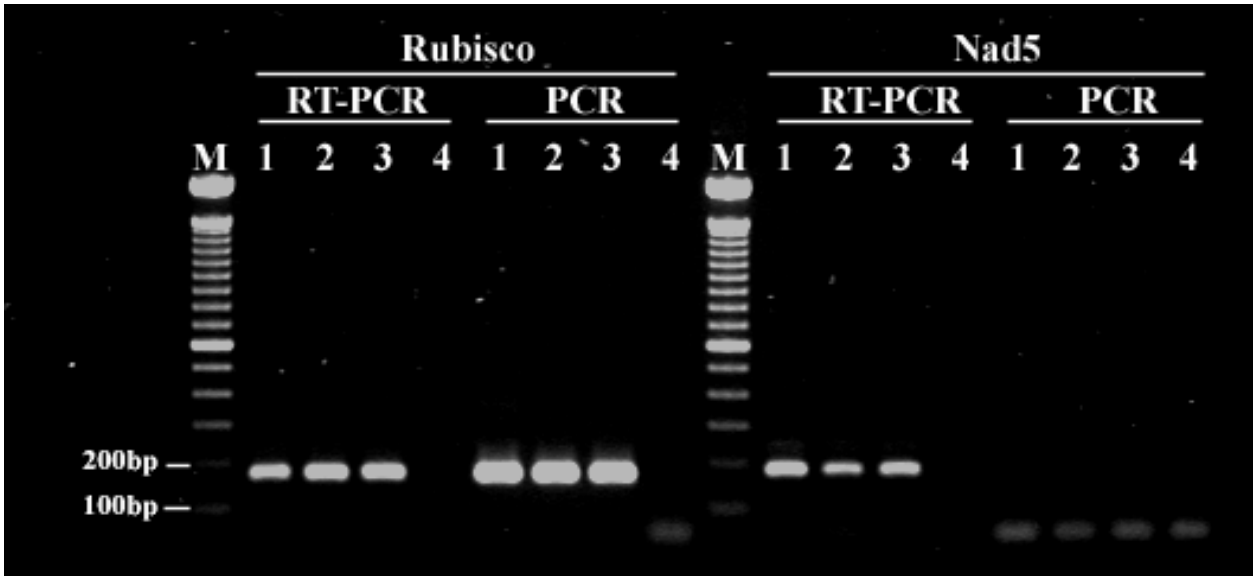

Fig. 4. Amplification of RubiscoL and *nad5* gene sequences by RT-PCR and PCR. DNA extracts from cassava (lane 1), grapevine (lane 2) and *Nicotiana benthamiana* (lane 3) were used to amplify RubiscoL- and nad5-specific fragments using primer pairs RBCL-F535 and RBCL-R705 and nad5-s and nad5-as, respectively. Lane 4 represents control with no sample. Lane M represents 100 bp DNA ladder (Invitrogen). The sizes of the DNA ladder are indicated on the left hand side.

3.4. Multiplex PCR detection of ACMV and EACMCV in plant DNA extracts from CMD-affected cassava leaves

Fig. 5a shows a comparison of uniplex and multiplex PCR detection of ACMV and EACMV in plant DNA extracts prepared from cassava leaves infected with one or both viruses. The primer pair CMBRep/F and ACMVRep/R amplified a 368 nt fragment specific to ACMV (lanes 1 and 5) and the primer pair CMBRep/F and EACMVRep/R amplified a 650 nt fragment specific to EACMCV (lanes 3 and 5) in both uniplex and multiplex formats. Distinct sizes enabled the differentiation of virus-specific DNA bands by agarose gel electrophoresis. No such bands were amplified from healthy samples (lanes 2, 4 and 6). These results showed specificity of Rep specific primers in simultaneous detection of ACMV and EACMCV. Amplification of 171 nt DNA fragment specific to the RubiscoL gene in both healthy and virus-infected samples indicated reliability of the PCR. Multiplex PCR was then validated for the detection of ACMV and EACMCV in DNA extracts made from cassava leaf samples with or without symptoms collected from farmers' fields in Nigeria. As shown in Fig. 5a, lanes 7–28, both ACMV and EACMCV were detected in 16 of the 22 symptomatic leaves and ACMV alone in three symptomatic leaves revealing mixed infection in a majority of the field samples. Three asymptomatic samples tested negative for both viruses. Concurrent detection of the 171 nt fragment of the RubiscoL in all these samples shows the specificity and reliability of the PCR results. Similar results were obtained when primers specific for RubiscoL were not included in the multiplex PCR (Fig. 5b). CP-specific primer pairs CMBCP/F and ACMVCP/R and CMBCP/F and EACMVCP/R amplified a 655 nt and 524 nt fragment specific to ACMV (Fig. 6a, lanes 1 and 5) and EACMCV (Fig. 6a, lanes 3 and 5), respectively, either in uniplex or multiplex formats. The CP specific primers detected both viruses in 13 of the 22 symptomatic

samples and ACMV alone in six symptomatic samples and none in three asymptomatic leaves (lanes 7–28). Concurrent detection of the 171 bp fragment specific to RubiscoL in all samples validated the specificity and reliability of PCR results. Similar results were obtained when primers specific for RubiscoL were not included in the multiplex PCR (Fig. 6b). The above results indicate that both Rep-specific primers (Fig. 5) and CP-specific primers (Fig. 6) can be used for uniplex or multiplex PCR detection of ACMV and EACMCV in cassava. However, Rep-specific primers amplified both viruses with similar efficiency, as shown by equal intensity of DNA bands. In contrast, ACMV specific band was over amplified when compared to amplification of EACMCV-specific band with CP-specific primers. In addition, CP-specific primers amplified non-specifically high mol. wt. DNA bands in some samples (Fig. 6, lanes 5 and 7). Furthermore, a 286 bp difference between ACMV- and EACMCV-specific DNA fragments obtained with Rep-specific primers makes better discrimination of the two viruses in agarose gel electrophoresis as opposed to a difference of 131 bp between virus-specific DNA fragments obtained with the CP-specific primers. These results indicate a comparative advantage of Rep-specific primers over CP-specific primers in multiplex PCR detection of ACMV and EACMCV.

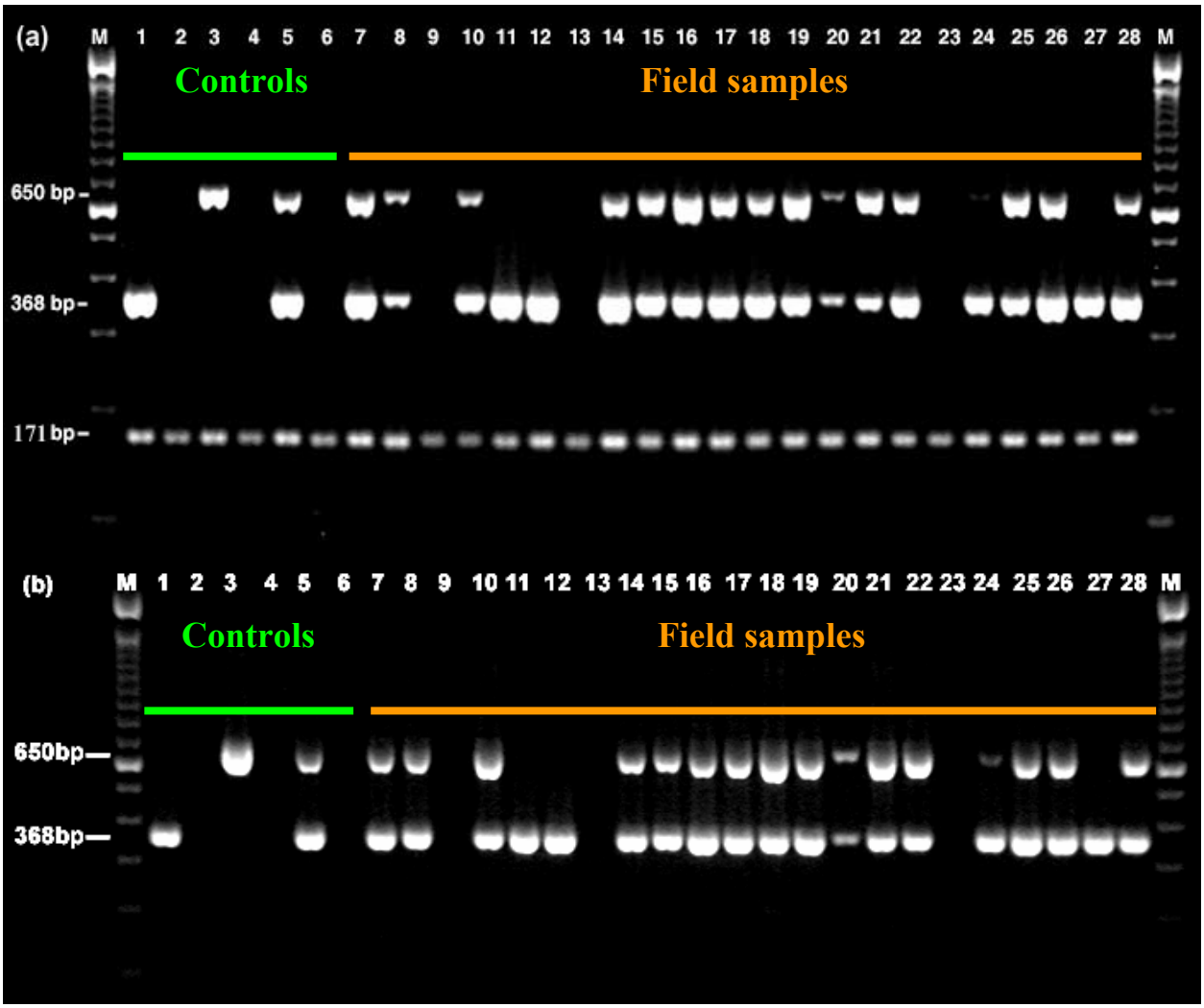

Fig. 5. Multiplex PCR detection of ACMV and EACMCV using replicase gene-specific primers. DNA extracted from cassava leaves were tested by uniplex (lanes 1-4) and multiplex (lanes 5-28) PCR using virus-specific primers in the presence (a) and absence (b) of RubiscoL-specific primers as an internal control. Lanes 1, 3, and 5 represent DNA from ACMV and EACMCV infected leaves; lanes 2, 4, and 6 represent DNA from healthy leaves, and lanes 7-28 represent DNA from field-collected leaf samples exhibiting CMD symptoms. Lane M shows 100 bp DNA ladder (Invitrogen). DNA bands specific to ACMV (368 bp), EACMCV (650 bp) and RubiscoL (171 bp) are indicated on the left hand side.

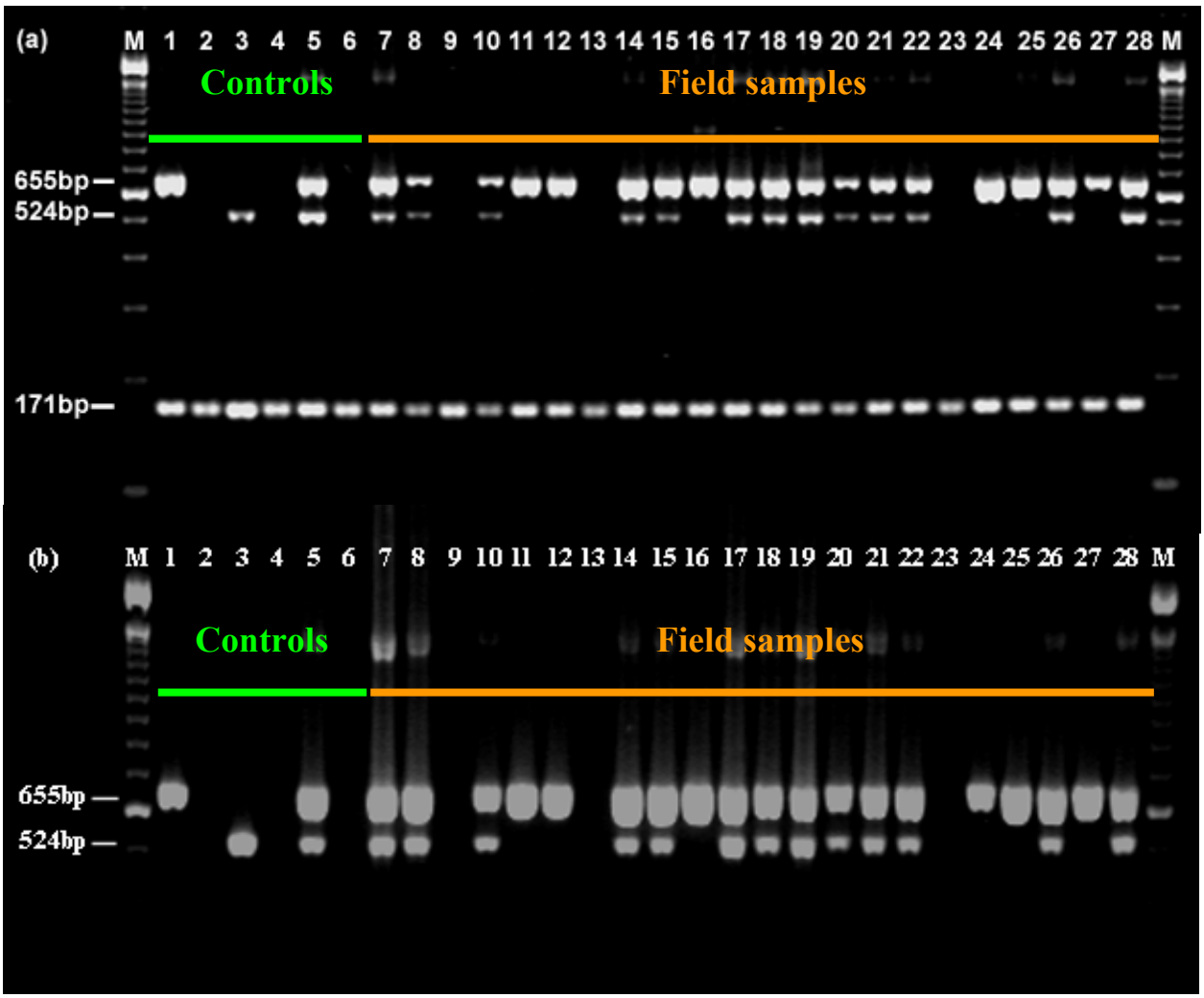

Fig. 6. Multiplex PCR detection of ACMV and EACMCV using coat protein-specific primers. DNA extracted from cassava leaves were tested by uniplex (lanes 1-4) and multiplex (lanes 5-28) PCRs using virus-specific primers in the presence (a) and absence (b) of RubiscoL-specific primers as an internal control. Lanes 1, 3, and 5 represent DNA from ACMV and EACMCV infected leaves; lanes 2, 4, and 6 represent DNA from healthy leaves, and lanes 7-28 represent DNA from field-collected leaf samples exhibiting CMD symptoms. Lane M shows 100 bp DNA ladder (Invitrogen). DNA bands specific to ACMV (655 bp), EACMCV (524 bp) and RubiscoL (171 bp) are indicated on the left hand side.

3.5. Validation of a simplified sample preparation method for multiplex PCR detection of ACMV and EACMCV

DNA extracted from cassava leaves are used in many laboratories as a template for PCR diagnosis of CMBs. Since this method involves several steps for DNA preparation, a simple and rapid sample preparation method was sought as an alternative to multistep plant DNA preparation protocol for use in multiplex PCR detection of ACMV and EACMCV. In initial experiments, multiplex PCR conditions described in Section 2.5 failed to amplify virus-specific DNA bands when denatured leaf extracts were used as a template in place of DNA extracts. Therefore, conditions were optimized against a range of buffer concentrations and annealing and extension temperatures to achieve amplification of virus-specific DNA bands by multiplex PCR. Decreasing the buffer concentration to 1X with other parameters being similar to the protocol in Section 2.5 gave satisfactory amplification of ACMV and EACMCV with denatured leaf extracts, as opposed to higher buffer strength when DNA was used as a template to achieve comparable results (data not shown). Using these optimized conditions, the Rep- and CP-specific primers amplified DNA fragments of expected size specific to ACMV and EACMCV from extracts made from virus-infected but not healthy leaves in either uniplex or multiplex PCR (Fig. 7). The usefulness of sample extraction method in multiplex PCR detection of these viruses was validated using CMD-affected cassava leaves collected from farmers' fields in Nigeria. As shown in Fig. 7, lanes 7-16, five samples revealed the presence of mixed infection of ACMV and EACMCV and another five samples revealed single infection of ACMV. The amplification of 171 bp DNA band specific to RubiscoL indicates the reliability of multiplex PCR for diagnosing ACMV and EACMCV in single and mixed virus infections in field samples.

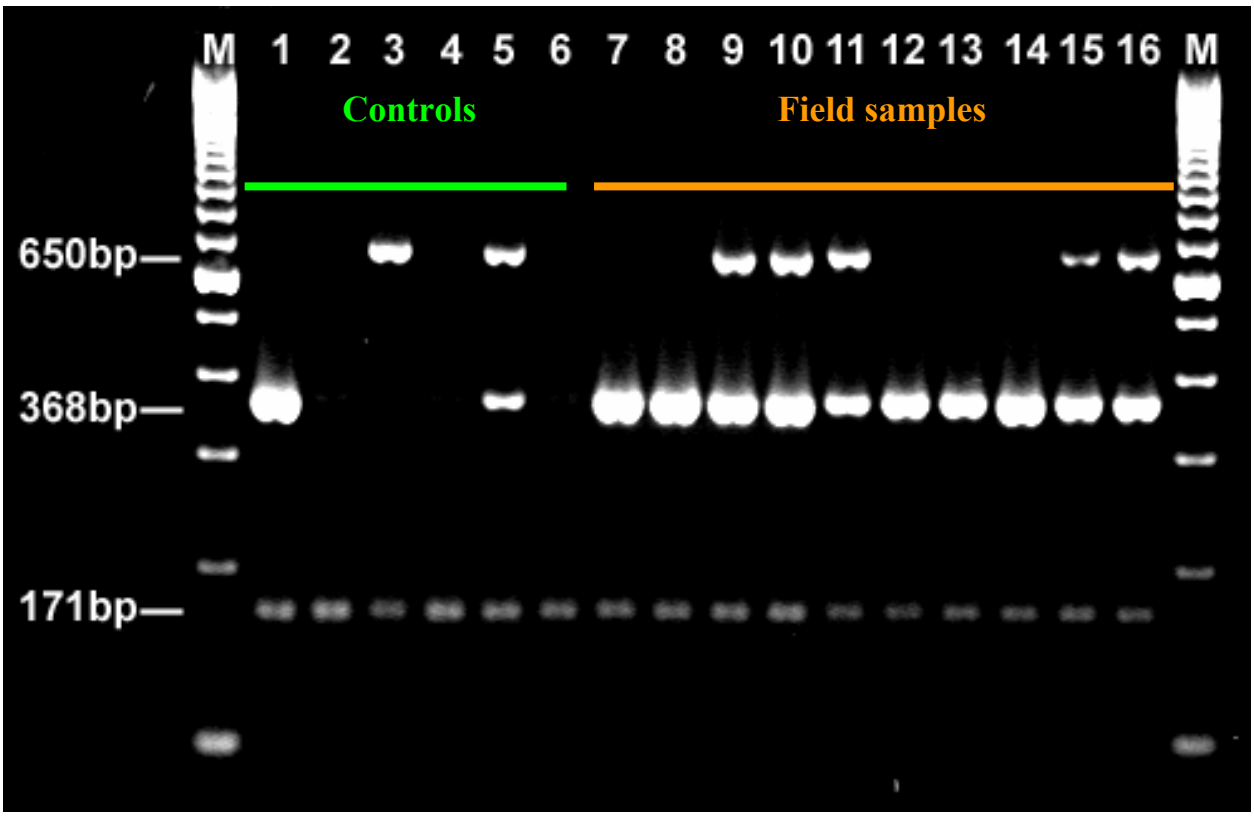

Fig. 7. Multiplex PCR detection of ACMV and EACMCV in extracts prepared from field-collected cassava leaves exhibiting CMD symptoms. The leaf extracts were tested by uniplex (lanes 1-4) and multiplex (lanes 5-16) PCR using Rep-specific primers in the presence of RubiscoL-specific primers as an internal control. Lanes 1, 3, and 5 are extracts from ACMV and EACMCV infected leaves; lanes 2, 4, and 6 are extracts from healthy leaves, and lanes 7-16 are extracts from CMD-affected cassava leaves from farmers' fields in Nigeria. Lane M represents 100 bp DNA ladder (Invitrogen). The sizes of ACMV-, EACMCV- and RubiscoL-specific DNA bands are indicated on the hand left.

3.6. Comparison of sensitivity between uniplex and multiplex PCR

In multiplex PCR, competition between different primers could influence the sensitivity and efficiency of amplification of the target molecules. To address this issue, a 10-fold serial dilution of sample extracts were made and an aliquot from each dilution was simultaneously tested in uniplex and multiplex PCR for amplifying viral DNA. As shown in Fig. 8a, ACMV was consistently detected in sample dilutions up to 10^{-4} and EACMCV (Fig. 8b) was detected in sample dilutions up to 10^{-5} in uniplex PCRs. In multiplex PCRs (Fig. 8c), both viruses were detected in sample dilutions up to 10^{-4}. These results indicate that multiplex PCR is equally sensitive compared with uniplex PCR.

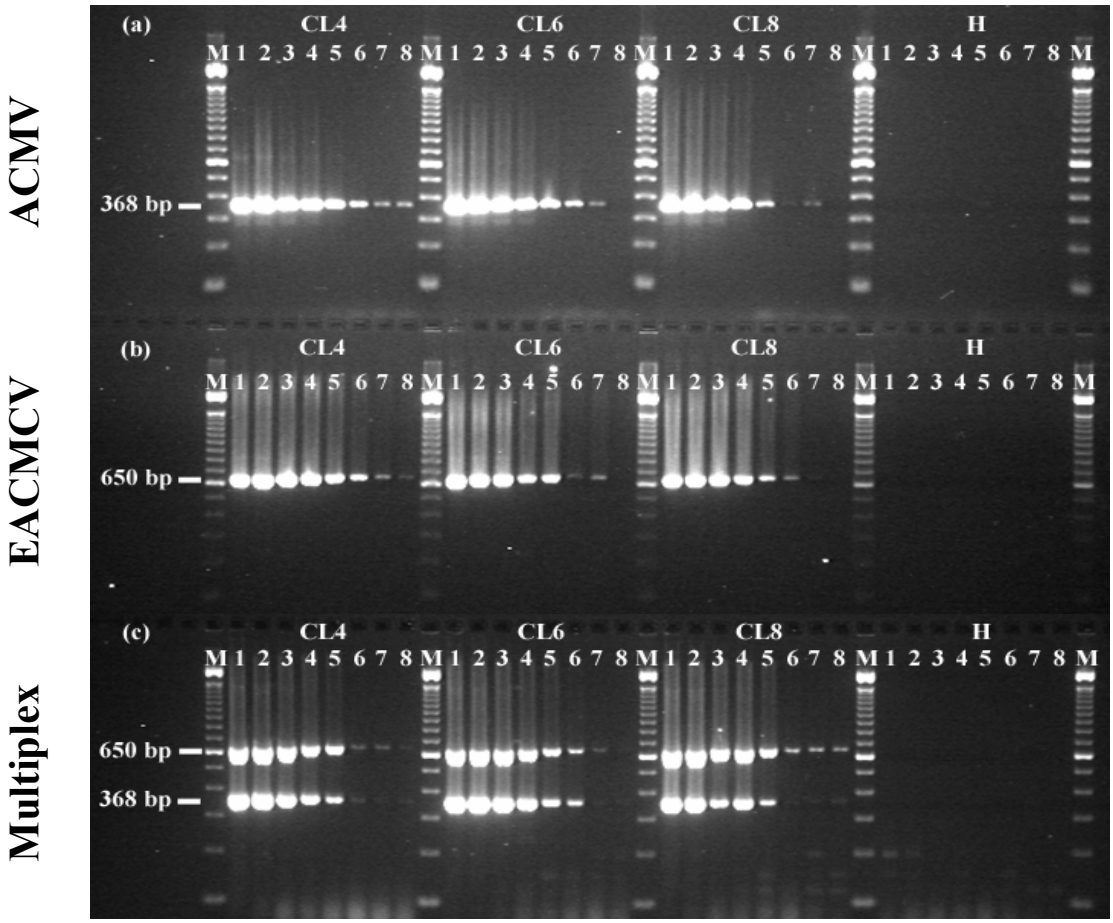

Fig. 8. A comparison of sensitivity between uniplex and multiplex PCRs for the detection of (a) ACMV, (b) EACMCV and (c) both viruses together in extracts prepared from leaves exhibiting CMD symptoms. A 10-fold serial dilution of sample extracts from CMD-affected (CL4, CL6, and CL8) and healthy (H) leaves were made for virus detection. Lanes 1-8 contain 10-fold serial dilution of each sample, with lane 1 representing original extract. LaneMrepresents 100 bp DNA ladder (Invitrogen). The sizes of ACMV- and EACMCV- specific DNA bands are shown on the left hand side.

4. DISCUSSION

This study describes the development of a multiplex PCR for rapid detection of ACMV and EACMCV in CMD-affected cassava leaves. The results indicated that Rep-specific primers gave better results with balanced intensity of DNA fragments amplified for each virus relative to multiplex PCR based on CP-specific primers (Figs. 5 and 6). Thus, Rep-specific primers are more versatile in multiplex PCR detection of ACMV and EACMCV using either plant DNA (Fig. 5) or leaf extracts (Fig. 7). A 'degenerate' upstream primer and two virus-specific downstream primers designed in this study permitted the use of less number of primers for PCR amplification of DNA fragments of distinct size thereby allowing easy discrimination of the two viruses by agarose gel electrophoresis. Although this study focused only on two viruses, the usefulness of multiplex PCR can be extended for the detection of other CMBs in cassava by a combination of a common upstream primer based on conserved sequences in the majority of CMBs and species-specific downstream primer such that different size products amplified in multiplex PCR assay would allow discrimination of each virus thereby.

The uniplex and multiplex PCRs using Rep-specific primers produced similar results for the detection of ACMV and EACMCV (Fig. 8) thus underscoring the benefits (economy in resource utilization and time taken for conducting the assays) of multiplex PCR detection of the two viruses. Another advantage is that these primers offer improved virus diagnosis without non-specific bands (Fig. 3), when compared to primers that have been designed previously (Zhou *et al.*, 1997). This could be due to high sequence identity of Rep specific primers designed in this study. In contrast to HMA used for the discrimination of CMBs and their strains (Berry and Rey, 2001), the multiplex PCR method provides simplicity facilitating large scale screening of field-collected cassava samples in a relatively short period of time.

Multiplex RT-PCR has been used for the detection of a range of plant viruses in plants, seed and insect vectors (Bariana *et al.*, 1994; Deb and Anderson, 2008; Du *et al.*, 2006; He *et al.*, 2006; Menzel *et al.*, 2002; Nassuth *et al.*, 2000). In many of these assays, co-amplification of a house keeping gene(s) of host origin has been used as an internal control for reliable detection of plant viruses. Previously, a considerable number of studies demonstrated that PCR can be used for single virus detection in cassava leaves (Fondong *et al.*, 2000; Ndunguru *et al.*, 2005; Ogbe *et al.*, 2003, 2006; Okao-Okuja *et al.*, 2004; Pita *et al.*, 2001; Sseruwagi *et al.*, 2004a,b). In all these assays, no house-keeping gene sequences were included as an internal control making the interpretation of negative results difficult. Our results indicate that RubiscoL primers would be a useful internal control for reliable detection of ACMV and EACMCV in cassava samples (Fig. 4). As reported by Menzel *et al.* (2002), the nad5 primers were designed for the specific amplification of mRNA of the mitochondrial *nad5* gene. This explains the amplification of nad5-specific fragment only in the presence of reverse transcriptase. A lack of amplification of specific fragment using primers designed for the detection of plant mRNAs encoding malate dehydrogenase (MDH), in the presence of *Taq* polymerase was also observed in a wide range of plant species (Nassuth *et al.*, 2000). Since CMBs are DNA viruses, the use of RubiscoL-specific primers as internal control makes it suitable for reliable interpretation of results in the detection of ACMV and EACMCV.

Considering that the number of steps from sample preparation to combined detection of two different viruses is reduced, the risk of cross-contamination leading to false positive results is minimized by multiplex PCR. The simplified sample extraction method that we have adapted for cassava has improved the practical advantages of multiplex PCR. The method allows reliable detection of ACMV and EACMCV in 1 day (approximately 2 h of processing of about 50

samples, 3 h of multiplex PCR, 2.5 h of electrophoresis) whereas conventional PCR requires a minimum of 2 days for DNA extraction using traditional method (Dellaporta *et al.*, 1983) and uniplex PCR detection in the same number of samples. Thus, a quick 'turnaround' time makes the multiplex PCR well suited for processing a large number of cassava samples in a relatively short period of time. Due to its simplicity, the multiplex PCR assay we have developed permits high throughput diagnosis of CMBs in field samples in many laboratories in Africa.

The multiplex PCR developed in this study can be used for strategic epidemiological studies like relation between mixed infections of ACMV and EACMCV and the temporal pattern of disease spread by the whitefly vector and to develop control strategies against CMD in cassava-growing regions in Africa. The assay has the ability to detect and discriminate geographically diverse isolates of ACMV and EACMCV as well as other CMBs reported from Sub-Saharan Africa. The limitation of the multiplex PCR assays described in this study, however, is its inability to detect recombinant CMBs like EACMZV with Rep-specific primers, EACMV-UG2 with CP-specific primers and SACMV with both primers due to sequence variation in the region selected for designing the PCR primers. With the availability of new sequence information on different CMBs and their variants, additional primer(s) can be designed to improve the robustness of the assay for virus detection in plants. In addition, primers CMBRep/F and ACMVRep/R have sequence identities in Rep gene of *Indian cassava mosaic virus* (ICMV, GenBank accession no. NC 001932) to amplify a 365 bp fragment, thereby extending the potential of multiplex PCR for the detection of CMBs outside continental Africa.

In conclusion, the multiplex PCR in conjunction with a simplified sample preparation method is reliable, rapid, sensitive, specific and cost-effective for the diagnosis of CMBs in cassava plants. The assay is versatile since it can also be used for single virus detection using

virus-specific primers. Consequently, this method is suitable for a wide variety of applications in many African countries for reliable assessment of the prevalence of CMBs in epidemiological studies and for crop improvement, quarantine, eradication and certification programs.

ACKNOWLEDGEMENTS

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CHAPTER 5

GRAPEVINE AND THE RUGOSE WOOD DISEASE COMPLEX

Wine grapes and their importance to the Pacific Northwest region of the USA

Grapevine (*Vitis* spp.) is the most widely cultivated fruit crop worldwide, encompassing about 8 million hectares of arable land (Vivier and Pretorius, 2002) with about 67,200 kilotons produced in 2007 (FAOSTAT, 2009). Since their first appearance over 65 million years ago in Eurasia (Mediterranean region, central Europe, and southwestern Asia; de Saporta, 1879), grapes from the species, *V. vinifera* L., have been used extensively for making wine throughout the world (This *et al.*, 2006). Other members of the family *Vitaceae* used in wine grape breeding programs, especially for breeding rootstocks and inter-specific hybrids, include *Muscadinia rotundifolia*, *V. aestivalis*, *V. amurensis*, *V. berlandieri*, *V. candicans*, *V. caribaea*, *V. champinii*, *V. cinerea*, *V. cordifolia*, *V. labrusca*, *V. longii*, *V. riparia*, *V. rupestris* and *V. simpsonii* (This *et al.*, 2006). Wine, the main product of wine grapes, has been made for millennia (Clarke and Rand, 2001). Besides their economic importance as alcoholic beverages, wines also have ancient historical connections with the development of human culture (McGovern, 2004). In recent years, the health benefits of wine consumption in moderate amounts have been recognized (German and Walzem, 2000) thus reinforcing the so-called 'French Paradox' phenomenon (Renaud and de Lorgeril, 1992; 1993).

The United States is ranked 4th internationally in grape production and accounts for 10 % of the world's production on 5 % of land area. In the U.S., grape production is the 6th most economically important crop behind corn, soy, wheat, cotton and tomatoes (FAOSTAT, 2009).

Although the grape berry is used for multiple purposes, wine and distilled liquor produced from cultivars of *V. vinifera* have the highest economic value (Mullins *et al.*, 1992). In 2005, the economic value of wine production, grapes, grape products and their related industries to the American economy was put at $162 billion besides employing over half a million people (MKF Research LLC, 2007).

Winemaking in Oregon (OR) and Washington (WA), the two leading wine grape producers in the Pacific Northwest (PNW) region of the United States of America (USA), dates back to 1825 as a consequence of the influx of European immigrants and settlers. Although Idaho is often considered a 'new frontier' of wine grape growing areas in the U.S., historical anecdotes indicate that the first set of wineries in the PNW was located in this state (http://www.idahowines.org/winehistory.cfm). However, growth of the industry in PNW was slowed down during the prohibition era of the 1920's. In recent decades, the industry has expanded in the region. Currently, wine grapes are grown in about 14,000 hectacres (ha) in Washington, about 6,000 ha in Oregon and about 800 ha in Idaho. Wine grapes are largely grown as own-rooted vines in Washington and Idaho and on rootstocks in Oregon. Currently, Washington and Oregon rank second and fourth largest producers of premium wine in the U.S., respectively. Washington produces 5 % of the nation's total wine, juice, and table grapes based on both tonnage and hectarage (WASS, 2008).

The wine industry in the PNW contributes significantly to the regional economy. For example, the wine industry in Washington State contributes $3 billion plus to the State's economy and has a national economic impact of $4.7 billion per year (www.washingtonwine.org; MKF Research LLC, 2007). Wine grape bearing acreage was 32,000 in 2008, and production in 2008 totaled 145,000 tons, a 14 percent increase from 2007

and 21 percent above 2006 (www.usda.gov/nass/). Growers received a record high average of $1,030 per ton for all varieties in 2008, up $76.00 from 2007. Of the total wine grapes produced in 2008, 48 percent were red-fruited varieties and 52 percent were white-fruited varieties. The top four varieties of wine grapes (white varieties: Chardonnay and White Riesling; Red varieties: Cabernet Sauvignon and Merlot) accounted for 74 percent of Washington's production. The top four varieties of wine grapes (white grapes: Chardonnay and White Riesling; Red grapes: Cabernet Sauvignon and Merlot) accounted for 75 % of the state's total production.

The rapid expansion of the wine grape industry within the past two decades has predisposed the viticultural enterprise to several debilitating virus diseases. Due to the negative impact of viruses: decreased lifespan of vineyards, reduced yield of grapevines, and delayed ripening and poor quality of grapes, the Washington State Wine Advisory Committee ranked virus diseases as one of the high priority areas for research and development in their 2008 Viticulture Research Survey. Because grapevines are vegetatively propagated, spread of many debilitating viruses occurs through cuttings resulting in economic losses to growers. Since virus diseases cannot be controlled by economically feasible chemical agents similar to fungicides and bactericides, strategies aimed at the management of grapevine viruses are largely directed at preventing virus spread by utilizing virus-tested planting material.

Virus diseases of the grapevine

On a worldwide basis, the grapevines appear to be infected with more viruses than any other perennial woody species (Martelli, 1993; Walter and Martelli, 1996). Currently, the International Council for the Study of Virus and Virus-like Diseases of the Grapevine (ICVG) recognized about 60 viruses belonging to 20 different genera (Martelli, 2000; Martelli, 2003;

Martelli and Boudon-Padieu, 2006). The 'traditional' virus diseases such as fanleaf, leafroll, ruguose wood and fleck represent a group of well-known disorders in several grape-growing countries around the world (Hewitt, 1954; Walter and Martelli, 1996; Martelli and Walter, 1998; Martelli, 1999; Krake *et al.*, 1999), while many of the other virus and virus-like disorders are of limited geographic distribution. The etiology of many of these diseases remains largely unsolved.

Rugose Wood disease complex

The ICVG currently groups all graft-transmissible disorders of the woody trunk under the name Rugose Wood (RW) disease complex, one of the major disease complexes of the grapevine. RW complex derived its name from the Latin word rūgōsus (from rūga, wrinkle) that describes the characteristic rough, wrinkled surface of the woody cylinder of affected grapevines. The woody cylinders of affected vines are typically marked by pits and/or grooves (Fig. 1). These alterations may occur on the scion, rootstock or both according to the cultivar and rootstock combination. Since its first report over 40 years ago from southern Italy and its description as a graft-transmissible disease of grapevine (Graniti *et al.*, 1965), RW complex has been found to have a worldwide distribution (Martelli, 1993; Martelli and Boudon-Padieu, 2006; Minafra and Boscia, 2003).

RW complex is categorized into four disorders based on the type of symptoms elicited on specific indicator hosts (Table 1; Fig. 1). They are Rupestris stem pitting (RSP), Kober stem grooving (KSG), LN33 stem grooving (LNSG) and Corky bark (CB). All four disorders elicit similar symptoms in *V. vinifera* cultivars and cannot be readily distinguished in the field. In general, affected vines may be dwarfed and less vigorous than normal and may have delayed bud

opening in spring. Some vines decline and die within a few years after planting (Savino *et al.*, 1985; Credi *et al.*, 1991; Credi and Babini, 1996; Tomažič *et al.*, 2005).

At least four distinct phloem-limited viruses belonging to two genera in the family *Flexiviridae* (Adams *et al.*, 2005) have been found associated with the RW complex (Martelli and Boudon-Padieu, 2006). They include *Grapevine rupestris stem pitting-associated virus* (GRSPaV; genus *Foveavirus*; Martelli & Jelkmann, 1998) and four members of the genus *Vitivirus* namely *Grapevine virus A* (GVA; Conti *et al.*, 1980), *Grapevine virus B* (GVB; Bonavia *et al.*, 1996) *Grapevine virus C* (GVC, Monette and James, 1991) and *Grapevine virus D* (GVD; Abou-Ghanem *et al.*, 1997). However, in a recent study (Masri *et al.*, 2006), GVC was shown to be serologically related to *Grapevine leafroll-associated virus-2* (GLRaV-2) and suggested to be either closely related to or the same virus as the latter. More recently, another virus was characterized from an apparently healthy Japanese table grape *V. labrusca* cultivar Pione and was tentatively named *Grapevine virus E* (GVE; Nakaune *et al.*, 2008), although there is no biological evidence that GVE is associated with RW complex. GVA was consistently found associated with KSG (Garau *et al.*, 1994), GVB with CB (Bonavia *et al.*, 1996) and GRSPaV with RSP (Zhang *et al.*, 1998). Recently, GRSPaV was also found to be associated with veinal necrosis symptom on 110 Richter rootstock (*Vitis rupestris* × *V. berlandieri*) (Bouyahia *et al.* 2005) and may also be associated with Syrah decline. Although GVD was detected in a vine showing corky rugose wood symptoms (Abou-Ghanem, *et al.*, 1997), its role in the RW complex is currently not clear.

Genome organization of viruses associated with RW complex

The genome of viruses associated with RW complex is composed of positive-sense; single-stranded RNA of variable size and gene content (Fig. 2). The genome of GRSPaV is 8725 nucleotides (nt) in length, excluding the polyA tail, and encodes five open reading frames (ORFs, Meng et al. 1998; Zhang et al. 1998). The genomes of GVA, GVB and GVE, all vitiviruses, are 7851, 7599 and 7564 nt in size, respectively (Minafra et al., 1994; Goszczynski, 2007). Only partial coat protein and RNA-dependent RNA polymerase sequences of GVD are currently available (Abou-Ghanem et al., 1997). Similar to GRSPaV, the vitiviruses associated with RW complex also encode five slightly overlapping ORFs (Adams et al., 2005). Although some differences exist in the type and size of the genes encoded by the vitiviruses and GRSPaV, the first and largest ORF is always the replicase gene. Within the replicase ORF, both GRSPaV and the vitiviruses share four conserved domains - methyltransferase, AlkB, RNA helicase and RNA-dependent RNA polymerase (Martelli et al., 2007). Two additional domains, OTu-like peptidase and papain-like protease, are present in the replicase ORF of GRSPaV. Another notable difference between GRSPaV and the vitiviruses is the nature of the ORFs coding for movement function. While GRSPaV possess a set of three partially overlapping ORFs known as a triple gene block (TGB) of movement proteins, the vitiviruses have a single movement protein that is a member of the 'p30-like' superfamily typified by the 30-kDa movement protein of *Tobacco mosaic virus* (Melcher, 2000). Also while the coat protein on GRSPaV is 3'-most ORF, that of the vitiviruses is penultimate since they encode an RNA-binding protein which is 3'-most in their genomes. The specific functions of the individual ORFs and the associated domains have been well reviewed (Martelli et al., 2007).

Table 1. Disorders of the rugose wood (RW) disease complex

Disorder	Indicator grapevine‡			Typical symptom
	Vitis rupestris	LN 33	Kober 5BB	
Rupestris stem pitting	+	-	-	Distinct basipetal pitting limited to a band extending downward from point of inoculation
Corky bark	+	+	-	Grooving and pitting of entire surface of stem, severe stunting of LN 33 accompanied by rolling and reddening of leaves, intermodal swelling of canes
Kober stem grooving	-	-	+	Marked grooving on the stem
LN 33 stem grooving†	-	+	-	Stem grooving

†Grooves similar to corky bark but no intermodal swelling of the shoots or foliar discolorations are present; LN 33 (Courdec 1613 x Thompson seedless) and Kober 5BB (*V. berlandieri* x *V. riparia*) are hybrid cultivars.
‡+ = symptomatic; - = asymptomatic

Rupestris stem pitting (cv. St. George)

LN33 stem grooving (cv. LN33)

Kober stem grooving (cv. Kober 5BB)

Corky bark (cv. St. George & LN33)

Source of images:
Dr. Deborah Golino,
University of California-Davis

Fig. 1. Symptoms associated with four disorders of the Rugose Wood disease complex.

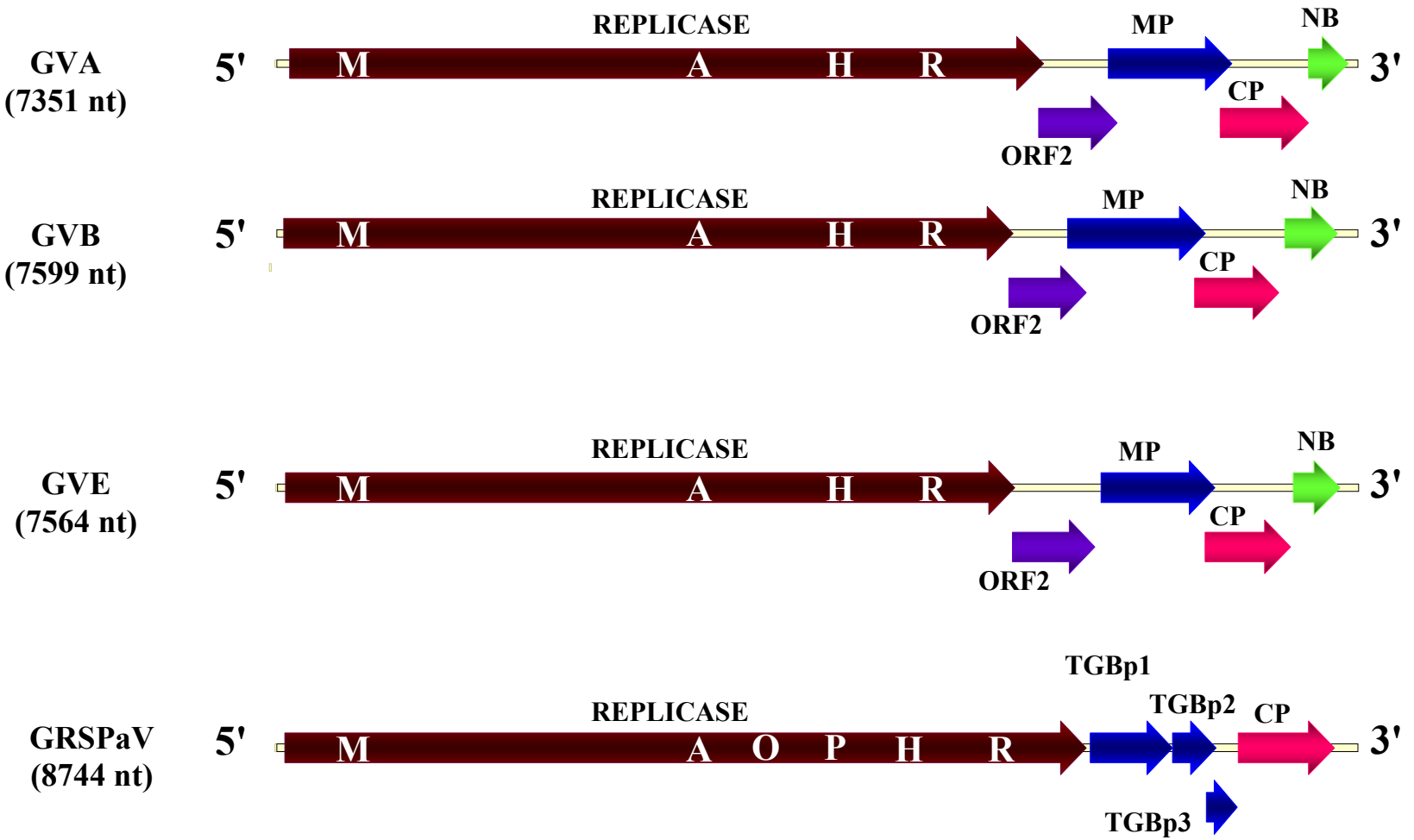

Figure 2. Genome organization of viruses associated with the rugose wood disease complex. Each block arrow represents one open reading frame (ORF). ORFs with same colors have similar functions. MP = movement protein, TGBp = Triple gene block of movement proteins, CP = coat protein, NB = RNA-binding protein). Domains of the replicase (M= methyltransferase, A = AlkB, O = OTu-like peptidase, P = papain-like protease, H = RNA helicase, R = RNA-dependent RNA polymerase) are shown. The map for each virus was drawn based on their complete genome sequences available in GenBank (Accession Numbers X75433, X75448, AB432910 and AF057136 for GVA, GVB, GVE and GRSPaV, respectively).

Spread of viruses associated with RW complex

Similar to most viruses infecting perennial plants, long distance spread of viruses associated with RW complex is primarily through contaminated grapevine cuttings and through grafting and top-working of infected scion and/or rootstock materials. GVA, GVB and GVE have been reported to be transmitted semi-persistently by pseudococcid mealybugs and/or scale insects (Martelli and Boudon-Padieu, 2006). Most of the transmission studies have been conducted with GVA transmitted by *Pseudococcus longispinus*, *Pseudococcus affinis*, *Pseudococcus comstocki*, *Planococcus ficus*, *Planococcus citri*, *Heliococcus bohemicus*, *Neopulvinaria innumerabilis* and *Parthenolecanium corni* (Rosciglione *et al.* 1983; Rosciglione and Castellano 1985; Agran *et al.* 1990; Engelbrecht and Kasdorf 1990; Pedroso *et al.* 1991; Garau *et al.* 1995; La Notte *et al.*, 1997; Goszczynski and Jooste 2003; Nakano *et al.* 2003; Zorloni *et al.* 2004, 2006; Fortusini *et al.* 1997; Hommay *et al.*, 2008). GVB has also been reported to be transmitted by *Pseudococcus longispinus*, *Pseudococcus affinis* and *Planococcus ficus* (Kuniyuki *et al.*, 2006; Martelli and Boudon-Padieu, 2006), while GVE was reported to be transmitted by *Pseudococcus comstocki* (Nakaune *et al.*, 2008). So far, no arthropod vector has been reported for GRSPaV.

The natural host range of viruses associated with RW complex is currently restricted to the genus *Vitis* although several herbaceous experimental hosts have been reported for GVA and GVB. GVA and GVB have been shown to be transmissible by mechanical inoculations to herbaceous hosts (Monette *et al.*, 1990; Goszczynski *et al.*, 1996). GRSPaV was recently shown to be transmissible by seed (Lima *et al.*, 2007) and reported to be pollen-borne (Martelli and Boudon-Padieu, 2006).

Diagnosis of viruses associated with RW complex

Biological, serological and nucleic acid-based assays are available for the reliable detection of viruses associated with RW complex. Traditionally, the disorders associated with these viruses are distinguished based on biological indexing using specific indicators such as *V. rupestris*, Kober 5BB and LN 33 (Table 1b). However, the limitation of biological indexing lies in its lack of definitive identification of specific virus involved in the disease complex. It is also time consuming, labor intensive and requires elaborate field facilities. Moreover, biological indexing is of limited value for virus indexing programs due to the presence of virus strains that cause latent infections and also because of variations that could occur in symptom expression depending on the environmental factors. Due to the limitations of biological indexing, considerable efforts have been devoted to the development of serology- and nucleic acid-based approaches for the detection of viruses associated with RW complex. Currently, antibodies are available for the detection of GVA, GVB and GVD by ELISA (Bonavia *et al.*, 1996; Boscia *et al.*, 1994; Rubinson, 1997; Boscia *et al.*, 2001). Diagnostic assays based on reverse-transcription polymerase chain reaction (RT-PCR) are routinely used for the detection of GVA, GVB, GVD, GVE and GRSPaV (Nolasco *et al.*, 2000; 2006; Gribaudo *et al.*, 2006; Nakaune *et al.*, 2008; Kominek *et al.*, 2008; Osman and Rowhani, 2008).

Status of RW complex and associated viruses in the PNW

GRSPaV was reported in several wine grape cultivars in a survey conducted in early 2000 (Martin *et al.*, 2005). Recent studies have documented the presence of GVA and GVB along with GRSPaV in several wine grape cultivars, where they are found occurring as mixed

infections with grapevine leafroll-associated viruses (Naidu *et al.*, 2006; 2009; Mekuria *et al.*, 2009). The presence of GVD and GVE are not yet documented in the region.

Management of viruses associated with RW complex

Similar to other viruses transmitted via vegetative propagation, sanitation and distribution of virus-tested planting materials are effective strategies in preventing the introduction and dissemination of viruses associated with RW complex. The use of meristem tip culture, heat therapy and somatic embryogenesis have also been practiced in eliminating virus from infected planting materials (Gribaudo *et al.*, 2006).

Conclusions and perspectives

The etiology of RW complex and the role of GRSPaV, GVA, GVB, GVD, and GVE in eliciting different disorders remains unresolved, although a considerable body of knowledge has been accumulated on the biology and molecular biology of these viruses. Distribution of planting materials has largely contributed to dissemination of these viruses between different grape-growing regions worldwide. Viticultural practices such as grafting on suitable rootstocks or topworking can increase the potential for mixed infections of RW complex-associated viruses and other economically important viruses like grapevine leafroll-associated viruses (Credi and Giunchedi, 1996; Martin *et al.*, 2005; Mekuria *et al.*, 2009). Such mixed infections often result in synergistic interactions (Tomažič *et al.*, 2005). In addition, mixed infections in perennial crops could provide opportunities for recombinations to occur between different strains of a virus, thereby increasing genetic diversity within virus populations. These recombination events appear to be common in the genomes of viruses infecting vegetatively propagated perennial crops such

as citrus (Rubio *et al.*, 2001; Weng *et al.*, 2007). In a broader context, recombination in viruses infecting perennial crops offer potentially significant advantages for their increased genetic diversity and adaptability. Deleterious mutations accumulated by the virus due to the lack of proofreading activity of RNA-dependent RNA polymerase can be offset by recombination of the error-free parts of co-infecting genomes (García-Arenal *et al.*, 2001; Vives *et al.*, 2005).

The documentation of GRSPaV, GVA, GVB, GVD and GVE and their genetic variants as well as an assessment of their impact, either individually or in concert, needs to be appraised in detail. Due to differences in viticultural practices in the PNW (viz. grafting scion cultivars on rootstocks in Oregon vs. ownrooted planting in Washington and Idaho) and frequent exchanges of planting material among the growers in the region, such information will aid the development of improved diagnostic tools for accurate detection of these viruses and their genetic variants, and aid in formulating strategies for mitigating their negative impact on the sustainability of wine grape industry in the region.

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CHAPTER 6

SEQUENCE DIVERSITY, POPULATION GENETICS AND POTENTIAL RECOMBINATION EVENTS IN *GRAPEVINE RUPESTRIS STEM PITTING-ASSOCIATED VIRUS* IN PACIFIC NORTHWEST VINEYARDS

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SUMMARY

Grapevine rupestris stem pitting-associated virus (GRSPaV; genus *Foveavirus*; family *Flexiviridae*) is present in many grape-growing regions of the world. A total of 84 full-length coat protein (CP) sequences and 57 sequences representing the helicase-encoding region (HR) of the RNA-dependent RNA polymerase were obtained from wine grape cultivars grown in the Pacific Northwest (PNW) of the United States and their molecular diversity compared with corresponding sequences previously reported from other grape-growing regions. In pairwise comparisons, the CP sequences from PNW showed identities ranging between 80 and 100 % at the nucleotide (nt) level and the HR sequences showed identities between 79 and 100 %. A global phylogenetic analysis of the CP and HR sequences revealed segregation of GRSPaV isolates into four major lineages with isolates from PNW distributed in all four lineages, indicating a lack of clustering by geographical origin. Scion cultivars grafted onto rootstock were found to contain mixtures of more genetic variants belonging to different lineages than own-rooted cultivars. Assessment of population genetic parameters found that the CP was more variable than the HR region. The discordant gene phylogenies obtained for some CP and HR sequences and the identification of potential recombination events involving parents from

different lineages provided strong evolutionary evidence for genetic diversity among GRSPaV isolates. These results underscore the highly variable nature of the virus with implications for grapevine health status and distribution of virus-tested planting materials. This study also contributes to an increased understanding of molecular population genetics of viruses infecting deciduous woody perennials.

INTRODUCTION

Grapevine rupestris stem pitting-associated virus (GRSPaV; genus *Foveavirus*; family *Flexiviridae*; Martelli & Jelkmann, 1998; Adams *et al.*, 2004; 2005) is a graft-transmissible virus widely distributed in many grape-growing regions around the world (Minafra & Boscia, 2003). The positive-sense, single-stranded RNA of GRSPaV is 8725 nucleotides (nt) in length, excluding the poly A tail, and encodes five open reading frames (ORFs; Meng *et al.* 1998; Zhang *et al.* 1998). The 3'-most ORF encodes a 28 kDa coat protein (CP), whereas the 5'-most ORF encodes the viral replicase polyprotein of 244 kDa. The ORFs 2, 3 and 4 encode polypeptides of 24, 13 and 8 kDa, respectively, which together constitute the 'triple gene block' (TGB) proteins TGBp1, TGBp2 and TGBp3, respectively (Meng *et al.*, 1998). Recent studies have shown that TGBp1 has both a cytosolic and nuclear distribution, whereas both TGBp2 and TGBp3 are associated with the endoplasmic reticulum (Rebelo, *et al.*, 2008). The replicase polyprotein of GRSPaV contains two characteristic domains conserved in the alphavirus-like superfamily of the positive-strand RNA viruses (Koonin & Dolja, 1993) and two novel domains, AlkB and OTU, that are present in several other members of the *Flexiviridae* (Makarova *et al.*, 2000; Martelli *et al.*, 2007). A putative ORF6 encoding a 14kDa protein of unknown functions, partially

overlapping the CP at the C terminus, has been reported in some variants of GRSPaV (Meng *et al.*, 1998; Zhang *et al.*, 1998; Lima *et al.*, 2006).

Grapevine (*Vitis* spp.) is the only known natural host of GRSPaV. The virus can be spread via vegetative propagation and grafting (Minafra & Boscia, 2003) and possibly through seeds of infected grapevine (Lima *et al.*, 2007). Although GRSPaV has been detected in the pollen of infected grapevines (Rowhani *et al.*, 2000b), its spread through pollen is not confirmed. The virus is not transmissible by mechanical inoculations and no biological vector has been reported. Studies have indicated the biological association of GRSPaV with rupestris stem pitting (RSP), one of the four disorders of the rugose wood complex (Martelli, 1993). RSP shows pitting symptoms on the woody cylinder below the graft union in 'St. George' grapevines (*V. rupestris* Scheele). The aetiological relationship of GRSPaV with RSP, however, remains to be elucidated. Previous studies have shown that GRSPaV can occur as distinct variants (Rowhani *et al.*, 2000b; Habili *et al.*, 2006; Meng *et al.*, 2006; Nolasco *et al.* 2006; Nakaune *et al.*, 2008), some of which may not elicit RSP symptoms when graft-inoculated on the indicator 'St George' (Meng *et al.*, 1999; Meng *et al.*, 2006; Habili *et al.*, 2006). In addition, some variants that are latent in *Vitis vinifera* cultivars and in most American *Vitis* species and hybrids can produce necrosis of the veinlets when graft-inoculated on *V. rupestris* x *Vitis. berlandieri* 110 Richter (Bouyahia, *et al.*, 2005). Recently, GRSPaV, along with a novel marafivirus and several viroids, has been documented in Californian Syrah grapevines showing decline symptoms, although the role of GRSPaV in Syrah decline is yet to be resolved (Al Rwahnih *et al.*, 2009).

The Pacific Northwest (PNW) states of the United States (Washington, Oregon and Idaho) are collectively emerging as the second largest wine grape-growing region in the country. Wine grapes are largely grown as own-rooted vines in Washington and Idaho and on rootstocks in Oregon. Since exchange of planting material occurs among growers in the region, we have been carrying out studies to document grapevine viruses and their genetic variants for assessing the sanitary status of vineyards. Such information will aid the development of strategies for mitigating their negative impact on the sustainability of the wine grape industry in the region. Towards this objective, we assessed genetic diversity of natural GRSPaV populations from PNW and compared them with isolates from other grape-growing regions. The results indicated that GRSPaV is highly variable in chronically infected, vegetatively propagated grapevines. The study provides evidence for the first time of the occurrence of potential recombination events in a foveavirus. The occurrence of mixed infection of genetically divergent variants within a single grapevine and recombination events identified in this study could, in part, contribute to extensive sequence diversity and evolution that could impact grapevine health status and diagnosis of GRSPaV in grapevines. On a wider scale, the results contribute to an increased understanding of molecular population genetics of viruses infecting deciduous woody perennials.

METHODS

Virus isolates. Seventy three isolates of GRSPaV from different grapevine cultivars were included in this study (Table 1). The virus derived from a single grapevine was considered as one isolate. The GRSPaV isolates were collected between 2005 and 2007 from different vineyards (names withheld due to grower confidentiality) in Washington and Oregon. Leaf petioles were

randomly collected from different parts of individual grapevines and pooled for extractions to minimize possible variations due to uneven distribution of the virus in infected tissues. Samples from eastern Washington (i.e. eastern side of the Cascade Range) originated from own-rooted wine grape cultivars and those from western Washington (i.e. western side of the Cascade Range) and Oregon were from grapevines grafted onto a rootstock. Other samples were obtained from a collection maintained at the NorthWest Grape Foundation Service, Prosser, WA.

Sample extraction and RT-PCR. Petiole sample extracts prepared with the aid of a HOMEX 6 homogenizer (BIOREBA AG, Reinach BL1, Switzerland) were used in one step-single tube RT-PCR assay (Rowhani *et al.*, 2000a) for amplification of two distinct regions of the virus genome (Fig. 1). Two sets of primers, designed based on the consensus sequence of multiple viral variants reported previously (Meng *et al.*, 2006; Nolasco *et al.*, 2006, Nakaune *et al.*, 2008), were used to amplify the two regions of the virus genome. The primer pair RSP4373F (5'-GATGAGGTCCAGTTGTTTCC-3') and RSP4711R: (5'-ATCCAAAGGACCTTTTGACC-3') correspond to nt 4373-4711 (AF057136) within the helicase-encoding region (HR) of ORF1 and the primer pair RSP52F (5'-TGAAGGCTTTAGGGGTTAG-3') and RSP53R (5'-CTTAACCCAGCCTTGAAAT-3') correspond to nt 7709-8613 (AF057136), encompassing the entire CP gene and flanking sequences upstream in the TGBp3 and downstream in the 3' untranslated region (UTR).

Cloning, sequencing and sequence analysis. Amplicons were cloned into pCR2.1 vector (Invitrogen Corp., Carlsbad, CA, USA) and transformed into *Escherichia coli*. Plasmid DNA was purified from positive recombinant clones using the QIAprep spin miniprep kit (Qiagen Inc.,

Valencia, CA, USA). Three independent clones per isolate were sequenced in both orientations. Sequence identity above 99 % between the three clones of an isolate was considered as a single sequence. Sequences derived from additional clones of an isolate were included in the analyses when differences were greater than 2 %. Alignments of nucleotide sequences were done using CLUSTALW (Thompson *et al.*, 1994) with default settings and sequence identities were obtained using Vector NTI Advance 11 program (Invitrogen Corp., USA). Distribution of the percentages of pairwise divergence (p-distance, nucleotide identity) within and between GRSPaV sequences and another foveavirus, *Apple stem pitting virus* (ASPV) were plotted using Micosoft Excel spreadsheet (Microsoft Office 2000, Microsoft Corporation, USA).

Phylogenetic analysis. Evolutionary relationships were inferred from multiple sequence alignments calculated by CLUSTALW using the Minimum evolution (ME) method (Rzhetsky & Nei, 1992) with 1 000 bootstrap replications. The evolutionary distances were computed using the Kimura two-parameter model (Kimura, 1980). The ME trees were searched using the Close Neighbor-Interchange algorithm (Nei & Kumar, 2000) at a search level of 1. The Neighbor Joining algorithm (Saitou & Nei, 1987) was used to generate the initial tree, for which all positions containing gaps and missing data were eliminated from the dataset. These phylogenetic analyses tools were implemented by the molecular evolutionary genetics analysis (MEGA) software version 4.0 (Tamura *et al.*, 2007). Corresponding sequences of GRSPaV isolates available in GenBank (Table 2) were included in these analyses and corresponding sequences of ASPV (GenBank accession no. D21829) were used as an outgroup.

RNA polymorphism and evolution. DnaSP version 4.90.1 (Rozas *et al.* 2003) was used to estimate Tajima's D (Tajima, 1989), Fu & Li's D and F (Fu & Li, 1993) statistical tests to examine the hypothesis of neutral selection operating on the CP and HR sequences. We also estimated several population genetic parameters including nucleotide polymorphism (π; estimated by the average number of nucleotide differences between two random sequences in a population), haplotype diversity (Hd; the frequency and number of haplotypes in a population), the statistic θ from the number of segregating sites (S) (Watterson, 1975) and the average number of nonsynonymous (d_N) and synonymous (d_S) substitutions. The distribution of d_S and d_N along the coding regions was analyzed using the SNAP program (http://www.hiv.lanl.gov; Korber, 2000).

Recombination analysis. The occurrence of recombination events in CP and HR sequences was investigated by using the suite of programs included in the Recombination Detection Program version 3 (RDP3) Beta 27 (Martin *et al.*, 2005).

RESULTS

Genetic diversity of GRSPaV populations from the Pacific Northwest

Primers RSP52F & RSP53R amplified a DNA fragment of approx. 905 base pairs (bp) encompassing the entire CP and 62 and 63 bp upstream and downstream of the CP, respectively (Fig. 1). However, the flanking sequences were removed and only the CP gene (780 bp) sequences were used for analyses. The primers RSP4373F & RSP4711R amplified a fragment of

approx. 339 bp specific to the HR and after removing the primer sequences, only 299 nt was used for further analyses. Both CP- and HR-specific fragments were amplified from a total of 34 grapevines, whereas a CP- or HR-specific fragment only was amplified from additional 20 and 19 grapevines, respectively. Thus, a total of 84 CP sequences and 57 HR sequences derived from 54 and 53 individual grapevines, respectively, were included for the genetic diversity analyses. These sequences were deposited in GenBank under the accession numbers FJ943274- FJ943357 and FJ943358- FJ943414 for CP and HR, respectively (Table 1).

In pairwise comparisons, the 84 CP sequences from PNW showed identities ranging between 80 and 100 % at the nt level and between 86 and 100 % at the aa level (Fig 2a). The percent identities among the 57 HR sequences from the PNW ranged between 79 and 100 % and between 86 and 100 % at the nt and aa level, respectively (Fig 2b). Similar ranges of values were obtained when comparisons were made with corresponding sequences in GenBank (Table 2). These values are within the limits of species demarcation criteria in the family *Flexiviridae*, where isolates sharing greater than 72 % nt or 80 % aa sequence identities between their CP or polymerase genes are considered one species (Adams *et al.*, 2005). Based on these results, it can be concluded that CP and HR sequences are specific to GRSPaV and divergent variants of the virus are present in different wine grape cultivars grown in PNW vineyards.

Table 1. Origin, cultivar-type and GenBank accession numbers of Pacific Northwest region isolates of *Grapevine rupestris stem pitting-associated virus* obtained in the study and the analyzed genomic regions.

Virus isolate	Origin*	Cultivar	Region†	Accession nos.‡
BCScB1	BC	Schonburger	HR	FJ943401
BCZR1	BC	Zweigelt rebe	HR, CP	FJ943358, FJ943274
EWSY1	E-WA	Syrah	HR, CP	FJ943359, FJ943275
EWSY2	E-WA	Syrah	HR, CP	FJ943360, FJ943276
EWSY3	E-WA	Syrah	HR, CP	FJ943361, FJ943277
EWSY4	E-WA	Syrah	HR, CP	FJ943362, FJ943278
EWCH1	E-WA	Chardonnay	HR, CP	FJ943364, FJ943285
EWCH2	E-WA	Chardonnay	HR, CP	FJ943365, FJ943286
EWCH3	E-WA	Chardonnay	HR, CP	FJ943366, FJ943287
EWCH4	E-WA	Chardonnay	HR, CP	FJ943367, FJ943288
EWCH5	E-WA	Chardonnay	HR, CP	FJ943368, FJ94393-4
EWCH6	E-WA	Chardonnay	HR, CP	FJ943369, FJ943289
EWCH7	E-WA	Chardonnay	HR, CP	FJ943370, FJ943290
EWCH8	E-WA	Chardonnay	CP	FJ943291
EWCH9	E-WA	Chardonnay	HR, CP	FJ943371, FJ943292
EWSY5	E-WA	Syrah	HR	FJ943376
EWSY6	E-WA	Syrah	HR	FJ943379
EWSY7	E-WA	Syrah	HR	FJ943377
EWSY8	E-WA	Syrah	HR	FJ943378
EWMR1	E-WA	Merlot	HR, CP	FJ943392, FJ943333-5
EWSY9	E-WA	Syrah	HR, CP	FJ943400, FJ943341
EWSY10	E-WA	Syrah	CP	FJ943338-40
EWCS1	E-WA	Cabernet Sauvignon	HR, CP	FJ943405, FJ943344
EWCS2	E-WA	Cabernet Sauvignon	HR, CP	FJ943406, FJ943345
EWCS3	E-WA	Cabernet Sauvignon	HR, CP	FJ943407, FJ943346
EWCS4	E-WA	Cabernet Sauvignon	HR, CP	FJ943408, FJ943347
EWSY11	E-WA	Syrah	HR	FJ943409
WWPN1	W-WA	Pinot Noir	HR, CP	FJ943410, FJ943348-9
WWPN2	W-WA	Pinot Noir	HR, CP	FJ943411, FJ943350
WWPN3	W-WA	Pinot Noir	HR, CP	FJ943412, FJ943351
WWPN4	W-WA	Pinot Noir	HR, CP	FJ943413, FJ943352

*BC, British Columbia; E-WA, Eastern Washington; FB, Foundation Block; ID, Idaho; OR, Oregon; W-WA, Western Washington. All isolates from E-WA and ID were obtained from vines growing on their own roots while those from other regions were obtained from vines growing on rootstocks

†HR, sequences obtained from helicase domain of the replicase gene; CP, full-length coat protein gene sequence; HR/CP, sequences obtained from both HR and CP

‡Acession nos., GenBank accession numbers

Table 1 (contd.). Origin, cultivar-type and GenBank accession numbers of Pacific Northwest region isolates of *Grapevine rupestris stem pitting-associated virus* obtained in the study and the analyzed genomic regions.

Virus isolate	Origin*	Cultivar	Region†	Accession nos.‡
WWPN5	W-WA	Pinot Noir	HR, CP	FJ943414, FJ943353
FBCL1	FB	Chelois	CP	FJ943284
FBFH1	FB	Foch	CP	FJ943296-7
FBGW1	FB	Gertwurtz	HR, CP	FJ943375, FJ943313
FBPB1	FB	Pinot Blanc	HR	FJ943398
FBPB2	FB	Pinot Blanc	HR	FJ943399
FBSB1	FB	Sauvignon Blanc	CP	FJ943342-3
FBSR1	FB	SummerRoyal	HR	FJ943402
FBVN1	FB	Venus	HR	FJ943403
IDKH1	ID	Kashishi	HR	FJ943380
ORCH1	OR	Chardonnay	HR, CP	FJ943363, FJ943279-83
ORPN1	OR	Pinot Noir	HR, CP	FJ943373, FJ943298-301
ORPN2	OR	Pinot Noir	CP	FJ943302
ORPN3	OR	Pinot Noir	CP	FJ943303
ORPN4	OR	Pinot Noir	CP	FJ943304
ORPN5	OR	Pinot Noir	CP	FJ943305-6
ORPN6	OR	Pinot Noir	CP	FJ943307-8
ORPN7	OR	Pinot Noir	CP	FJ943309-10
ORPN8	OR	Pinot Noir	CP	FJ943311
ORPN9	OR	Pinot Noir	HR, CP	FJ943381, FJ943314-7
ORPN10	OR	Pinot Noir	HR	FJ943382
ORPN11	OR	Pinot Noir	HR	FJ943383
ORPN12	OR	Pinot Noir	CP	FJ943318
ORPN13	OR	Pinot Noir	CP	FJ943319-20
ORPN14	OR	Pinot Noir	HR, CP	FJ943384-5, FJ943321
ORPN15	OR	Pinot Noir	HR, CP	FJ943386, FJ943322
ORPN16	OR	Pinot Noir	HR	FJ943387
ORPN17	OR	Pinot Noir	HR	FJ943388-90
ORPN18	OR	Pinot Noir	CP	FJ943323-4
ORPN19	OR	Pinot Noir	CP	FJ943325
ORPN20	OR	Pinot Noir	HR	FJ943391
ORPN21	OR	Pinot Noir	CP	FJ943326-9
ORPN22	OR	Pinot Noir	CP	FJ943330-1

*BC, British Columbia; E-WA, Eastern Washington; FB, Foundation Block; ID, Idaho; OR, Oregon; W-WA, Western Washington. All isolates from E-WA and ID were obtained from vines growing on their own roots while those from other regions were obtained from vines growing on rootstocks

†HR, sequences obtained from helicase domain of the replicase gene; CP, full-length coat protein gene sequence; HR/CP, sequences obtained from both HR and CP

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Table 1 (contd.). Origin, cultivar-type and GenBank accession numbers of Pacific Northwest region isolates of *Grapevine rupestris stem pitting-associated virus* obtained in the study and the analyzed genomic regions.

Virus isolate	Origin*	Cultivar	Region†	Accession nos.‡
ORPN23	OR	Pinot Noir	CP	FJ943332
ORPN24	OR	Pinot Noir	CP	FJ943354-7
ORPGB1	OR	Pinot Gris (Becker FR 49-207)	HR, CP	FJ943396, FJ943336
ORPGR1	OR	Pinot Gris (RulaN/ter 2/15 GM)	HR, CP	FJ943397, FJ943337
WWDC1	W-WA	Dornfelder-Cloud	HR, CP	FJ943372, FJ943295
WWGB1	W-WA	Goluboc	HR, CP	FJ943374, FJ943312
WWMC1	W-WA	Muscat	HR	FJ943393-4
WWPL1	W-WA	Perle	HR	FJ943395
WWVD1	W-WA	Volga Don	HR	FJ943404

*BC, British Columbia; E-WA, Eastern Washington; FB, Foundation Block; ID, Idaho; OR, Oregon; W-WA, Western Washington. All isolates from E-WA and ID were obtained from vines growing on their own roots while those from other regions were obtained from vines growing on rootstocks

†HR, sequences obtained from helicase domain of the replicase gene; CP, full-length coat protein gene sequence; HR/CP, sequences obtained from both HR and CP

‡Acession nos., GenBank accession numbers

Table 2. Name, origin and accession numbers of GenBank isolates of *Grapevine rupestris stem pitting-associated virus* used in phylogenetic analyses.

Isolate	Origin	Region†	Accession no. ‡
420A	Brazil	CP	EU040204
CF195	Brazil	CP	EF636803
CF195-2	Brazil	CP	EU204913
CF207	Brazil	CP	EF636804
CF208	Brazil	CP	EF690383
CF210	Brazil	CP	EF690384
DQ443732	Brazil	CP	DQ443732
MG	Brazil	CP	EF690380
MH	Brazil	CP	EF690382
PN	Brazil	CP	EF690381
GRSPaV	California, USA	CP	AF026278
SY	California, USA	CP	AY368590
SY	California, USA	HR	AY368590
BS	Canada	CP	AY881627
BS	Canada	HR	AY881627
Kober5BB	Canada	HR	DQ278621
Mlld14	Canada	HR	DQ278622
Niagara2	Canada	HR	DQ278623
Niagara9	Canada	HR	DQ278624
Paulsen	Canada	HR	DQ278620
Canino	Foundation vineyard, Italy	HR	DQ278629
Pgt14	Foundation vineyard, Italy	HR	DQ278628
Pgt5	Foundation vineyard, Italy	HR	DQ278627
Tbb1	Foundation vineyard, Italy	HR	DQ278625
Tbb5	Foundation vineyard, Italy	HR	DQ278626
ALB3SH	Italy	CP	DQ364993
BL1	Italy	CP	DQ364979
BL11A	Italy	CP	DQ364980
BOS3MTR	Italy	CP	DQ364991
CF6	Italy	CP	DQ364994
CF8/2	Italy	CP	DQ364995
CNO15	Italy	CP	DQ364982
CNO9	Italy	CP	DQ364981
Doberdo	Italy	CP	DQ364988
LS3	Italy	CP	DQ364983
NE423PMIV	Italy	CP	DQ364989
NE423TPI	Italy	CP	DQ364990
PGD2	Italy	CP	DQ364984

†HR, sequences obtained from helicase domain of the replicase gene; CP, full-length coat protein gene sequence; HR/CP, sequences obtained from both HR and CP
‡GenBank accession number

Table 2 (contd.). Name, origin and accession numbers of GenBank isolates of *Grapevine rupestris stem pitting-associated virus* used in phylogenetic analyses.

Isolate	Origin	Region†	Accession no. ‡
RSS6	Italy	CP	DQ364985
TRCV1	Italy	CP	DQ364986
TRCV4	Italy	CP	DQ364987
VER84SH	Italy	CP	DQ364992
Hai1	Japan	CP	AB331440
Ham1	Japan	CP	AB331441
His1-2	Japan	CP	AB331438
His4-2	Japan	CP	AB331439
Hiz1	Japan	CP	AB331431
Hiz12-1	Japan	CP	AB331436
Hiz12-2	Japan	CP	AB331437
Hiz3	Japan	CP	AB331432
Hiz5	Japan	CP	AB331433
Hiz6	Japan	CP	AB331434
Hiz7	Japan	CP	AB331435
OB1	Japan	CP	AB331418
OC5	Japan	CP	AB331419
OC8	Japan	CP	AB331420
OD1	Japan	CP	AB331421
OD5	Japan	CP	AB331422
OE11	Japan	CP	AB331424
OE8	Japan	CP	AB331423
OH11	Japan	CP	AB331426
OH4	Japan	CP	AB331425
OK4	Japan	CP	AB331427
OK7	Japan	CP	AB331428
OT26	Japan	CP	AB331429
OT28	Japan	CP	AB331430
Cbl1	New York, USA	HR	DQ278630
Cbl3	New York, USA	HR	DQ278631
GG	New York, USA	HR	DQ278632
Merlot1	New York, USA	HR	DQ278633
Merlot2	New York, USA	HR	DQ278634
PN1	New York, USA	HR	DQ278635
PN8	New York, USA	HR	DQ278636
RSPaV-1	New York, USA	HR, CP	AF057136
Rv1	New York, USA	HR	DQ278639
Rv6-2	New York, USA	HR	DQ278640

†HR, sequences obtained from helicase domain of the replicase gene; CP, full-length coat protein gene sequence; HR/CP, sequences obtained from both HR and CP
‡GenBank accession number

Table 2 (contd.). Name, origin and accession numbers of GenBank isolates of *Grapevine rupestris stem pitting-associated virus* used in phylogenetic analyses.

Isolate	Origin	Region†	Accession no. ‡
Rv6-4	New York, USA	HR	DQ278641
Rv6-6	New York, USA	HR	DQ278642
Rv7-6	New York, USA	HR	DQ278643
SG1	New York, USA	HR, CP	AY881626
Svd2	New York, USA	HR	DQ278637
Svd8	New York, USA	HR	DQ278638
Svl11	New York, USA	HR	DQ278648
Svl18	New York, USA	HR	DQ278649
Svl19	New York, USA	HR	DQ278650
Svl2	New York, USA	HR	DQ278644
Svl4	New York, USA	HR	DQ278645
Svl7	New York, USA	HR	DQ278646
Svl9	New York, USA	HR	DQ278647
A8.4	Portugal	CP	AY927670
B10-1	Portugal	CP	AY927680
B10-3	Portugal	CP	AY927681
B1-1	Portugal	CP	AY927682
B11-2	Portugal	CP	AY927679
B1-2	Portugal	CP	AY927683
Cl29	Portugal	CP	AY927678
D10	Portugal	CP	AY927672
E105-G	Portugal	CP	AY927676
E156	Portugal	CP	AY927677
M31-35	Portugal	CP	AY927673
M31-37	Portugal	CP	AY927674
M37-12	Portugal	CP	AY927675
M5-G	Portugal	CP	AY927671
Vs279-2	Portugal	CP	AY927684
Vs284-21	Portugal	CP	AY927685
Vs284-23	Portugal	CP	AY927686
SL38-20	Slovenia	CP	AY927687
SL48-14	Slovenia	CP	AY927688
SG2	New York, USA	HR	DQ278617
SG3	New York, USA	HR	DQ278618
ASPV	Germany	HR, CP	D21829

†HR, sequences obtained from helicase domain of the replicase gene; CP, full-length coat protein gene sequence; HR/CP, sequences obtained from both HR and CP
‡GenBank accession number

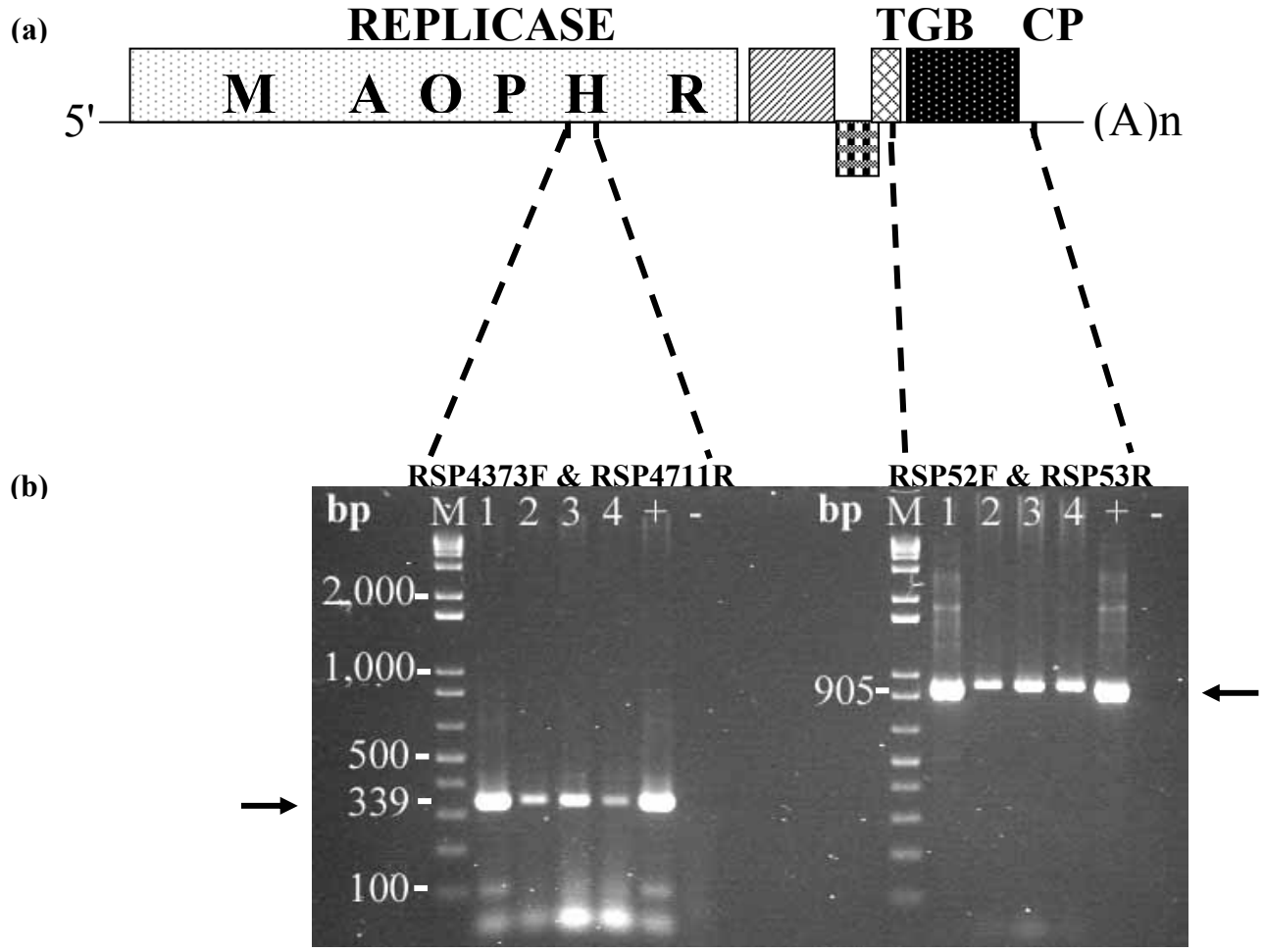


Fig. 1. (a) Genome organization of GRSPaV (drawing not according to scale) and (b) agarose gel electrophoresis of RT-PCR products specific to HR (left) and CP (right). M, Methyl transferase; A, AlkB; O, OTU-like peptidase; P, papain-like protease; H, RNA helicase; R, RNA-dependent RNA polymerase; TGB, triple gene block; and CP, coat protein. Lane M, 1 Kb Plus DNA ladder (Invitrogen Corp., Carlsbad, CA, USA), lanes 1-4, field samples, and lane + and - are positive and negative controls, respectively. The approximate locations of primer sequences used for RT-PCR are indicated by the dotted lines, the sizes of the DNA fragments amplified are shown by arrows.

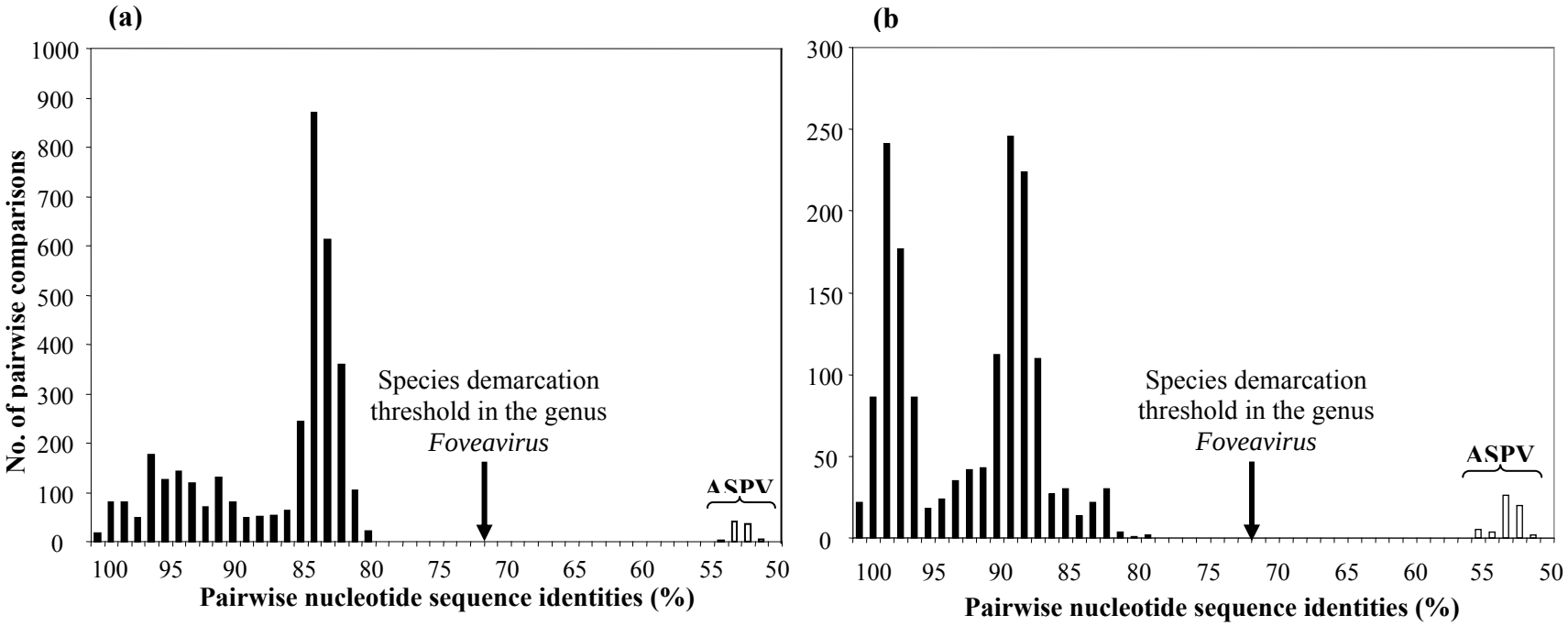


Fig. 2. Distribution of the percentages of pairwise identities (p-distance, nucleotide identity) among 84 CP (a) and fifty-seven HR (b) sequences of GRSPaV isolates. Each bar represents the total number of pairwise nucleotide comparisons sharing the same percentage of nucleotide sequence identity. Solid bars refer to comparisons between RSPaV sequences while open bars refer to comparisons between a GRSPaV sequence and the homologous sequence from ASPV (GenBank accession no. D21829), used as an outgroup. The species demarcation threshold is according to criteria established by the International Committee on Taxonomy of Viruses (Adams et al., 2004; 2005).

GRSPaV isolates from the Pacific Northwest comprise four major genetic lineages

Initially, all 159 sequences of the CP (84 from this study and 75 in GenBank) and 94 sequences of the HR (57 from this study and 37 in GenBank) were included in assessing phylogenetic relationship of GRSPaV isolates. A comparison of CP- and HR-based phylogenetic trees revealed inconsistencies in the placement of 19 GRSPaV isolates (18 from PNW and 1 from GenBank) in different lineages with poor bootstrap support suggesting that their genomic RNA might have originated from potential recombination events between different lineages. These events are described below to explain the inconsistencies. We omitted these interlineage recombinants and recalculated trees from the CP and HR sequences of other isolates together with sequences available in GenBank as described above, and the inferred phylogenetic trees are shown in Fig. 3.

The CP-based phylogenetic relationships of GRSPaV isolates from PNW were compared among themselves and with corresponding sequences from other grape-growing regions. This analysis, using the minimum evolution (ME) and neighbor-joining (NJ) methods with ASPV CP as an outgroup, included a total of 140 CP sequences, 66 from PNW and 74 from GenBank. The results showed segregation of GRSPaV CP sequences into four major lineages (Fig. 3a). We designated each of these lineages with a reference isolate to maintain a standardized nomenclature of GRSPaV sequence variant groups in analogy with a previous report by Meng *et al.* (2006). Thus, GRSPaV-1, GRSPaV-SG1, GRSPaV-BS and GRSPaV-VS lineages correspond to groups 2b, 2a, 3 and 1, respectively, as proposed by Nolasco *et al.* (2006) and Nakaune *et al.* (2008). The GRSPaV-1, GRSPaV-SG1, GRSPaV-BS and GRSPaV-VS lineages contained 11,

12, 21 and 22 sequences from PNW, respectively, and 20, 10, 15 and 29 from GenBank, respectively. These results indicate that GRSPaV sequences from the PNW were distributed in all four lineages with no geographical structuring, suggesting that they are polyphyletic. However, a greater number of sequences from PNW were aligned with GRSPaV-VS lineage, followed by GRSPaV-BS, GRSPaV-SG1 and GRSPaV-1 lineages. The mean genetic distance between the four groups ranged from 0.107 to 0.189, and those within each group ranged from 0.038 to 0.082.

A total of 74 sequences - 38 from PNW vineyards and 36 from GenBank - specific to HR were analyzed phylogenetically. HR sequences also segregated into four lineages (Fig. 3b) with each lineage designated in a manner similar to that described for the CP-based phylogram. The GRSPaV-1, GRSPaV-SG1, GRSPaV-BS and GRSPaV-VS lineages contained 25, 12, 0 and 1 isolates from PNW, respectively, and 10, 16, 7 and 3 from GenBank, respectively. In contrast with CP sequences, many of the HR sequences from PNW clustered with the GRSPaV-1 lineage, followed by the GRSPaV-SG1 lineage and and only one sequence was aligned with GRSPaV-VS lineage and none with GRSPaV-BS lineage. The mean genetic distance between the four lineages ranged from 0.118 to 0.215, and those within each lineage range from 0.031 to 0.080.

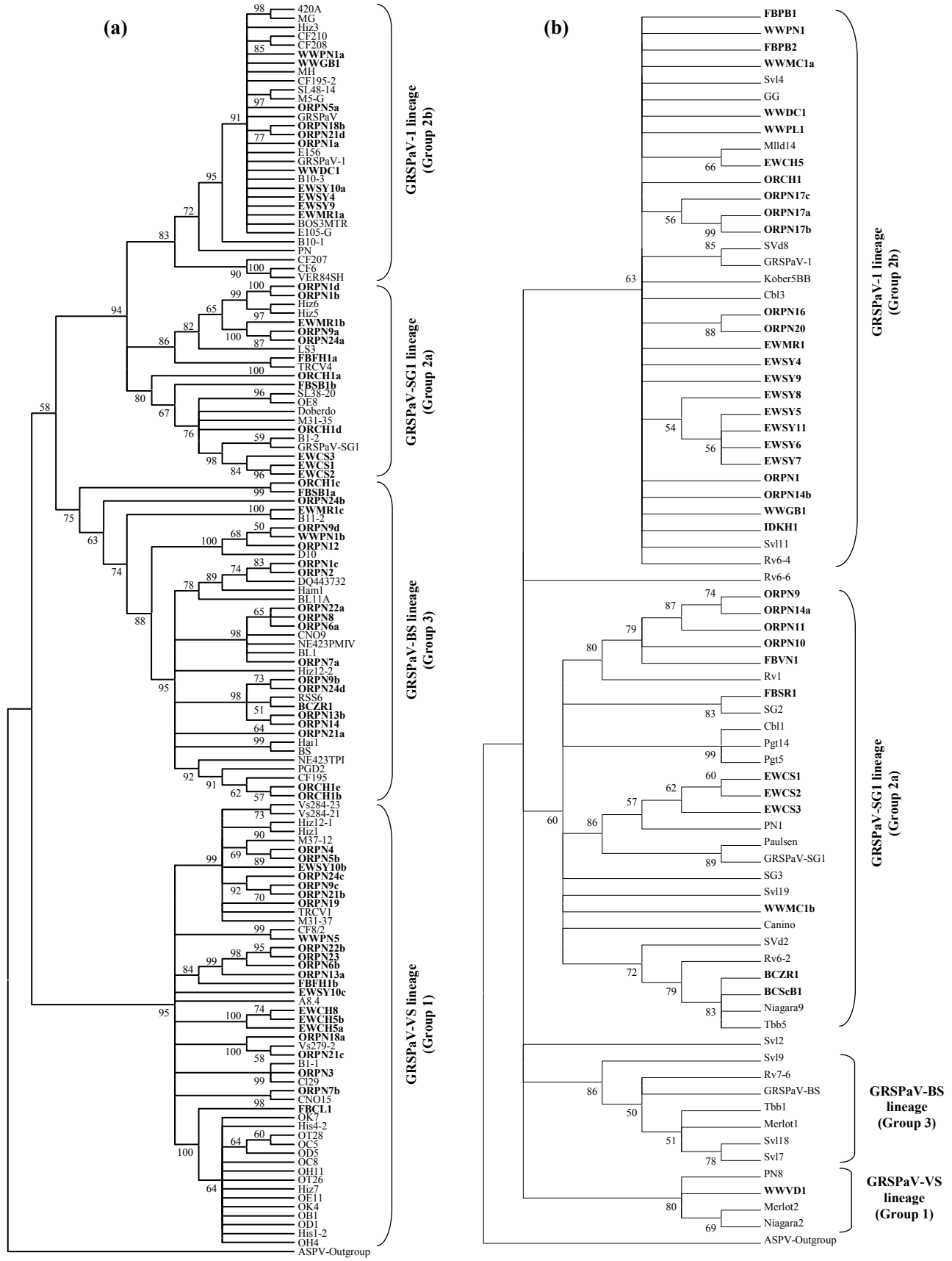

(a)
420A
MG
Hiz3
CF210
CF208
WWPN1a
WWGB1
MH
CF195-2
SL48-14
M5-G
ORPN5a
GRSPaV
ORPN18b
ORPN21d
ORPN1a
E156
GRSPaV-1
WWDC1
B10-3
EWSY10a
EWSY4
EWSY9
EWMR1a
BOS3MTR
E105-G
B10-1
PN
CF207
CF6
VER84SH
ORPN1d
ORPN1b
Hiz6
Hiz5
EWMR1b
ORPN9a
ORPN24a
LS3
FBFH1a
TRCV4
ORCH1a
FBSB1b
SL38-20
OE8
Doberdo
M31-35
ORCH1d
B1-2
GRSPaV-SG1
EWCS3
EWCS1
EWCS2
ORCH1c
FBSB1a
ORPN24b
EWMR1c
B11-2
ORPN9d
WWPN1b
ORPN12
D10
ORPN1c
ORPN2
DQ443732
Ham1
BL11A
ORPN22a
ORPN8
ORPN6a
CNO9
NE423PMIV
BL1
ORPN7a
Hiz12-2
ORPN9b
ORPN24d
RSS6
BCZR1
ORPN13b
ORPN14
ORPN21a
Hai1
BS
NE423TPI
PGD2
CF195
ORCH1e
ORCH1b
Vs284-23
Vs284-21
Hiz12-1
Hiz1
M37-12
ORPN4
ORPN5b
EWSY10b
ORPN24c
ORPN9c
ORPN21b
ORPN19
TRCV1
M31-37
CF8/2
WWPN5
ORPN22b
ORPN23
ORPN6b
ORPN13a
FBFH1b
EWSY10c
A8.4
EWCH8
EWCH5b
EWCH5a
ORPN18a
Vs279-2
ORPN21c
B1-1
ORPN3
CI29
ORPN7b
CNO15
FBCL1
OK7
His4-2
OT28
OC5
OD5
OC8
OH11
OT26
Hiz7
OE11
OK4
OB1
OD1
His1-2
OH4
ASPV-Outgroup
GRSPaV-1 lineage (Group 2b)
GRSPaV-SG1 lineage (Group 2a)
GRSPaV-BS lineage (Group 3)
GRSPaV-VS lineage (Group 1)
(b)
FBPB1
WWPN1
FBPB2
WWMC1a
Svl4
GG
WWDC1
WWPL1
Mlld14
EWCH5
ORCH1
ORPN17c
ORPN17a
ORPN17b
SVd8
GRSPaV-1
Kober5BB
Cbl3
ORPN16
ORPN20
EWMR1
EWSY4
EWSY9
EWSY8
EWSY5
EWSY11
EWSY6
EWSY7
ORPN1
ORPN14b
WWGB1
IDKH1
Svl11
Rv6-4
Rv6-6
ORPN9
ORPN14a
ORPN11
ORPN10
FBVN1
Rv1
FBSR1
SG2
Cbl1
Pgt14
Pgt5
EWCS1
EWCS2
EWCS3
PN1
Paulsen
GRSPaV-SG1
SG3
Svl19
WWMC1b
Canino
SVd2
Rv6-2
BCZR1
BCScB1
Niagara9
Tbb5
Svl2
Svl9
Rv7-6
GRSPaV-BS
Tbb1
Merlot1
Svl18
Svl7
PN8
WWVD1
Merlot2
Niagara2
ASPV-Outgroup
GRSPaV-1 lineage (Group 2b)
GRSPaV-SG1 lineage (Group 2a)
GRSPaV-BS lineage (Group 3)
GRSPaV-VS lineage (Group 1)

Fig. 3. Phylogenetic analysis of (GRSPaV) isolates based on CP (a) and HR (b) sequences. The trees were constructed by the ME method using NJ algorithm implemented by MEGA4. The trees exclude interlineage recombinants identified in this study. The trees were rooted by using (ASPV) sequences as an outgroup. Bootstrap values (1 000 replicates) are given at the branch nodes. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are collapsed. The optimal trees with the sums of branch lengths (2.03681006 for HR and 2.58111728 for CP) are shown. Isolates from the Pacific Northwest region are in bold.

Population genetic analysis show variation in the two genomic regions of GRSPaV

The population genetic parameters are listed in Table 3. Haplotype diversity (Hd) values for CP and HR sequences are close to 1.000 and π values (estimated by the average number of nucleotide differences between two random sequences in a population) for the two genomic regions ranged from 0.027 to 0.128. The highest genetic variation was observed in the CP (π = 0.128) indicating that CP was the more variable of these two genomic regions. However, lower π values for each lineage suggest less variability within individual lineages, although differences in π values suggest genetic variation between them.

Nucleotide polymorphisms in the GRSPaV CP and HR populations were evaluated using Tajima's D (Tajima, 1989) and Fu and Li's D and F (Fu & Li, 1993) statistical tests to assess the influence of demographic forces on the population (Hey & Harris, 1999; Tajima, 1989; Tsompana *et al.*, 2005). The significantly negative values of Tajima's D and Fu and Li's D and F statistical tests (Table 3) for CP sequences discount the neutral hypothesis but suggest the occurrence of demographic expansion of GRSPaV populations. This trend seemed less marked for the HR sequences than for the CP since the Tajima's D and Fu and Li's D and F values for HR did not significantly deviate from zero except for isolates belonging to GRSPaV-VS lineage.

The rate of non-synonymous (d_N) to synonymous (d_S) substitutions for the CP and HR was less than 1.0 (0.037 and 0.027, respectively, Table 3), implying that these regions are under predominantly purifying selection. The distribution profiles of d_N and d_S substitutions were analyzed separately for HR and CP (Fig. 4). The d_S substitution curves for HR shows a

generally consistent slope that implies that these substitutions were almost evenly distributed (Fig. 4a). The d_S substitution curve for the CP shows reduced number of substitutions in the last 100 aa compared with the first 150 aa, suggesting that substitutions in the CP are not selectively neutral (Fig. 4b). In the HR, few d_N substitutions were observed between codons 10 and 45 with a fairly constant rate of substitutions in other portions. In the CP, a higher frequency of d_N substitutions was observed in the first 100 codons of the N-terminal portion than in the other portion of the gene. These differences in d_N substitution frequencies indicated a bias in the distribution of these changes in the CP and HR.

Table 3. Population genetic parameters and neutrality tests calculated for the HR and CP coding regions of GRSPaV

n, Number of isolates; S, number of segregating sites; Hd, haplotype diversity; π, nucleotide diversity estimated by the average number of differences per site between two sequences; θ, statistic from S; d_S, number of synonymous substitutions per site; d_N, number of nonsynonymous substitutions per site.

Region	Lineage	n	S	Hd	π	θ	d_S	d_N	d_N/d_S	Tajima's D	Fu & Li's D	Fu & Li's F
HR (299 nt)	All	94	145	0.998	0.092	0.098	0.695	0.019	0.027	-0.974	-1.645	-1.630
	GRSPaV-1	5	34	1.000	0.054	0.055	0.294	0.008	0.027	-0.147	-0.147	-0.159
	GRSPaV-SG1	32	89	0.992	0.070	0.077	0.356	0.021	0.059	-0.849	-1.159	-1.249
	GRSPaV-BS	10	36	1.000	0.031	0.043	0.199	0.010	0.049	-1.272	-1.236	-1.406
	GRSPaV-VS	47	94	0.995	0.032	0.071	0.483	0.020	0.041	-2.144*	-3.128*	-3.302†
CP (780 nt)	All	159	427	0.999	0.128	0.097	0.874	0.032	0.037	-0.318	-2.640*	-1.817
	GRSPaV-1	63	258	0.995	0.045	0.070	0.205	0.010	0.046	-1.595‡	-2.591*	-2.631*
	GRSPaV-SG1	25	215	1.000	0.067	0.073	0.089	0.011	0.125	-0.768	-1.076	-1.150
	GRSPaV-BS	33	182	0.998	0.027	0.058	0.330	0.013	0.038	-2.188†	-2.672*	-2.971*
	GRSPaV-VS	38	277	1.000	0.069	0.085	0.353	0.013	0.038	-1.209	-1.128	-1.378

*Significant values that reject the null hypothesis of selective neutrality; P<0.05.
†Significant values that reject the null hypothesis of selective neutrality; P<0.02.
‡Significant values that reject the null hypothesis of selective neutrality; P<0.10.

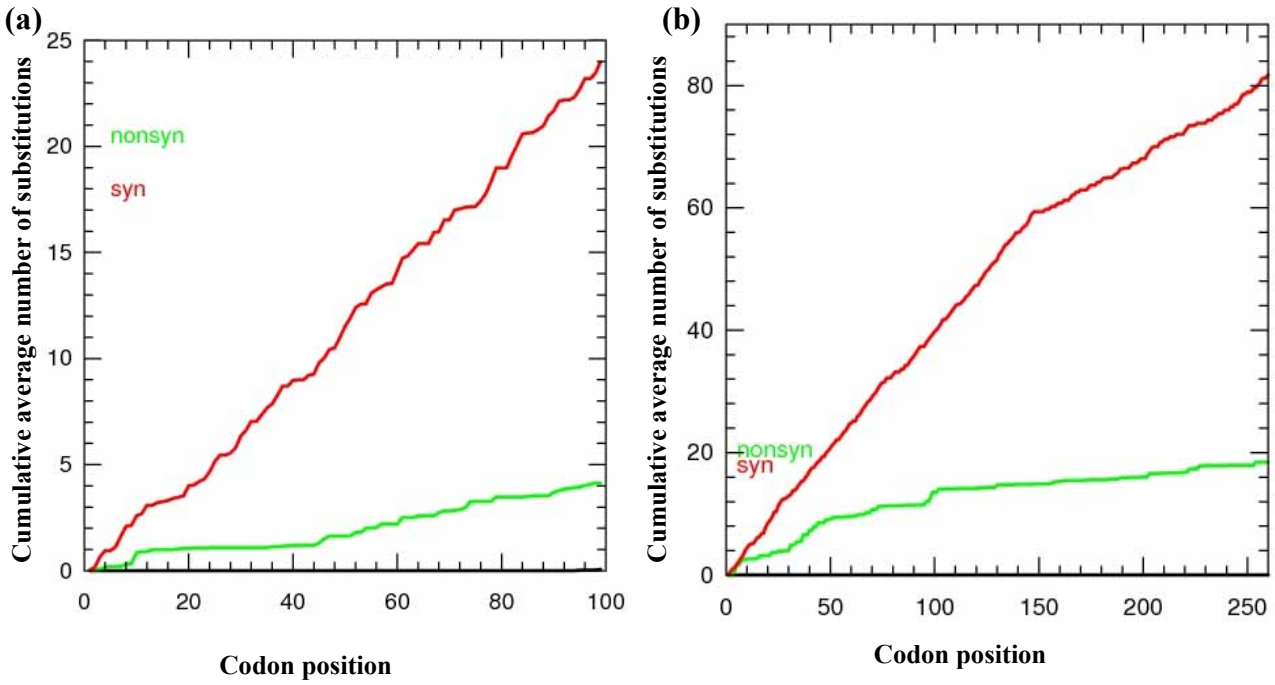


Fig. 4. Amount and distribution of synonymous (d_S) and nonsynonymous (d_N) substitutions for (a) HR and (b) CP sequences of GRSPaV. The d_S and d_N values were computed using a total of 159 CP sequences and 95 HR sequences. Gaps were removed from the sequence alignments to avoid indels. The Y-axis shows cumulative average number of substitutions found for all possible pairwise sequence comparisons, a total of 12,561 comparisons for 159 CP and 4,465 for 94 HR sequences.

GRSPaV CP sequences show intra-plant genetic variation with respect to viticultural practices

In a previous study (Meng *et al.*, 2006), the population structure of GRSPaV was found to be different between grapevine scion and rootstock cultivars, with the former harboring mixtures of distinct variants. Since the wine grape scion cultivars are grown on rootstocks in Oregon and as own-rooted plants in eastern Washington, we assessed the status of CP sequence diversity in cultivars grown under different viticultural practices (grafting vs. own-rooting). Our results indicated that distinct sequences of CP occurred as mixtures in 10 of 54 grapevines. Further analysis of these sequences from the 10 grapevines revealed that they belonged to two or more lineages (Table 4). Eight of these grapevines were from Oregon grown on rootstocks and only two were from eastern Washington grown as own-rooted plants. The variant sequences obtained from these 10 grapevines belonged to two or three different groups and they occurred in various proportions with one variant sequence predominating over the others in a given plant. Although the majority of samples in Oregon came from one cultivar (cv. Pinot Noir), samples from one other cultivar (cv. Chardonnay) also had genetically distinct variants in a single grapevine. We did not find the occurrence of all four variant groups in the same grapevine in this study. In contrast, the majority of CP sequences derived from grapevine samples collected from own-rooted scion cultivars in eastern Washington revealed no such intra-plant diversity. Only two samples, one each from cvs. Syrah and Merlot contained variant sequences belonging to two and three different lineages, respectively. These results indicate propensity of the occurrence of mixtures of distinct variants of GRSPaV in grafted plants. The profile of virus variants in individual grapevines revealed no specific correlation between variant groups and scion cultivars.

Table 4. Proportions of GRSPaV variant mixtures present in individual grapevine isolates

The CP coding region was assessed. EWMR1 and EWSY10 were grown on their roots; all other isolates were grown on rootstock

Isolate‡	Sequence variant groups†				Nt identity (%)
	Group 1 (GRSPaV-VS)	Group 2a (GRSPaV-SG1)	Group 2b (GRSPaV-1)	Group 3 (GRSPaV-BS)	
EWMR1	-	1	11	1	82-91
EWSY10	10	-	4	-	84
ORCH1	-	1	-	9	82-92
ORPN1	-	7	1	2	80-90
ORPN7	4	-	-	1	85
ORPN9	2	1	-	5	82-84
ORPN13	2	-	-	3	85
ORPN21	3	-	7	1	82-84
ORPN22	10	-	-	2	83
ORPN24	6	1	-	23	83-86

‡Details of each isolate are provided in Supplementary Table S1
†The lineage of the corresponding sequence variant group is shown in parenthesis and the numbers in each column shows the number of clones obtained for each GRSPaV group in a given plant; -, absent

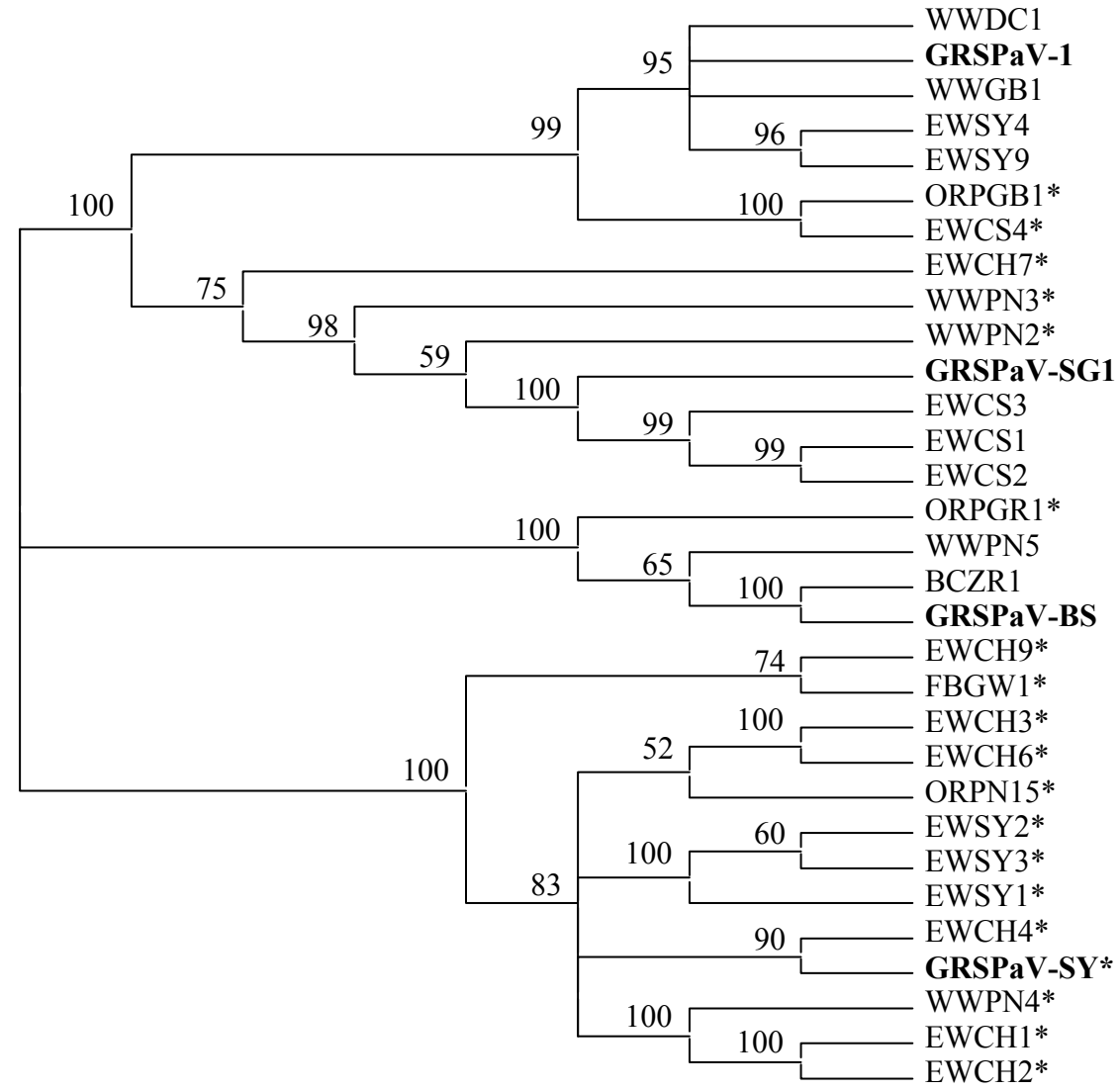
Discordance between the CP and HR phylogenies suggest recombination among GRSPaV sequence variants

Among the 34 isolates of GRSPaV from PNW vineyards, from which both CP and HR sequences are available, a group of 27 isolates contained a single variant. Phylogenetic relationships of these 27 isolates based on HR and CP concatenated sequences showed four major lineages (Fig. 5a) analogous to the tree presented in Fig. 3. The CP-based tree showed the same groupings as for the concatenated sequences (Fig.5c), whereas the HR-based tree differed with respect to the position of 19 isolates (Fig. 5b). Since sequences of each of the 19 isolates comprised a single variant, their discordant phylogenies would suggest potential recombination events involving parents from different lineages. We investigated this possibility with the suite of programs included in the RDP3 package using concatenated sequences of the HR and CP from all 27 isolates.

A total of 19 putative recombination events were detected by at least four programs of the RDP3 software. The recombinant isolates and their potential 'parental sequences' are listed in Table 5. The cross-over sites were identified at different locations for these recombinants, with 17 and 2 involving inter- and intra-lineage recombination events, respectively. The majority of these recombination events involved parental isolates from GRSPaV-1 and GRSPaV-VS lineages generating 10 recombinant sequences (EWSY1, EWSY2, EWSY3, EWCH1, EWCH2, EWCH3, EWCH4, EWCH6, ORPN15, and WWPN4). The other recombination events involved GRSPaV-SG1 and GRSPaV-1 lineages generating five recombinant sequences (EWCH7, ORPGB1, EWCS4, WWPN2 and WWPN3) and one each involving GRSPaV-1 and GRSPaV-BS, and

GRSPaV-VS and GRSPaV-BS resulting in ORPGR1 and GRSPaV-SY, respectively. In addition, intra-lineage recombination events occurred between 'parental' sequences from GRSPaV-BS lineage resulting in two progeny sequences (EWCH9 and FBGW1). A closer look at the various recombination events indicated proclivity for such events occurring between lineages GRSPaV-1 and GRSPaV-VS. In addition, 11 and 2 of 19 crossover sites were present in CP and HR sequences, respectively. Four crossover sites were observed between concatenated sequences of the HR and CP, indicating possible recombination events in portions of the virus genome between the HR and CP.

(a)


WWDC1
GRSPaV-1
WWGB1
EWSY4
EWSY9
ORPGB1*
EWCS4*
EWCH7*
WWPN3*
WWPN2*
GRSPaV-SG1
EWCS3
EWCS1
EWCS2
ORPGR1*
WWPN5
BCZR1
GRSPaV-BS
EWCH9*
FBGW1*
EWCH3*
EWCH6*
ORPN15*
EWSY2*
EWSY3*
EWSY1*
EWCH4*
GRSPaV-SY*
WWPN4*
EWCH1*
EWCH2*
95
99
96
100
100
75
98
59
100
99
99
100
65
100
74
100
52
100
60
100
83
90
100
100

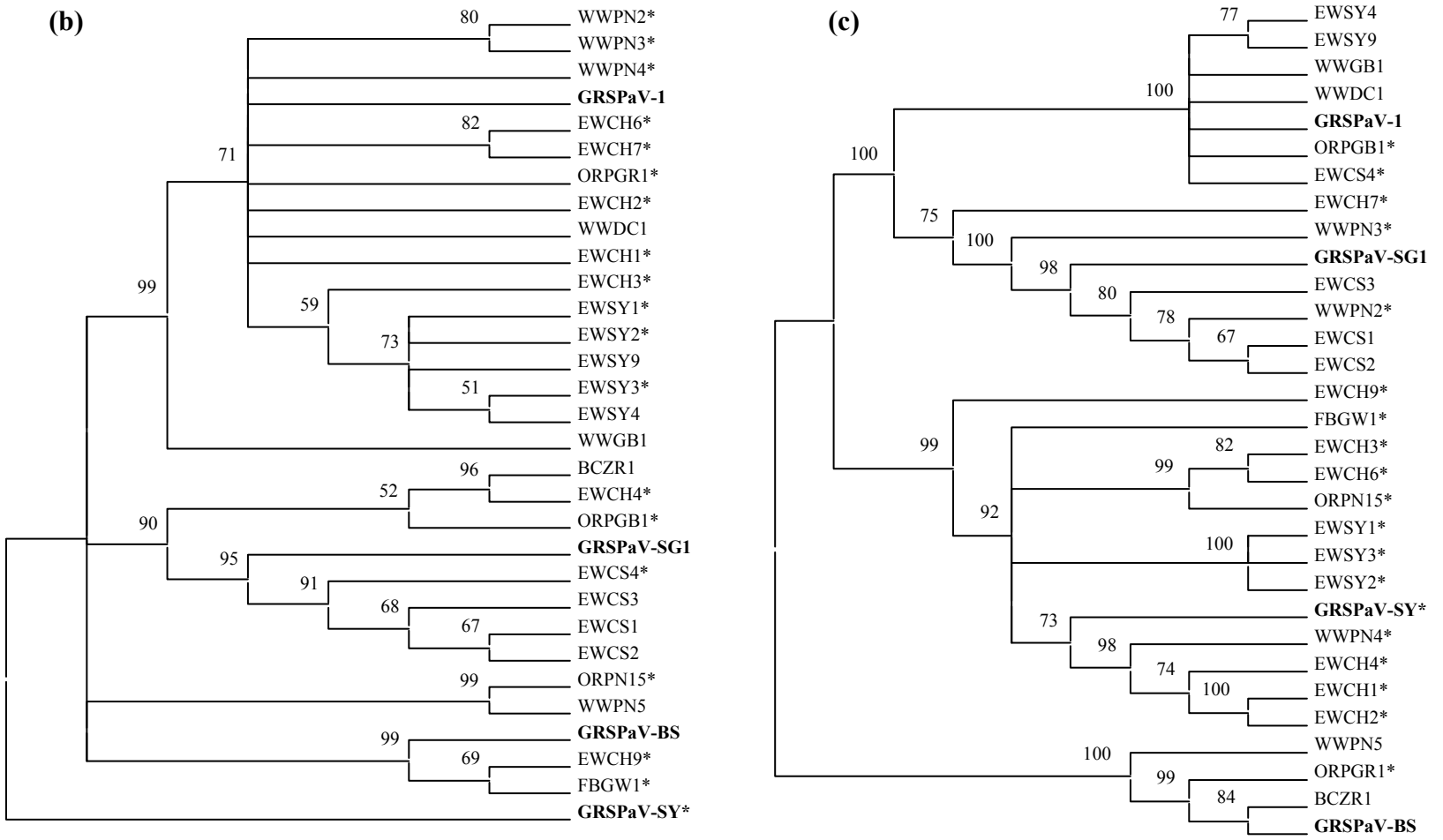


Fig. 5. Unrooted nucleotide maximum-likelihood phylogenetic tree calculated from HR and CP concatenated (a) HR (b) and (c) CP sequences of 27 isolates of *Grapevine rupestris stem pitting-associated virus* (GRSPaV) from PNW. Representative isolates for each lineage is in bold. Recombinant isolates detected by the RDP3 software are indicated with *. Bootstrap values (1,000 replicates) are given at the branch nodes. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Horizontal branch length indicate 0.1 nt replacements per site and is drawn to scale.

Table 5. List of GRSPaV isolates showing putative recombination events in concatenated sequences of CP and HR

Recombinant and parental isolates obtained from PNW are in bold and the sources of isolates are indicated in Supplementary Tables S1 and S2. The suite of recombination detection programs used for the detection of recombination events and the corresponding average *P*-values for each event were: R, RDP; G, GeneConv; B, Bootscan; M, MaxChi; C, CHIMAERA; S, SiScan; 3S, 3SEQ. Bootscan, RDP and SiScan are phylogeny-based methods, GeneConv, MaxChi, CHIMAERA and LARD are substitution-based methods. No recombination events were detected using LARD. *P* values >0.05 are considered significant; -, no recombination event detected.

Recombinant isolate	**Parental isolates**[b]		***P*-value**						
	Major	**Minor**	**R**	**G**	**B**	**M**	**C**	**S**	**3S**
EWSY1	GRSPaV-1 (*GRSPaV-1*)	GRSPaV-SY (*GRSPaV-VS*)	$1x10^{-05}$	$3x10^{-05}$	$1x10^{-04}$	$6x10^{-10}$	$3x10^{-07}$	$2x10^{-21}$	$3x10^{-26}$
EWSY2	GRSPaV-1 (*GRSPaV-1*)	GRSPaV-SY (*GRSPaV-VS*)	$1x10^{-05}$	$3x10^{-05}$	$1x10^{-04}$	$6x10^{-10}$	$3x10^{-07}$	$2x10^{-21}$	$3x10^{-26}$
EWSY3	GRSPaV-1 (*GRSPaV-1*)	GRSPaV-SY (*GRSPaV-VS*)	$1x10^{-05}$	$3x10^{-05}$	$1x10^{-04}$	$6x10^{-10}$	$3x10^{-07}$	$2x10^{-21}$	$3x10^{-26}$
EWCH1	GRSPaV-1 (*GRSPaV-1*)	GRSPaV-SY (*GRSPaV-VS*)	$1x10^{-05}$	$3x10^{-05}$	$1x10^{-04}$	$6x10^{-10}$	$3x10^{-07}$	$2x10^{-21}$	$3x10^{-26}$
EWCH2	GRSPaV-1 (*GRSPaV-1*)	GRSPaV-SY (*GRSPaV-VS*)	$1x10^{-05}$	$3x10^{-05}$	$1x10^{-04}$	$6x10^{-10}$	$3x10^{-07}$	$2x10^{-21}$	$3x10^{-26}$
EWCH4	GRSPaV-1 (*GRSPaV-1*)	GRSPaV-SY (*GRSPaV-VS*)	$1x10^{-05}$	$3x10^{-05}$	$1x10^{-04}$	$6x10^{-10}$	$3x10^{-07}$	$2x10^{-21}$	$3x10^{-26}$
EWCH3	GRSPaV-1 (*GRSPaV-1*)	GRSPaV-SY (*GRSPaV-VS*)	$1x10^{-05}$	$3x10^{-05}$	$1x10^{-04}$	$6x10^{-10}$	$3x10^{-07}$	$2x10^{-21}$	$3x10^{-26}$
EWCH6	GRSPaV-1 (*GRSPaV-1*)	GRSPaV-SY (*GRSPaV-VS*)	$1x10^{-05}$	$3x10^{-05}$	$1x10^{-04}$	$6x10^{-10}$	$3x10^{-07}$	$2x10^{-21}$	$3x10^{-26}$
ORPN15	GRSPaV-1 (*GRSPaV-1*)	GRSPaV-SY (*GRSPaV-VS*)	$1x10^{-05}$	$3x10^{-05}$	$1x10^{-04}$	$6x10^{-10}$	$3x10^{-07}$	$2x10^{-21}$	$3x10^{-26}$
WWPN4	GRSPaV-1 (*GRSPaV-1*)	GRSPaV-SY (*GRSPaV-VS*)	$1x10^{-05}$	$3x10^{-05}$	$1x10^{-04}$	$6x10^{-10}$	$3x10^{-07}$	$2x10^{-21}$	$3x10^{-26}$
EWCH7	**EWCS3** (*GRSPaV-SG1*)	GRSPaV-1 (*GRSPaV-1*)	-	$1x10^{-02}$	$8x10^{-04}$	$4x10^{-05}$	$3x10^{-05}$	$2x10^{-07}$	-
EWCH9	**WWPN5** (*GRSPaV-BS*)	GRSPaV-BS (*GRSPaV-BS*)	$3x10^{-06}$	$5x10^{-04}$	$2x10^{-06}$	$8x10^{-03}$	$7x10^{-03}$	$7x10^{-03}$	-
FBGW1	**WWPN5** (*GRSPaV-BS*)	GRSPaV-BS (*GRSPaV-BS*)	$3x10^{-06}$	$5x10^{-04}$	$2x10^{-06}$	$8x10^{-03}$	$7x10^{-03}$	$7x10^{-03}$	-
GRSPaV-SY	**FBGW1** (*GRSPaV-VS*)	Unknown (*GRSPaV-BS*)	-	$1x10^{-07}$	$3x10^{-02}$	-	-	$4x10^{-14}$	$5x10^{-06}$
ORPGR1	**EWSY9** (*GRSPaV-1*)	GRSPaV-BS (*GRSPaV-BS*)	-	-	-	$1x10^{-03}$	$2x10^{-03}$	$7x10^{-16}$	$2x10^{-10}$
ORPGB1	**EWCS2** (*GRSPaV-SG1*)	GRSPaV-1 (*GRSPaV-1*)	-	-	$4x10^{-03}$	$2x10^{-05}$	$1x10^{-08}$	$6x10^{-16}$	$2x10^{-16}$
EWCS4	**EWCS2** (*GRSPaV-SG1*)	GRSPaV-1 (*GRSPaV-1*)	-	-	$4x10^{-03}$	$2x10^{-05}$	$1x10^{-08}$	$6x10^{-16}$	$2x10^{-16}$
WWPN2	GRSPaV-1 (*GRSPaV-1*)	**EWCS1** (*GRSPaV-SG1*)	-	$1x10^{-02}$	$2x10^{-04}$	$3x10^{-07}$	$3x10^{-08}$	$6x10^{-13}$	$1x10^{-10}$
WWPN3	GRSPaV-1 (*GRSPaV-1*)	**EWCS1** (*GRSPaV-SG1*)	-	$1x10^{-02}$	$2x10^{-04}$	$3x10^{-07}$	$3x10^{-08}$	$6x10^{-13}$	$1x10^{-10}$

'Minor' and 'Major' parents refer to the parental isolates contributing the smaller and larger fractions of the recombinant's sequence, respectively. Lineages of parental isolates (in italics) are derived from Fig. 3(a). An 'unknown' parent is so called probably because isolates distantly resembling one of the recombinant's parents have been sampled.

DISCUSSION

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In this study, the molecular diversity of field isolates of GRSPaV in wine grape cultivars grown in the PNW region was assessed relative to virus isolates from other grape-growing regions. A phylogenomic approach was used to analyze a total of 159 full-length CP sequences (84 from this study and 75 from GenBank) and 94 sequences specific to the helicase region of the RNA-dependent RNA polymerase (RdRp) (57 from this study and 37 from GenBank). As only a few isolates from the USA and none from the PNW were included in previous studies, the work represents an analysis of GRSPaV isolates at a global level, extending previous investigations conducted in other grape-growing regions of the world (Meng *et al.* 2006; Nolasco *et al.* 2006; Habili *et al.* 2006; Lima *et al.* 2006; Nakaune *et al.* 2008). These comparative results show that the genetic diversity of GRSPaV in the PNW vineyards is considerably greater than reported in other regions, probably as a result of the introduction of planting materials from several sources outside the region.

The results reinforce that the global GRSPaV population is very diverse, with numerous, disparate strains segregating into four distinct lineages (Fig. 3a, b). Several factors could contribute to the perpetuation of such a complex and dynamic population structure. The perennial and clonal propagation of grapevines, chronic infection for many years without host mortality, and absence of a biological vector provides a conducive environment for the evolution of a population of genetically related variants, in the absence of selection pressure or a bottleneck for different sequence variants generated by short replication times and the error-prone nature of the RdRp (Garcia-Arenal *et al.*, 2001). That grapevines often remain in the field for several years or decades coupled with cultural practices such as grafting onto rootstocks and topworking to

new cultivars could mean that new variants are introduced into a plant providing additional opportunities for changes in the viral population dynamics within individual grapevines. The presence of genetically distinct variants in infected grapevines in the PNW and the presence of sequences from different genetic lineages within a scion cultivar grafted onto rootstock validate this argument.

Relatively higher π values in the CP than in the HR (Table 3) suggest that the CP is the more variable among the two coding regions. Overall, the high level of π of GRSPaV isolates in both coding regions is largely due to the accumulation of d_S substitutions (Fig. 4). Although the d_N/d_S ratio obtained for both CP and HR sequences were below 1.0, it was 1.4 times higher for CP (0.037) than for the HR (0.027), suggesting a stronger purifying selection operating on the HR. Similar high levels of nucleotide sequence variability yet low values of d_N/d_S have been reported in different genomic regions for other members of the family *Flexiviridae* (Chare & Holmes, 2004, 2006; Shi *et al.*, 2004; Teycheney *et al.*, 2005), further indicating that the attribute is common to members of this family. Considering the critical role of the helicase domain in viral RNA replication and its possible involvement in RNA unwinding and cap formation (Martelli *et al.*, 2007), it is likely that HR is less tolerant of mutations that could affect its function. In contrast, a lack of biological vector for transmission makes the CP of GRSPaV more tolerant to sequence variability, since the capsid proteins of vector-borne plant viruses are subject to greater purifying selection on amino acid changes than viruses transmitted by other routes (Chare & Holmes, 2004; Rubio *et al.*, 2001). It is also plausible that the C-terminal third of the CP is less tolerant of mutations due to assembly or particle structural limitations. Given the high degree of the global genetic variation among GRSPaV sequences, it is unlikely that the estimated error rate

of 1 x 10^{-4} to 2 x 10^{-5} errors per base pair for Taq polymerase (Lundberg *et al.*, 1991; Bracho *et al.*, 1998) would have contributed to the apparent nucleotide divergence obtained in this study and previous reports (Meng *et al.* 2006; Nolasco *et al.* 2006; Habili *et al.* 2006; Lima *et al.* 2006; Nakaune *et al.* 2008).

Phylogenetic analysis of GRSPaV isolates characterized in this study further supports the existence of the same four phylogroups (Meng *et al.*, 2006; Nolasco *et al.* 2006; Nakaune *et al.* (2008), irrespective of the protein employed for the analysis (Fig. 3). These isolates were distributed in all four groups, confirming a lack of clustering by geographical origin. Such a situation could be due to the spread of GRSPaV exclusively through the distribution of vegetative cuttings, in contrast to geographical delineation of virus variants in some vector-borne viruses (Karan *et al.*, 1994; Varsani *et al.*, 2008). Although the phylograms derived in this study using the entire CP were similar to HR-based phylograms, the placement of some GRSPaV isolates showed differences between CP and HR-based phylograms due to possible recombination events in the genome (discussed below). In view of such discordant gene phylogenies among GRSPaV isolates, it seemed appropriate to use one of these two genes for comparative sequence analysis and we favor the whole CP gene sequences as the best representation of the phylogeny of global isolates of GRSPaV. Such CP-based phylogeny has been used for the classification of several different virus groups (Karan *et al.*, 1994; Pfosser & Baumann, 2002; Fajardo *et al.*, 2007).

The discordant gene phylogenies obtained for CP and HR sequences of some GRSPaV isolates (Fig. 5) provided strong evolutionary evidence for recombination. Factors that can cause

different genes to give different topologies have been well reviewed (Jeffroy *et al.*, 2006; Rokas & Carroll, 2006; Castresana, 2007). These include the stochastic nature of mutation, lineage sorting, phylogenetic reconstruction artifacts and methodological problems related to the assessment of homology. Hence, similar, rather than identical, trees should be expected from different genes (Penny *et al.*, 1982; Huerta-Cepas *et al.*, 2007). In order to investigate recombination events among GRSPaV isolates, the two genomic regions were concatenated and analyzed by a suite of programs representing phylogenetic-, and substitution-based methods for robust assessment of definitive evidence of recombination. Several potential breakpoints for recombination have been identified in CP and HR sequences. It is unlikely that these recombinants were RT-PCR artifacts, since a minimum of three independent clones originated from different PCR products were sequenced to avoid artificial results arising from RT, PCR and cloning. Besides, the occurrence of nearly identical recombinant breakpoints in different isolates and the highly significant statistical support provided for each event strongly suggests that the recombinants are genuine. In addition, Nolasco *et al.* (2006) reported a recombination event involving GRSPaV isolates belonging to two of the four CP variant groups from Portugal and Slovenia. However, support for this putative recombinant event came from only 4 of the 8 algorithms implemented by the RDP program.

As the CP and HR sequences represent only about 12 % of the GRSPaV genome, the likelihood of recombination events in the portions of virus genome not covered by present analyses cannot be excluded. Thus, the actual proportion of recombination events may be much greater and the extent of recombination in the global GRSPaV population can be realized when the full genomic sequences of a representative set of isolates from different grape-growing regions are available.

A possible explanation for recombination events observed in this study is that the scion culitvar could be carrying one variant and certain rootstocks could be carrying another and, after grafting, these variants might have mixed together in the same grapevine. Mixed infections provide the opportunity for recombination to occur between different strains and are therefore important in increasing the diversity of GRSPaV within populations. Such recombination events appear to be common in the genomes of viruses infecting vegetatively propagated perennial crops such as citrus (Rubio *et al.*, 2001; Weng *et al.*, 2007). In a broader context, recombination in viruses infecting perennial crops offer potentially significant advantages for an increased genetic diversity and adaptability because deleterious mutations accumulated due to the lack of proofreading activity of an RdRp can be offset by recombination of the error-free parts of co-infecting genomes (García-Arenal *et al.*, 2001; Vives *et al.*, 2005). Thus, RNA recombination is likely to play a significant role in GRSPaV variation and evolution. Consequently, every field isolate of GRSPaV could be unique in each gene sequence due to the highly variable nature of the virus.

Previous studies have documented genetic diversity at the intra-host level in perennial woody hosts (Magome *et al.*, 1998; D'Urso *et al.*, 2000). A recent study has shown that *Plum pox virus* has evolved into several distinct populations to the point that in different parts of the same *Prunus* tree, the composition of these populations was different over a period of 13 years after inoculation (Jridi *et al.*, 2006). It will be interesting to pursue similar studies with GRSPaV to better understand the dynamics of genetic diversity and the potential association between different *Vitis* spp. and cultivars and virus population structures.

The role of GRSPaV in the etiology of RSP disorder is still unresolved (Nakaune *et al.*, 2008), and therefore, the possible biological implications of genetic variants of GRSPaV are difficult to assess presently. A recent study in Australia (Habili *et al.*, 2006) has shown no association between the type of symptom expressed on the indicator host (cv. St. George) and the sequence diversity in the virus genome. This is further compounded by the presence of GRSPaV isolates causing latent infections in the *V. rupestris* sources widely used for biological indexing (Minafra *et al.*, 2000; Meng *et al.*, 1999; Petrovic *et al.* 2000). It is likely that such latent infections could be the result of antagonism between genetically divergent variants present as mixed infections in a grapevine resulting in erroneous conclusions of biological indexing assays. Conversely, GRSPaV variants present at low frequencies might play an important biological role, for example in virus pathogenesis. Recent studies have shown the association of GRSPaV with *Grapevine rupestris vein-feathering virus* and *Grapevine Syrah virus-1* in grapevines showing Syrah decline symptoms (Al Rwahnih, *et al.*, 2009). Thus, from a practical point of view, the results presented in this study provide a foundation for further exploring the biological role of GRSPaV sequence variants in disorders like Syrah decline and veinal necrosis. The likely recombination events in the CP and HR sequences as reported in this study and frequent occurrence of GRSPaV as mixed infections with other known and currently uncharacterized graft-transmissible agents (Credi, 1997; Komar *et al.*, 2006; Naidu *et al.*, 2006; Prosser *et al.*, 2007) add additional layers of complexity in assessing the biological behavior of different genetic variants of GRSPaV. The availability of an infectious clone of GRSPaV would provide additional possibilities for establishing the aetiological relationship between GRSPaV and RSP and understanding the biological implications of molecular diversity in the virus genome.

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CHAPTER 7

GENERAL CONCLUSIONS

Due to their perennial nature and vegetative propagation, management of virus diseases in cassava and grapevine requires strategies largely different from management of virus diseases in annual crops. Prevention of spread via distribution of infected vegetative cuttings is considered as an effective first line of defense against virus diseases in both crops. Management of insect vectors is an additional strategy adapted to minimize secondary spread of viruses in perennial crops, since an infected cutting could offer a constant source of inoculum for the vector to spread virus disease to new plantings in the field. Conversely, a 'healthy' plant can be infected with a virus transmitted by a vector from external sources in the vicinity and cuttings derived from such plants will likely result in dissemination of the 'new' virus to other areas. In addition, the perennial nature of cassava and grapevine offers potentially significant advantages, due to the lack of bottle necks or purifying selection, for an increased genetic diversity of viruses in these crops. The research presented in Chapters 2-4 generated new knowledge on (i) the epidemiology and molecular biology of cassava mosaic begomoviruses (CMBs) infecting cassava (*Manihot esculenta* Crantz) in Nigeria and (ii) genetic diversity of *Grapevine rupestris stem pitting-associated virus* (GRSPaV) infecting wine grapes (*Vitis vinifera* L.) in the Pacific Northwest (PNW) region of the United States of America. The information and methodologies described in these chapters will be valuable for improved diagnostics in quarantine and certification programs and crop improvement strategies. The data presented in this study also contributed to an increased understanding of molecular population genetics of viruses infecting deciduous woody perennials.

Cassava mosaic disease (CMD) is the most economically important viral disease of cassava in Nigeria, and indeed in the entire Sub-Saharan Africa (SSA) region. By carrying out nucleotide sequence analysis of the entire DNA A genomic components, the identity of *African cassava mosaic virus* (ACMV) and *East African cassava mosaic Cameroon virus* (EACMCV) in cassava infected with cassava mosaic disease (CMD) has been confirmed. A definitive identification of EACMCV, therefore, clarified the inconsistency with regard to misidentification of this virus as *East African cassava mosaic virus* in previous studies and underscored the need to carry out nucleotide sequence analysis to alleviate problems associated with diagnosis of viruses associated with CMD exclusively by serology and/or PCR. One of the key gaps in knowledge on the epidemiology of CMD in Nigeria, and indeed on a regional scale, is the potential role of non-cassava plant species as alternative/ reservoir hosts in the perpetuation of cassava mosaic begomoviruses (CMBs). By completing nucleotide sequence analysis of the entire DNA A genomic component of ACMV and EACMCV, this study revealed that both viruses can infect non-cassava plant species. They were found as single or mixed infections in both weed hosts (*S. occidentalis* and *C. confertum*) and crop plants (*R. communis*, *L. leucocephala*, and *G. max*). These results underscore the significance of documenting the natural host range of different CMBs for an improved understanding of the role of indigenous non-cassava plant species in the epidemiology of CMD. Future studies on the ability of the whitefly vector to transmit CMBs from non-cassava hosts to cassava and vice versa would help to determine the implications of these findings in developing sustainable strategies for the management of CMD in Nigeria and in the wider Sub-Saharan Africa region.

Since both ACMV and EACMCV often occur in mixed infections under field conditions, the availability of diagnostic assay for concurrent detection of both viruses in a cost-effective manner will enhance disease monitoring and quarantine capability in sub-Saharan Africa. Towards this end, a multiplex PCR assay was developed for simultaneous detection of ACMV and EACMCV in cassava. The multiplex PCR developed in this study, in conjunction with a simplified method of sample extraction, is reliable, rapid, sensitive, specific and cost-effective for diagnosing EACMV and EACMCV in cassava plants. The assay is versatile since it can also be used for single virus detection using virus-specific primers. Consequently, the multiplex PCR is suitable for reliable assessment of the prevalence of CMBs in epidemiological studies and for crop improvement, quarantine, eradication and certification programs in African countries. This protocol is currently being used for monitoring CMD in many African countries.

GRSPaV is a graft-transmissible virus widely distributed in many grape-growing regions around the world. Previous studies have indicated biological association of GRSPaV with rupestris stem pitting (RSP), one of the four disorders of the Rugose Wood complex that affects woody cylinder of the grapevine leading to poor growth, reduced yield and general decline. The PNW states of the United States (Washington, Oregon and Idaho) are collectively emerging as the second largest wine grape growing region in the country. Notable differences exist in the viticultural practices within the PNW region with wine grapes largely grown as own-rooted vines in Washington and Idaho and on rootstocks in Oregon. Since exchange of planting material occurs among growers in the region, studies are conducted to assess the sanitary status of vineyards by documenting grapevine viruses and their genetic variants. This information will aid the development of strategies for preventing dissemination of viruses and mitigating their

negative impact on sustainability of the wine grape industry in the region. Towards this objective, the genetic diversity of GRSPaV in PNW vineyards was assessed. A total of 84 full-length coat protein (CP) sequences and 57 sequences representing the helicase-encoding region (HR) of the RNA-dependent RNA polymerase were obtained from wine grape cultivars grown in PNW and their molecular diversity compared with corresponding sequences previously reported from other grape-growing regions. The results indicated that GRSPaV is highly variable in chronically infected, vegetatively propagated grapevines. The study also provided evidence for the occurrence of potential recombination events in a foveavirus. The occurrence of mixed infection of genetically divergent variants within a single grapevine and recombination events identified in this study could in part contribute to extensive sequence diversity. The results have shown that genetic diversity of GRSPaV in PNW vineyards is considerably greater than reported in other regions, probably as a result of the introduction of planting materials from several sources outside the region. The results are useful for developing improved diagnosis of different genetic variants of the virus in 'clean' plant programs.